The requirement for phospholipase A_2 for activation of the assembled NADPH oxidase in human neutrophils

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Phospholipase A_2 (PLA_2) inhibitors suppressed simultaneously, in a dose-dependent manner, the activation of NADPH oxidase and the release of ^{3}H-labelled arachidonic acid ([^{3}H]AA) stimulated by either phorbol 12-myristate 13-acetate (PMA) or opsonized zymosan (OZ) in human neutrophils. In spite of total inhibition of superoxide production in the presence of the PLA_2 inhibitors, 10 μM bromophenacyl bromide (BPB) or 20 μM quinacrine, a maximal phosphorylation of p47 and translocation of p47 and p67 to the neutrophil membranes induced by PMA or OZ was observed. Addition of 10 μM free AA, which by itself did not stimulate superoxide generation, restored oxidase activity in neutrophils treated with PLA_2 inhibitors. These findings indicate that phosphorylation and translocation of the cytosolic factors to the membranes are not sufficient for generating superoxide; a functional PLA_2 is also needed to stimulate the oxidase activity. The inhibition of PLA_2 activity did not prevent the phosphorylation of p47, suggesting that the location of PLA_2 is downstream of and does not activate protein kinase C.

INTRODUCTION

On ingestion of micro-organisms, phagocytic cells undergo a rapid burst of oxygen consumption, leading to the production of microbicidal oxidants. The biochemical basis for this respiratory burst is the activation of a superoxide-generating NADPH oxidase. This oxidase is dormant in resting cells and is capable of being activated by several different stimuli [1,2]. In human neutrophils, the activated enzyme is a multicomponent electron-transport chain composed of a membrane flavocytochrome b_{558} [3,4], containing haem, flavin and NADPH-binding sites [5,6] and three cytosolic components, p47, p67 and *rac* 2, a *ras*-related GTP-binding protein [7–12]. Several proteins are phosphorylated when the respiratory burst is activated [13], but the only phosphoproteins that are known to be definitely involved are the 91 kDa and 22 kDa subunits of flavocytochrome *b* [14] and the 47 kDa cytosolic oxidase protein p47 [15–19]. By using two-dimensional gel electrophoresis [18,19], a chain of increasingly phosphorylated p47 was demonstrated in the cytosol and membranes of phorbol 12-myristate 13-acetate (PMA)-activated neutrophils.

The stimulus–receptor-coupled responses of human neutrophils to a variety of agonists result in a complex array of metabolic events ultimately linked to the activation of the NADPH oxidase enzyme. Studies in whole cells show that the oxidase can be activated by a variety of stimuli and suggest that there are several pathways to oxidase activation [20–22]. A direct role for protein kinase C (PKC) in the activation of NADPH oxidase has been suggested, since PKC translocates from cytosol to cell membranes and mediates phosphorylation of p47 during stimulation with PMA [7,23,24]. However, use of PKC and phospholipase A_2 (PLA_2) inhibitors has clearly demonstrated that the mechanism of oxidase activation is more complicated [25]. In both neutrophils and macrophages, an increase in PLA_2 activity and a release of arachidonic acid (AA) has been reported to occur in association with stimuli which also activate the oxidase [25–28]. In a recent study, we have demonstrated the release of AA and the involvement of PLA_2 in the transduction pathway stimulating the NADPH oxidase activity in peripheral blood monocytes by lipoteichoic acid or opsonized zymosan (OZ) [29]. Furthermore, AA itself stimulates superoxide in intact cells, in a cell-free assay and in electropermeabilized cells [30–32]. Although much work during the past few years delineated an increasingly complex set of reactions exhibiting evidence of exquisite regulation, the exact mechanisms leading to activation of the oxidase and the relationships between the different transduction pathways are unclear. The present study focuses on the role and the location of PLA_2 activity in the transduction pathway(s) leading to activation of the NADPH oxidase complex in human neutrophils.

MATERIALS AND METHODS

Neutrophil isolation

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

Superoxide-anion measurements

The production of superoxide anion (O_2^-) by intact cells was measured as the superoxide dismutase-inhibitable reduction of acetyl-ferricytochrome *c* as described [33]. Cells were suspended (5×10^5 cells/well) in 100 μl in Hanks' Balanced Salts Solution containing 150 mM acetyl-ferricytochrome *c*. Cells were stimulated by addition of the appropriate stimulus, and the reduction of acetyl-ferricytochrome *c* was monitored by the change in A_{550} every 2 min on a Thermomax microplate reader (Molecular Devices, Melno Park, CA, U.S.A.). The maximal rates of

Abbreviations used: PLA_2, phospholipase A_2; AA, arachidonic acid; PMA, phorbol 12-myristate 13-acetate; OZ, opsonized zymosan; BPB, bromophenacyl bromide; PKC, protein kinase C.

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superoxide generation were determined by using the absorption coefficient $\epsilon_{550} = 21\ mM^{-1} \cdot cm^{-1}$.

