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Superoxide generation is inhibited by phospholipase A_2 inhibitors

Role for phospholipase A_2 in the activation of the NADPH oxidase

Lydia M. HENDERSON, J. Brian CHAPPELL and Owen T. G. JONES
Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K.

The stimulation of $O_2^{\cdot-}$ generation by phorbol 12-myristate 13-acetate (PMA) in human neutrophil-derived cytoplasts was inhibited by a variety of phospholipase A_2 inhibitors in a concentration-dependent manner. Inhibition was found to be independent of the order of addition of the inhibitor and PMA. The most potent inhibitor, RO 31-4639, inhibited $O_2^{\cdot-}$ generation with an IC_{50} value (concentration causing 50% inhibition) of 1.5 μM. The addition of either arachidonic acid or SDS, in the presence of the inhibitors, was able to restore $O_2^{\cdot-}$ generation. The results suggest that arachidonic acid, released by phospholipase A_2, is necessary for both the activation and the maintenance of $O_2^{\cdot-}$ generation by the NADPH oxidase.

INTRODUCTION

The NADPH oxidase is located in the plasma membrane of neutrophils and performs the single electron reduction of O_2 to superoxide ($O_2^{\cdot-}$), using NADPH as an electron donor [1]. The contribution of $O_2^{\cdot-}$ and its dismutation products to the bactericidal capacity of these cells is illustrated by the susceptibility to infection shown by patients with chronic granulomatous disease (CGD) [2]. The neutrophils from CGD patients are unable to generate $O_2^{\cdot-}$ as they lack a functional oxidase [3–6].

The oxidase is normally inactive, but it can be stimulated by a range of particulate and soluble stimuli. The signal transduction pathway leading to the activation of the oxidase is considered to consist of a receptor, a guanine-nucleotide-binding (G)-protein-activated, phosphatidylinositol-specific phospholipase C which generates InsP_3 and diacylglycerol. The InsP_3 induces a rise in the intracellular free Ca^{2+}, which in association with diacylglycerol results in the activation of protein kinase C (PKC) [1,7,8]. The activated PKC may directly activate the oxidase. The phosphorylation of several proteins has been reported by a number of authors [9–11]. The phosphorylation of a 47 kDa protein occurs in normal cells but has been reported to be absent in a patient with an autosomal recessive CGD [12]. This suggests that a protein kinase may be involved in the activation of the oxidase. The involvement of PKC in the activation of the oxidase is supported by the fact that only those phorbol esters which can stimulate the activity of PKC are activators of the oxidase [13].

There have been reports suggesting that phospholipase A_2 (PLA_2) is involved in the activation of a number of systems including the cardiac G-protein-activated K^+ channel [14,15] and transducin stimulation in the rod outer segments of bovine retina membrane [16]. An increase in PLA_2 activity [17] and a release of arachidonic acid (AA) has been reported to occur following the stimulation of the oxidase, by a range of stimuli, in both neutrophils and macrophages [18–20]. Both quinacrine (mepacrine) and bromophenacyl bromide (BPB), inhibitors of PLA_2 [21], have previously been reported to inhibit the activation of the oxidase [18–20]. In this paper we demonstrate that phorbol 12-myristate 13-acetate (PMA)-stimulated $O_2^{\cdot-}$ generation in cytoplasts is inhibited by a number of recently developed PLA_2 inhibitors, as well as by both quinacrine and BPB. The activity of the oxidase can be restored by the addition of either AA or SDS. The results support a direct role for PLA_2 in both the stimulation and the maintenance of NADPH oxidase activity.

MATERIALS AND METHODS

Compounds I and II were a gift from Dr. R. Melarange (Beecham Pharmaceuticals, Harlow, Essex, U.K.) and compounds RO 31-4639, RO 31-4493 and RO 31-4373 were gifts from Dr. P. D. Davis (Roche Products Limited, Welwyn Garden City, Herts., U.K.). The structures of these compounds are shown in Table 1. Stock solutions of the inhibitors were prepared in aqueous medium (Compounds I and II), in dimethyl sulphoxide (RO 31-4693) or in ethanol (RO 31-4493 and RO 31-4373). The sodium salt of AA was obtained from Sigma, Poole, Dorset, U.K. A fresh stock solution was prepared in 33% (v/v) ethanol.

Preparation of the neutrophils and cytoplasts

Neutrophils and cytoplasts were prepared from human buffy coats obtained from the South West Regional Blood Transfusion Centre, Southmead Hospital, Bristol, U.K., as previously described [22,23]. The cytoplasts were routinely resuspended in 150 mM-NaCl/1 mM-KCl/5 mM-Hepes/Tris/5.5 mM-glucose, pH 7.4 (Na^+ medium).

Determination of $O_2^{\cdot-}$ generation

The PMA-stimulated generation of $O_2^{\cdot-}$ was determined by the diphenylene iodonium (DPI)-sensitive re-

Abbreviations used: AA, arachidonic acid; BPB, 4-bromophenacyl bromide; CGD, chronic granulomatous disease; DCCD, dicyclohexyl carbodi-imide; G-protein, guanine-nucleotide-binding protein; PKC, protein kinase C; PLA_2 phospholipase A_2; PMA, phorbol 12-myristate 13-acetate.

duction of cytochrome *c*, as previously described [22]. All assays were performed in Na^+ medium at 37 °C.

RESULTS

Cytoplasts are enucleated cells which lack the internal organelles of the parent neutrophils [22,23]. They have been shown to retain the functional characteristics of neutrophils, including the capacity for $O_2^{\cdot-}$ generation. Due to their ease of handling and to retain continuity with earlier work, cytoplasts were used in this study.

Compounds I and II (Table 1) are both reported to inhibit the release of pre-incorporated [^{14}C]AA following stimulation of neutrophils by zymosan (R. Melarange, personal communication). The design of compounds RO 31-4639, RO 31-4493 and RO 31-4403 was based on the structure of the catalytic site of pig pancreatic PLA_2. All three compounds inhibited the activity of the enzyme, to varying extents (P. D. Davis, personal communication).

The effect of PLA_2 inhibitors on the stimulation of $O_2^{\cdot-}$ generation

In the presence of each of the inhibitors, the PMA-stimulated $O_2^{\cdot-}$ generation was inhibited in a concentration-dependent manner (Fig. 1). The IC_{50} values (concentrations giving half-maximal inhibition) of $O_2^{\cdot-}$ generation are given in Table 1. The inhibition by all of the compounds did not require a period of incubation with the cytoplasts prior to the addition of PMA. The IC_{50} values for the inhibition of the PMA-stimulated $O_2^{\cdot-}$ generation by the compounds RO 31-4639, RO 31-4493 and RO 31-4373 (Table 1) are lower, but similar to the values obtained for the inhibition by these compounds of the pig pancreatic PLA_2 (10, 25 and > 1000 μM respectively; P. D. Davis, personal communication). Both quinacrine and BPB inhibit [^{14}C]AA release at concentrations comparable with those required to inhibit $O_2^{\cdot-}$ generation [18,20].

Quinacrine has previously been reported to inhibit $O_2^{\cdot-}$ generation by the oxidase solubilized from pig neutrophils [24]. However, the IC_{50} value of 750 μM is far greater than that obtained for the inhibition of the activity in whole cells. As the oxidase is considered to contain a flavoprotein [1,7,8], the inhibition of the solubilized oxidase by high concentrations of quinacrine may be due, in part, to the ability of quinacrine to replace the FAD [25].

As the wide range of inhibitors of PLA_2 used in this study were found to inhibit the activity of the oxidase, this suggests that the activity of PLA_2 is important in the activation of the oxidase by PMA.