[^{32}P]P_i loading

Loading with [^{32}P]P_i was performed as described previously [19]. Neutrophils were suspended at 10^7 cells/ml in loading buffer (137 mM NaCl, 0.8 mM $MgCl_2$, 5.4 mM KCl, 5.6 mM glucose, 10 mM Hepes, pH 7.4) and treated with 5 mM di-isopropyl fluorophosphate for 30 min at room temperature. The cells were washed once, suspended at 10^8 cells/ml in the same buffer supplemented with 1 mCi/ml [^{32}P]P_i (200 mCi/mmol), and incubated with intermittent mixing for 90 min at 30 °C. The loaded neutrophils were pelleted by centrifugation, and suspended at 5×10^5 cells/ml in the same buffer at 37 °C, supplemented with 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$. The inhibitors were added 3 min before addition of the stimulants for 5 min. The reaction was terminated by a 5-fold dilution with iced loading buffer and the mixture was centrifuged at 500 *g* for 5 min.

Isolation of membrane and cytosol fractions

Membrane and cytosol fractions were prepared as described previously [7]. 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, 1 mM ATP, 10 mM Pipes, pH 7.4) containing 1 mM phenylmethanesulphonyl fluoride and 100 μM leupeptin at 4 °C were sonicated for 3×10 s, resulting in about 95% cell breakage. Nuclei, granules and unbroken cells were removed by centrifugation (2 min, 15600 *g*) and the post-nuclear supernatant was made 5 mM in EDTA, 1 mM in Na_3VO_4 and 5 mM in NaF from 20–100-fold-concentrated stock solutions of the phosphatase inhibitors. The supernate was centrifuged in a Beckman airfuge (30 min, 134000 *g*) to obtain a cell membrane pellet and a cytosol supernatant. Membranes were suspended at 10^9 cell equivalents/ml in 0.34 M sucrose/half-strength relaxation buffer containing 1 mM dithiothreitol. Solubilized membrane and cytosol were stored at −70 °C.

Immunoprecipitation, electrophoresis and autoradiography

These were performed as described previously [19]. Membranes or cytosol were added to 100 μl of solubilization buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.5, 1% sodium deoxycholate, 1% Nonidet P-40). Then 2 μl of rabbit antiserum raised against recombinant p47 was added and the mixture was incubated on ice overnight. The extract was adjusted to a volume of 0.4 ml in solubilization buffer containing 30 μl of 50% (w/v) slurry of recombinant Protein G–Sepharose. The mixtures of the extracts and the Protein G–Sepharose were tumbled end-over-end for 1 h, and washed with 2×1 ml of solubilization buffer containing 20% (w/v) sucrose and 0.15 (w/v) BSA, and then with 2×1 ml of solubilization buffer containing 20% sucrose. The samples were boiled in SDS sample buffer, and electrophoresed on an SDS/10%-polyacrylamide gel. Gels were stained with Coomassie Blue, dried and used for autoradiography with intensifying screens at −70 °C. Dried gels were also counted for radioactivity to access the relative ^{32}P content of resolved bands with a Betascope blot analyser (Betagen Corp., Waltham, MA, U.S.A.).

Immunoblot analysis

Immunoblot detection of cytosolic NADPH oxidase components was performed as described [33]. Samples were solubilized in $2 \times$ sample buffer (12% SDS, 8 M urea, 250 mM Tris, 8 mM EDTA, 0.2 mM leupeptin, 2 mM phenylmethanesulphonyl fluoride, pH 6.9). The amount of protein in each sample was quantified by the Pierce BCA protein assay (Pierce Chemical Co., Rockford, IL, U.S.A.) with BSA standards. Cytosols or membranes were analysed by PAGE. The resolved proteins were electrophoretically transferred to nitrocellulose, which was stained with Fast Green to detect protein banding, and then blocked in 5% (w/v) non-fat dry milk in Tris-buffered saline (TBS; pH 7.4). The blots were incubated in TBS/1% gelatin containing goat antiserum to either p47 or p67 (a gift from Dr. T. L. Leto). Immunoblots were incubated with 1 μg/ml peroxidase-conjugated rabbit anti-goat serum (Biomakor, Rehovot, Israel) and developed with 4-chloro-1-naphthol and H_2O_2.

The relative changes of each oxidase component were quantified by using a densitometer (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). These measurements are adequate to determine the changes in each individual oxidase component under different conditions, but not for comparison between the various components.

Measurement of [^{3}H]AA release

Incorporation and release of [^{3}H]AA were performed as previously reported [29]. Neutrophils (10^8/ml) were incubated for 30 min at 37 °C in Ca^{2+}/Mg^{2+}-free PBS containing 1 μCi of [^{3}H]AA (100 mCi/mmol). Neutrophils were washed once with PBS containing 0.1% fatty-acid-free human serum albumin (HSA) 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) with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$, containing 0.1% HSA. The incorporation of [^{3}H]AA was 81 ± 7.6% of the total radioactivity added to the cells after incubation for 30 min. Neutrophils (10^7/ml) were incubated in 37 °C with the appropriate stimulus in the presence or absence of inhibitors for 2.5, 5, 10 and 15 min. The reaction was terminated by centrifugation at 4 °C, and samples of the supernatants were counted for radioactivity by liquid-scintillation (Packard 1900CA spectrometry).

Statistical analysis

The differences between means were analysed by Student's *t* test. The plots were drawn as least-squares regression lines and tested by analysis of variance.

RESULTS

The effect of PLA_2 inhibitors on superoxide generation in neutrophils is shown in Figure 1. 4-Bromophenacyl bromide (BPB) or quinacrine inhibited superoxide generation in neutrophils activated by either 50 ng/ml PMA or 1 mg/ml OZ in a dose-dependent manner. The effect of BPB on PLA_2 activity was studied simultaneously with its effect on superoxide generation. As presented in Figure 2, BPB inhibited [^{3}H]AA release in neutrophils activated by 50 ng/ml PMA or 1 mg/ml OZ. The inhibition of PLA_2 activity by BPB was parallel to the inhibition of superoxide generation shown in Figure 1. Quinacrine showed similar results (results not shown).