Inhibition of $O_2^{\cdot-}$ generation following stimulation

PMA-stimulated $O_2^{\cdot-}$ generation was inhibited, in a concentration-dependent manner, by the addition of BPB (Fig. 2). The corresponding addition of all the other inhibitors resulted in the inhibition of the generation of $O_2^{\cdot-}$ (L. M. Henderson, J. B. Chappell & O. T. G. Jones, unpublished work). The ability of these compounds to inhibit $O_2^{\cdot-}$ generation, after the activity of the oxidase has been stimulated, suggests that continuous PLA_2 activity is necessary to maintain the oxidase in the active state.

The stimulation of the oxidase by the chemotactic peptide fMetLeuPhe is considered to follow the binding of the peptide to a receptor and the eventual stimulation

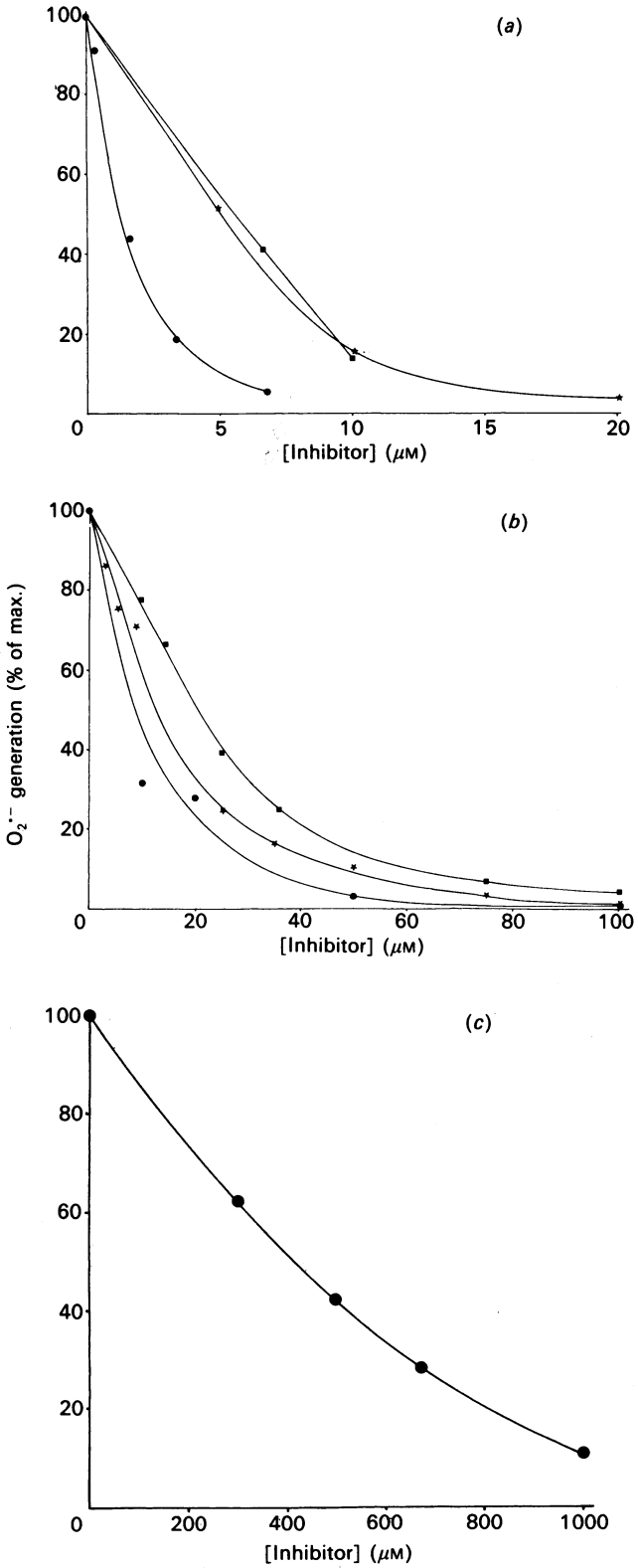
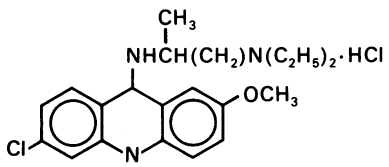
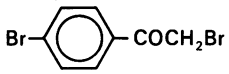
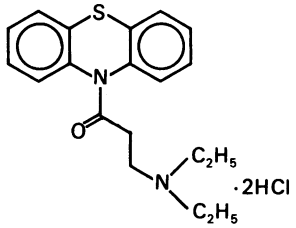
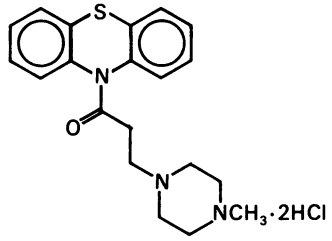
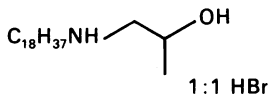
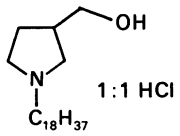
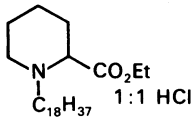