The inhibition of superoxide generation in the presence of PLA_2 inhibitors could be restored by addition of free AA. As shown in Figure 3, the inhibition of superoxide production by quinacrine in PMA-stimulated neutrophils could be reversed by addition of AA at a concentration of 10 μM, which by itself caused a limited production of superoxide. Addition of stearic,

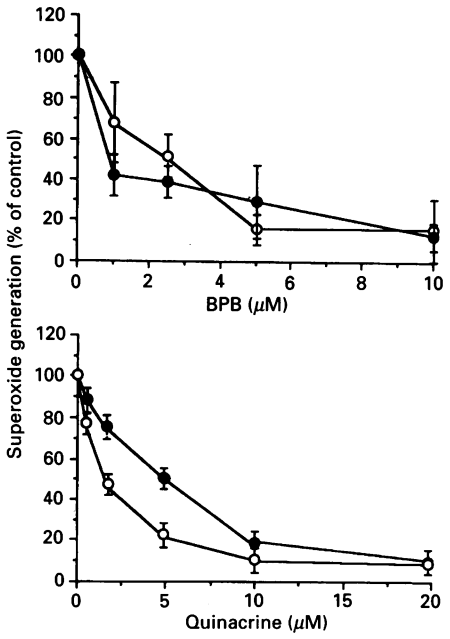

Figure 1 Effect of the PLA_2 inhibitors on superoxide generation in neutrophils stimulated by PMA or OZ

The results, expressed as percentages of control, are means ± S.E.M. from 7 experiments performed in duplicate. The neutrophils were incubated with the inhibitor for 5 min at 37 °C before addition of the stimulant. The maximal rates of superoxide production by neutrophils stimulated with 50 ng/ml PMA and with 1 mg/ml OZ in the absence of inhibitors were 25 ± 4.4 and 18.73 ± 5.4 nmol of O_2/10 min per 10^6 cells respectively. Symbols: ●, 50 ng/ml PMA; ○, 1 mg/ml OZ.

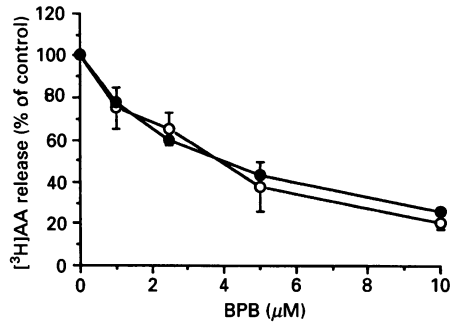

Figure 2 Effect of the PLA_2 inhibitor BPB on [^{3}H]AA release from neutrophils stimulated by PMA or OZ

The results, expressed as percentages of control, are means ± S.E.M. from 7 experiments performed in duplicate. The mean(± S.E.M.) [^{3}H]AA release in PMA-stimulated neutrophils in the absence of inhibitor was 5877 ± 595 c.p.m., and in OZ-stimulated neutrophils was 5704 ± 768 c.p.m. Symbols: ●, 50 ng/ml PMA; ○, 1 mg/ml OZ.

linoleic or oleic acids (up to 100 μM) did not cause a significant restoration of the activity. Similar results were obtained in OZ-stimulated neutrophils and with BPB as inhibitor (results not shown).

The effect of PLA_2 inhibitors on p47 phosphorylation and on translocation of the cytosolic factors to the membranes was studied. Figure 4 shows a representative immunoprecipitate of

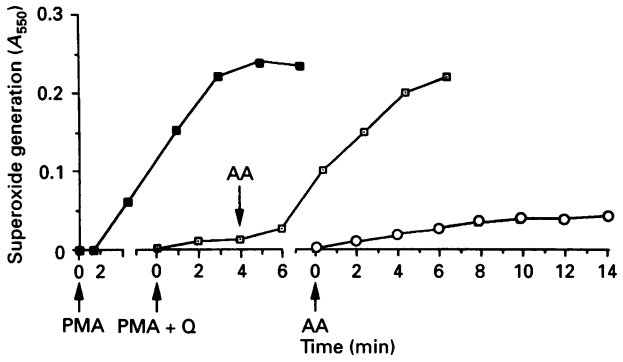

Figure 3 Effect of AA on the inhibition of superoxide generation by PLA_2 inhibitors in stimulated neutrophils

■, Generation of superoxide stimulated by 50 ng/ml PMA. ⊡, Superoxide production stimulated with 50 ng/ml PMA in the presence of 20 μM quinacrine (Q); the concentration of AA added was 10 μM. ○, Superoxide production stimulated by 10 μM AA. Results are shown from a representative experiment (4 other experiments show similar results).

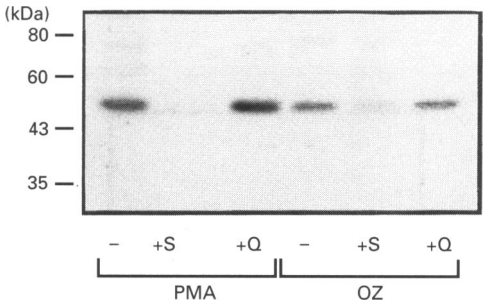

Figure 4 Effect of quinacrine on the phosphorylation of p47

Shown is an autoradiogram of phosphorylated p47 in membrane fractions. The PKC inhibitor staurosporine, which inhibits p47 phosphorylation, is shown as a control. The neutrophils were stimulated with 50 ng/ml PMA or 1 mg/ml OZ in the absence or presence of 20 μM quinacrine (Q) or 80 nM staurosporine (S).

Table 1 Effect of the PLA_2 inhibitor quinacrine on the phosphorylation of p47 in neutrophils stimulated by PMA or OZ

The degree of phosphorylation (c.p.m./10 h) of p47 immunoprecipitates from the membrane fractions is shown. The neutrophils were stimulated with 50 ng/ml PMA or 1 mg/ml OZ in the absence or presence of 20 μM quinacrine or 80 nM staurosporine. There are no significant differences between the absence or presence of quinacrine. There are significant differences between the absence or presence of staurosporine ($P < 0.001$). The results are means ± S.E.M. of 4 different experiments.

Addition	Stimulus	p47 phosphorylation
PMA	–	1400 ± 130
	Staurosporine (80 nM)	56 ± 15
	Quinacrine (20 μM)	1515 ± 100
OZ	–	1200 ± 100
	Staurosporine (80 nM)	150 ± 30
	Quinacrine (20 μM)	1150 ± 150

phosphorylated p47 in the membranes of neutrophils stimulated by PMA or OZ. Four other experiments showed similar results, and the means ± S.E.M. are summarized in Table 1. Quinacrine

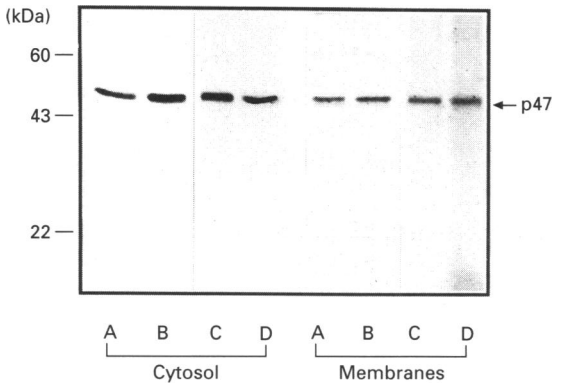

Figure 5 Effect of BPB on the phosphorylation of p47

Shown is a representative autoradiogram of phosphorylated p47 from the cytosol or membrane fractions. The neutrophils were stimulated with 50 ng/ml PMA in the absence (A) or presence (B) of 10 μM BPB and with 1 mg/ml OZ in the absence (C) or presence (D) of 10 μM BPB. Five other experiments showed similar results, and in all the experiments there were no differences in the amount of phosphorylation in p47 between the BPB-treated and untreated neutrophils.

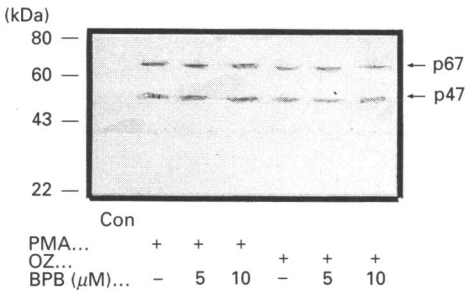

Figure 6 Representative SDS/PAGE immunoblot analysis of p47 and p67 in membrane fractions from neutrophils stimulated by 50 ng/ml PMA or 1 mg/ml OZ, in the presence or absence of BPB

Results are shown for resting cells (Con), PMA-stimulated cells in the absence (—) or in the presence of 5 μM BPB or 10 μM BPB, and OZ-stimulated cells in the absence (—) or in the presence of 5 μM BPB or 10 μM BPB: 1×10^7 cell equivalents of membranes was applied per lane.

(20 μM) did not cause any inhibition of the phosphorylation of p47. We used the PKC inhibitor staurosporine as a control which did inhibit the phosphorylation of p47. As shown in Figure 5, similar results were obtained by 10 μM BPB, which causes total inhibition of the oxidase activity, but did not affect the phosphorylation of p47 in the cytosol or the membranes. We then studied the effect of BPB on the translocation of the cytosolic factors p47 and p67 to neutrophil membranes by immunoblot analysis. We used 5 μM or 10 μM BPB, which respectively caused partial or total inhibition of superoxide production. As presented in the representative immunoblot (Figure 6) and the means ± S.E.M. of the densitometry from four experiments (Table 2), BPB did not inhibit the translocation of either p47 or p67 to membranes of neutrophils stimulated by PMA or OZ.

To explore further the involvement of AA in the oxidase activity, we examined simultaneously the time– and dose–

Table 2 Translocation of the cytosolic oxidase components p47 and p67 to the membranes of neutrophils stimulated by PMA or OZ in the absence or presence of BPB

The determinations by densitometry of the relative amounts of p47 and p67 in neutrophil membranes are averages from four experiments. Units are arbitrary units of density, with higher number representing a darker band on the immunoblot. The quantitative measurements are adequate to determine the relative changes of each individual oxidase component by the different conditions, but not for comparison between the two components, because of the different characteristics of the antibodies used. There are no significant differences in the results in the presence or absence of BPB.