Fig. 1. Inhibition of $O_2^{\cdot-}$ generation by PLA_2 inhibitors

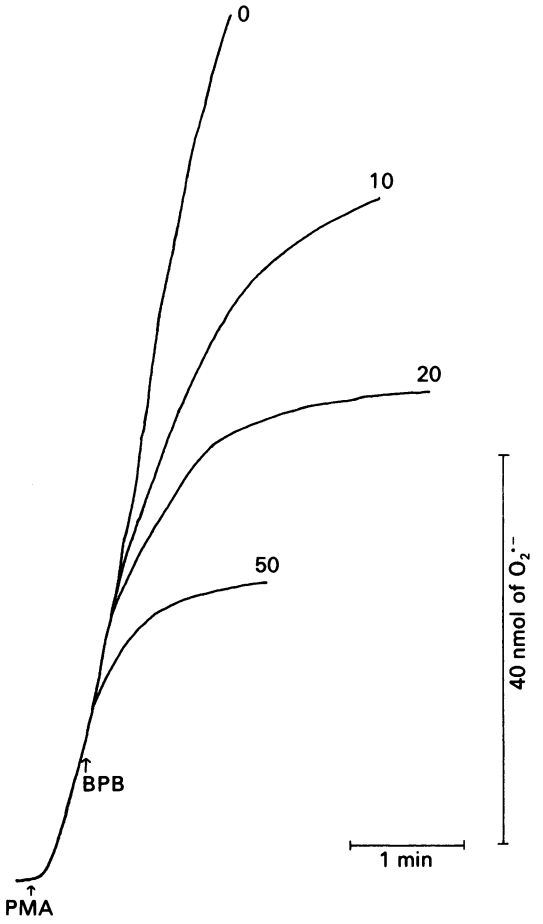
The compounds at a range of final concentrations were added, as indicated below, prior to the addition of 33 nM-PMA, and the generation of $O_2^{\cdot-}$ was recorded as described in the Materials and methods section. $O_2^{\cdot-}$-generation is expressed as a percentage of the untreated control cytoplasts to permit comparisons between different compounds. (*a*) ●, RO 31-4639; ■, RO 31-4493; ★, quinacrine. (*b*) ●, BPB; ★, Compound I; ■, Compound II. (*c*) ●, RO 31-4373.

Table 1. IC_{50} values for the inhibition of PMA-stimulated $O_2^{\cdot-}$ generation

The IC_{50} values for the compounds were obtained using the data presented in Figs. 1(*a*), 1(*b*) and 1(*c*).