	Oxidase protein	
Treatment	p47	p67
PMA	120 ± 9	101 ± 5
+5 μM BPB	120 ± 12	97 ± 7
+10 μM BPB	125 ± 6	110 ± 6
OZ	98 ± 5	92 ± 8
+5 μM BPB	93 ± 8	95 ± 6
+10 μM BPB	94 ± 7	94 ± 5

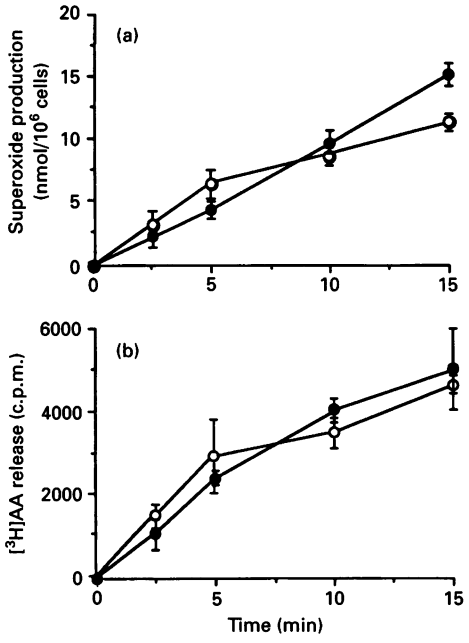

Figure 7 Time course for superoxide generation (a) and [^{3}H]AA release (b) in neutrophils stimulated by PMA or OZ

The results are means ± S.E.M. of three experiments performed in duplicate. Symbols: ●, 50 ng/ml PMA; ○, 1 mg/ml OZ.

responses of superoxide generation and [^{3}H]AA release in neutrophils activated by 50 ng/ml PMA or 1 mg/ml OZ. In these sets of experiments we took samples from the reaction mixture to analyse [^{3}H]AA release and assayed for cytochrome *c* reduction. As shown in Figure 7, the kinetics of [^{3}H]AA release occurred on a similar time-scale to the production of superoxide. In stimulus-dose–response studies, there is a high correlation between superoxide production and [^{3}H]AA release stimulated by PMA in the range 50–500 nM (Figure 8a) or by OZ in the range 0.001–1 mg/ml (Figure 8b).

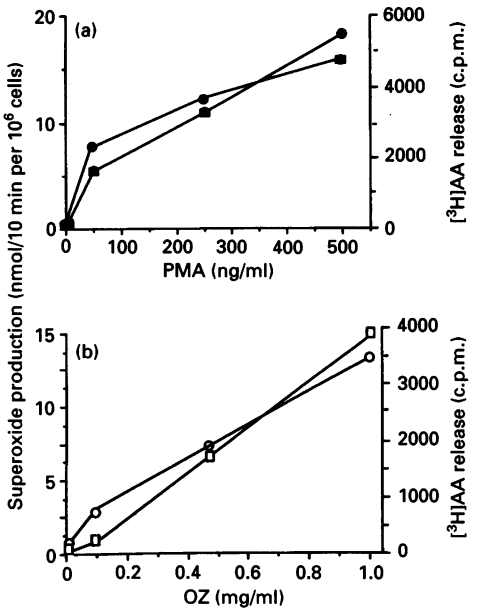

Figure 8 Stimulus-dose–response curves of superoxide production and [^{3}H]AA release in neutrophils measured simultaneously during 15 min

(**a**) PMA-stimulated neutrophils: ●, superoxide generation; ■, [^{3}H]AA release. (**b**) OZ-stimulated neutrophils: ○, superoxide generation; □, [^{3}H]AA release. The results are from a representative experiment performed in duplicate. Three other experiments showed similar results.

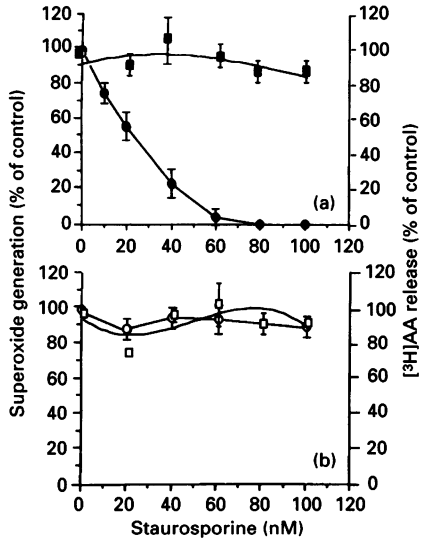

Figure 9 Effect of staurosporine on superoxide production and on [^{3}H]AA release from neutrophils stimulated by PMA or OZ during 15 min

The results, expressed as percentages of control, are means ± S.E.M. from seven experiments performed in duplicate. (**a**) PMA-stimulated neutrophils: ●, superoxide generation; ■, [^{3}H]AA release. (**b**) OZ-stimulated neutrophils: ○, superoxide generation; □, [^{3}H]AA release. The maximal rates of superoxide production by neutrophils stimulated with 50 ng/ml PMA or with 1 mg/ml OZ were 21.8 ± 1.5 or 19.4 ± 1.9 nmol of O_2/10 min per 10^6 cells respectively. The mean (± S.E.M.) [^{3}H]AA release in PMA-stimulated neutrophils was 4523 ± 271 c.p.m. and in OZ-stimulated neutrophils was 5487 ± 1178 c.p.m.

Since PKC is the major kinase responsible for the phosphorylation of the oxidase cytosolic factor p47 [18,19], we studied the effect of the PKC inhibitor staurosporine on PLA_2 activity. As shown in Figure 9(a), staurosporine in the range 20–100 nM caused a gradual inhibition of superoxide production, but did not affect the release of [^{3}H]AA induced by PMA. Staurosporine (20–100 nM) inhibited neither superoxide production nor release of [^{3}H]AA in neutrophils stimulated by OZ (Figure 9b). In other experiments (results not shown), a high concentration of staurosporine (1 μM) inhibited superoxide production stimulated by OZ or PMA (80 % and 96 % inhibition respectively) and [^{3}H]AA release (66 % and 82 % inhibition respectively).

DISCUSSION

The present study shows that with either PMA or OZ as the stimulus, both activation of the oxidase and the release of [^{3}H]AA were inhibited simultaneously by PLA_2 inhibitors, in a dose-dependent manner (Figures 1 and 2). The presence of PLA_2 inhibitors in OZ- or PMA-stimulated neutrophils did not affect the phosphorylation of p47 or the translocation of p47 and p67 to the membranes. In spite of maximal phosphorylation and translocation of these two cytosolic factors to the membranes, there was no oxidase activity in the presence of PLA_2 inhibitors; only when free AA was added the activity was regained. The effect of AA in restoring the oxidase activity was also shown [25] in PMA-stimulated neutrophil-derived cytoplasts in the presence of various PLA_2 inhibitors. These observations indicate that AA has a putative role in the activation of the oxidase and that phosphorylation and translocation, although necessary, are not sufficient for oxidase activity. In accordance with our findings, Lu et al. [34] reported that increased phosphorylation of p47 is not sufficient for superoxide production in cells treated with okadaic acid.

The dose-dependent and time-dependent patterns of superoxide production occurred on a similar time scale to the release of [^{3}H]AA (Figures 7 and 8) induced by OZ or PMA, suggesting that PLA_2 activity is required for both induction of NADPH oxidase activity and its maintenance over time.

In our recent study [35] we have suggested that AA may act as a positive modulator for the NADPH oxidase. This suggestion is based on the results demonstrating that very low concentrations of AA (10 μM) affect the assembled form of the oxidase in isolated cytosplasmic membranes and endosomes, by decreasing the K_m and increasing the $V_{max.}$ The effect of AA on the kinetic parameters of the enzyme is most readily explained by interaction of AA with the assembled oxidase or by affecting its close environment.

On the other hand, although PLA_2 is needed for oxidase activation, its activity alone is insufficient to activate the oxidase, since oxidase activity is inhibited in the presence of the PKC inhibitor staurosporine. It has been suggested [36] that NADPH oxidase may be activated by a PLA_2-mediated process without involvement of p47 phosphorylation. In addition, it has been shown recently [37] that the translocation of the cytosolic factors is independent of PKC activity and is linked to transmembrane signalling involving Ca^{2+} transients and production of lipidic second messengers. In contrast, we have recently shown [38] that in OZ-stimulated neutrophils in the presence of staurosporine there is a residual phosphorylation of p47 (also shown in Figure 4 and Table 1 in the present study), which is probably responsible for the oxidase activity obtained in these conditions. At a higher concentration of staurosporine both phosphorylation of p47 and superoxide generation induced by OZ were inhibited. Thus PLA_2

activity is a co-requirement in addition to the necessity for phosphorylation and translocation of the cytosolic factors.

The location of PLA_2 activity in the signal-transduction pathway leading to oxidase activation has not as yet been defined. PLA_2 activity may be involved in the activation of the oxidase at multiple sites: directly on PKC, downstream of PKC and mediated by PKC activity or at a site independent of PKC activity. We show here that phosphorylated p47 was found in cytosol and membranes of neutrophils induced by either PMA or OZ in the presence of PLA_2 inhibitors (Figures 4 and 5). These findings indicate that activation of PKC is not mediated by PLA_2 activity and rules out the possibility of PLA_2 having an effect on PKC during events leading to activation of the oxidase. Our results are in accordance with those of Henderson et al. [39], who have reported that AA does not activate protein phosphorylation. Although a number of publications have shown activation of PKC by AA [40–42], a major obstacle to assignment of its physiological role in this process is the very high concentration needed for enzyme activation (100–600 μM AA) [43,44]. It has been reported that these high concentrations of AA activate the α-isoform of PKC [43,44]. The γ-type enzyme, which is activated by very low, possibly physiological, concentrations of AA (10 μM), is found only in the central nervous system [45]. The predominant isoform present in human neutrophils, PKC-β [46], is activated by PMA or increased intracellular [Ca^{2+}] [47], is sensitive to conventional PKC inhibitors [48]), phosphorylates p47, and is involved in activation of the oxidase [18,19]. However, this PKC isoform responds very poorly to AA and other fatty acids [47,49]. Thus these findings are in agreement with our results demonstrating that, in events leading to oxidase activation, AA does not mediate PKC activity.

In accordance with work by others [35,50,51], our study demonstrates that [^{3}H]AA release stimulated by PMA or OZ is not affected by staurosporine in a range specific for PKC inhibition [52] which does inhibit PMA-stimulated oxidase activity (Figure 8). These results suggest that PKC does not mediate the activation of PLA_2 in the events leading to stimulation of the oxidase in human neutrophils. In contrast, several studies suggest that PKC activation mediates the release of [^{3}H]AA by PLA_2 in various cell types [53–55], since down-regulation of PKC by various PKC inhibitors or prolonged incubation with PMA prevented [^{3}H]AA release. The discrepancy between the results may be due to differences in the cell types and conditions studied.