Compound	Structure	IC_{50} (μM)
Quinacrine hydrochloride		4.4
p-Bromophenacyl bromide		8.0
Beecham Compound I		16.0
Beecham Compound II		22.0
Roche RO 31-4639		1.5
Roche RO 31-4493		5.8
Roche RO 31-4373		420.0

of PKC. RO 31-4639, BPB and quinacrine were all capable of inhibiting the activity of the oxidase which had been stimulated by fMetLeuPhe (Fig. 3). Inhibition of both the fMetLeuPhe-stimulated and the PMA-stimulated $O_2^{\cdot-}$ generation required similar concentrations of the inhibitors. Dicyclohexyl carbodi-imide (DCCD) has also been reported to stimulate the activity of the oxidase in bovine and guinea pig neutrophils [26]. The mechanism by which the oxidase is stimulated by DCCD is at present unknown. However, the stimulation of the oxi-



dase by DCCD is also inhibited by the subsequent addition of RO 31-4639 and quinacrine (L. M. Henderson, J. B. Chappell & O. T. G. Jones, unpublished work). Therefore the requirement for the continuous presence of PLA_2 activity is not restricted to the PMA-activated oxidase.

Restoration of $O_2^{\cdot-}$ generation by the addition of AA or SDS in the presence of the PLA_2 inhibitors

AA has been reported to stimulate $O_2^{\cdot-}$ generation in neutrophils [27–29]. This stimulation is independent of both the lipoxygenase and cyclo-oxygenase pathways [18,19,30]. Therefore it is probably due to AA itself. The phospholipids of the neutrophil plasma membrane are rich in AA, esterified to the *sn*-2 position of phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine [31,32]. The observation that there is a release of [^{14}C]AA in association with the stimulation of the oxidase [18–20] suggests that release of AA by PLA_2 may be required for the stimulation and maintenance of $O_2^{\cdot-}$ generation by the oxidase.

At concentrations greater than the IC_{50} value for both Compound II and RO 31-4639, the PMA-stimulated

Fig. 2. Inhibition of PMA-stimulated $O_2^{\cdot-}$ generation by BPB

The generation of $O_2^{\cdot-}$ by 1×10^7 cytoplasts was stimulated by the addition of 33 nM-PMA and recorded in a Na^+ medium at 37 °C, as described in the Materials and methods section. BPB was added at the final concentrations (μM) indicated in the Figure.