The nature of PLA_2 activated in neutrophils during stimulation of the oxidase is not yet clear. Since PLA_2 activity is not affected by the PKC inhibitor staurosporine in a range specific for PKC inhibition (Figure 9), we may suggest that in these conditions the cell-associated type 14 kDa PLA_2, found in neutrophils [56], shown to be activated by PMA [57] and which does not undergo phosphorylation [58], is responsible for the release of [^{3}H]AA during the stimulation of the oxidase. However, the cytosolic 85 kDa PLA_2 (cPLA_2) [59,60], which accounts for approx. 50% of the total PLA_2 in rabbit neutrophils [61], may also participate. cPLA_2 undergoes phosphorylation on serine residues [62] mediated by MAP (mitogen-activated protein) kinase [63]. The phosphorylation and activation of cPLA_2 is inhibited by 1 μM stauroporine [62], in accordance with the inhibition of [^{3}H]AA release by the same concentration of staurosporine obtained in our study.

In conclusion, the results presented in this study show that oxidase activity was inhibited in the presence of PLA_2 inhibitors, in spite of the p47 phosphorylation and the translocation of p47 and p67 to the membranes, suggesting a putative role for PLA_2 activity in the activation of NADPH oxidase. The types and mechanisms of activation of PLA_2 are unclear, and it is not clear whether the stimulation of PLA_2 activity by OZ or PMA is through the same mechanisms or through different ones.

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REFERENCES

1 Babior, B. M. (1984) J. Clin. Invest. **73**, 599–601
2 Korchak, H. M., Vienne, K., Rutherford, L. E. and Weissman, G. (1984) Fed. Proc. Fed. Am. Soc. Exp. Biol. **43**, 2749–2754
3 Gabig, T. G. and Lefker, B. A. (1984) Biochem. Biophys. Res. Commun. **118**, 430–436
4 Cross, A. R., Jones, O. T. G., Harper, A. M. and Segal, A. W. (1981) Biochem. J. **194**, 599–606
5 Rotrosen, D., Yeung, C. L., Leto, T. L., Malech, H. L., and Kowmg, C. H. (1992) Science **256**, 1459–1462
6 Segal, A. W., West, I., Wientjes, F., Nugent, J. H. A., Chavan, A. J., Haley, B., Garcia, R. C., Rosen, H. and Scrace, G. (1992) Biochem. J. **284**, 781–788
7 Nunoi, H., Rotrosen, D., Gallin, J. I. and Malech, H. L. (1988) Science **242**, 1298–1303
8 Volpp, B. D., Nauseef, W. M., Donelson, J. E., Moser, D. R. and Clark, R. A. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 7195–7199
9 Lomax, K. J., Leto, T. L., Nunoi, H., Gallin, J. I. and Malech, H. L. (1989) Science **245**, 409–412
10 Leto, T. L., Lomax, K. J., Volp, B. D., Nunoi, H., Sechler, J. M. G., Nauseef, W. M., Clark, R. A., Gallin, J. I. and Malech, H. L. (1990) Science **248**, 727–730
11 Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C. G. and Segal, A. W. (1991) Nature (London) **353**, 668–670
12 Abo, A. and Pick, E. (1991) J. Biol. Chem. **266**, 23577–23585
13 Babior, B. (1988) Arch. Biochem. Biophys. **264**, 361–367
14 Garcia, R. C. and Segal, A. (1988) Biochem. J. **252**, 901–904
15 Hayakawa, T., Suzuki, K., Suzuki, S., Andrews, P. C. and Babior, B. M. (1986) J. Biol. Chem. **261**, 9109–9115
16 Heyworth, P. G., Shrimpton, C. F. and Segal, A. W. (1989) Biochem. J. **260**, 243–248
17 Reibman, J., Korchak, H. M., Vosshall, L. B., Haines, K. A., Rich, A. M. and Weissmann, G. (1988) J. Biol. Chem. **263**, 6322–6328
18 Okamura, N., Curnutte, J. T., Roberts, R. L. and Babior, B. M. (1988) J. Biol. Chem. **263**, 6777–6782
19 Rotrosen, D. and Leto, T. L. (1990) J. Biol. Chem. **265**, 19910–19915
20 McPhail, L. C., Clayton, C. C. and Snyderman, R. (1984) J. Biol. Chem. **259**, 5768–5775
21 Rossi, F. (1986) Biochim. Biophys. Acta **853**, 65–89
22 Morel, F., Doussiere, J. and Vignais, P. V., (1991) Eur. J. Biochem. **201**, 523–546
23 Segal, A. W., Heyworth, P. G., Cockcroft, S. and Barrowman, M. M. (1985) Nature (London) **316**, 547–549
24 Kramer, L. M., Verhoeven, A. J., van der Bend, R. L., Weening, R. S. and Roos, D. (1988) J. Biol. Chem. **263**, 2352–2357
25 Henderson, L. M., Chappell, J. B. and Jones, O. T. G. (1989) Biochem. J. **264**, 249–255
26 Nakashima, S., Suganuma, A., Sato, M., Tohmatsu, T. and Nozawa, Y. (1989) J. Immunol. **143**, 1295–1302
27 Grinstein, S. and Furuya, W. (1988) J. Biol. Chem. **263**, 1779–1783
28 Kata, A., Ida, E., Tominaga, M. and Onoue, K. (1987) J. Immunol. **138**, 4353–4359
29 Tarsi-Tsuk, D. and Levy, R. (1990) J. Immunol. **144**, 2665–2670
30 Nuid, R. E., Twomey, B. and Dale, M. (1988) FEBS Lett. **234**, 235–239
31 Bromberg, Y. and Pick, E. (1983) Cell. Immunol. **70**, 240–252
32 Smolen, J. E. and Weissman, G. (1990) Biochem. Pharmacol. **29**, 533–538
33 Levy, R., Rotrosen, D., Nagauker, O., Leto, T. L., and Malech, H. L. (1990) J. Immunol. **145**, 2595–2601
34 Lu, D. J., Takai, A., Leto, T. L. and Grinstein, S. (1992) Am. J. Physiol. **262**, C39–C49
35 Rubinek, T. and Levy, R. (1993) Biochim. Biophys. Acta **1176**, 51–58
36 Badway, J. A., Robinson, J. M., Heyworth, P. G. and Curnutte, J. T. (1989) J. Biol. Chem. **264**, 20676–20682
37 Dusi, S., Bianca, V. D., Grzeskowiak, M. and Rossi, F. (1993) Biochem. J. **290**, 173–178
38 Levy, R. and Dana, R. (1992) Eur. J. Clin Invest. **22**, 40A
39 Henderson, L. M., Moule, S. K. and Chappell, J. B. (1993) Eur. J. Biochem. **211**, 157–162

40 MacEwan, D. J., Mitchell, R., Thomson, F. J. and Johnson, M. S. (1991) Biochim. Biophys. Acta **1094**, 346–354
41 Khan, W., Touny, S. E. and Hannun, Y. A. (1991) FEBS Lett. **292**, 98–102
42 Abramson, S. B., Leszczynska-Piziak, J. and Weissmann, G. (1991) J. Immunol. **147**, 231–236
43 McPhail, L. C., Clayton, C. C. and Snyderman, R. (1984) Science **224**, 622–625
44 Sekiguchi, K., Tsukuda, M., Ogita, K., Kikkawa, O. and Nishizuka, Y. (1987) Biochem. Biophys. Res. Commun. **145**, 797–802
45 Nishizuka, Y. (1988) Nature (London) **334**, 661–665
46 Majumdar, S., Rossi, M. W., Fujiki, T., Phillips, W. A., Disa, S., Queen, C. F., Johnston, R. B., Jr., Rosen, O. M., Corkey, B. E. and Korchak, H. M. (1991) J. Biol. Chem. **266**, 9285–9294
47 Naor, Z., Shearman, M. S., Kishimoto, A. and Nishizuka, Y. (1988) Mol. Endocrinol. **2**, 1043–1048
48 Huang, K. P. (1989) Trends Neurosci. **12**, 425–432
49 Naor, Z. (1991) Mol. Cell. Endocrinol. **80**, C181–C186
50 Svensson, U., Holst, E. and Sundler, R. (1991) Eur. J. Biochem. **200**, 699–705
51 Muler, S. and Nigam, S. (1992) Eur. J. Pharmacol. **218**, 251–258
52 Combadiere, C., Hakim, J., Giroud, J. P. and Perianin, A. (1990) Biochem. Biophys. Res. Commun. **168**, 65–70
53 Zor, U., Her, E., Harell, T., Fischer, G., Naor, Z., Braquet, P. and Ferber, E. (1991) Biochim. Biophys. Acta **1091**, 385–392
54 Gilliam, E. B., Schulam, P. G., Whelan, J. P., Rosenblatt, H. M. and Shearer, W. T. (1991) Cell. Immunol. **136**, 41–53
55 Parker, J., Daniel, L. W., and Waite, M. (1987) J. Biol. Chem. **262**, 5385–5393
56 Balsinde, J., Diez, E., Schuller, A. and Mollinedo, F. (1988) J. Biol. Chem. **263**, 1929–1936
57 Pernas, P., Masliah, J., Oliver, J. L., Salvat, C., Rybkine, T. and Bereziat, G. (1991) Biochem. Biophys. Res. Commun. **178**, 1298–1305
58 Mayer, R. J. and Marshal, L. A. (1993) FASEB J. **7**, 339–348
59 Clark, J. D., Lin, L. L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N. and Knopf, J. L. (1991) Cell **65**, 1043–1052
60 Sharp, J. D., White, D. L., Chiou, X. G., Goodson, T., Gamboa, G. C., McClure, D., Burgett, S., Hoskins, J., Skaturd, P. L., Sportsman, J. R., Becker, J. W., Kang, L. H., Roberts, E. F. and Kramer, R. M. (1991) J. Biol. Chem. **266**, 14850–14853
61 Fujimori, Y., Murakami, M., Kim, D. K., Hara, S., Takayama, K., Kudo, I. and Inoue, K. (1992) J. Biochem. (Tokyo) **111**, 54–60
62 Lin, L. L., Lin, A. Y. and Knopf, L. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 6147–6151
63 Lin, L. L., Wartma, M., Lin, A. Y., Knopf, L., Seth, A. and Davis, R. J. (1993) Cell **72**, 269–278

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