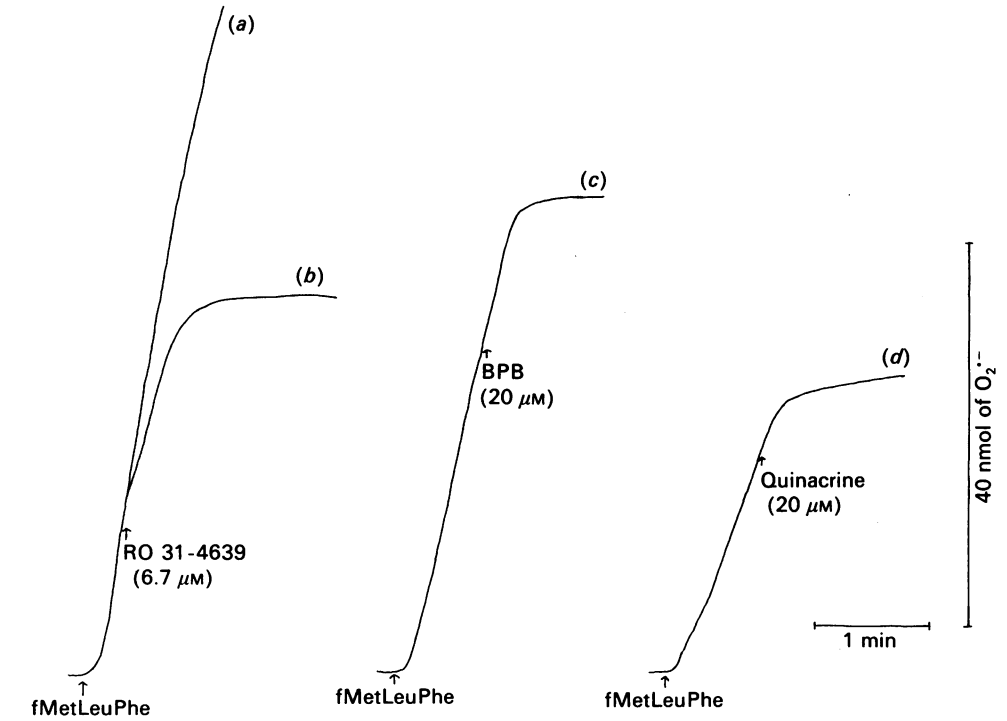


Fig. 3. Inhibition of fMetLeuPhe-stimulated $O_2^{\cdot-}$ generation

The generation of $O_2^{\cdot-}$ stimulated by the addition of 5 μM-fMetLeuPhe was recorded as described in the Materials and methods section. The final concentrations of the inhibitors were (*b*) 6.7 μM-RO 31-4639, (*c*) 20 μM-BPB and (*d*) and 20 μM-quinacrine. The cytoplasts in (*a*) were untreated.

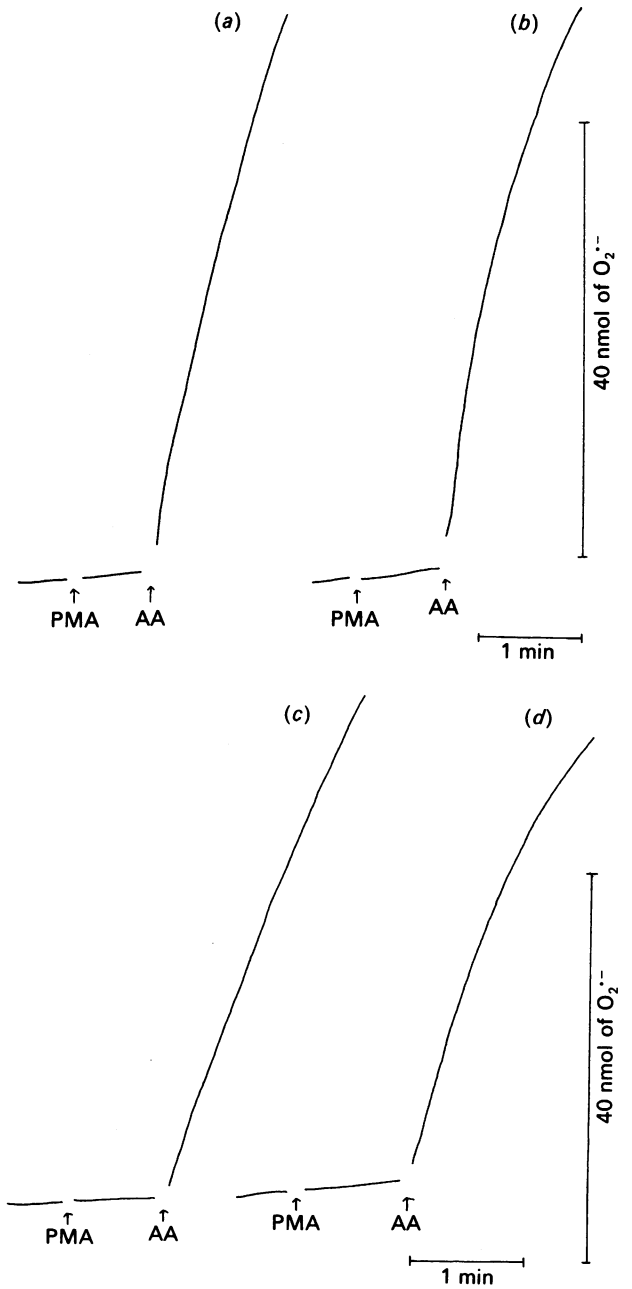


Fig. 4. Restoration of $O_2^{\cdot-}$ generation by the addition of AA to PMA-stimulated cytoplasts and neutrophils

The generation of $O_2^{\cdot-}$ was recorded as described in the Materials and methods section. PMA and AA were added, where indicated, at final concentrations of 33 nM and 50 μM respectively. The cytoplasts (*a*, *b*) and neutrophils (*c*, *d*) were treated with either 5 μM-RO 31-4639 (*a*, *c*) or 200 μM-compound II (*b*, *d*) prior to the addition of the PMA.

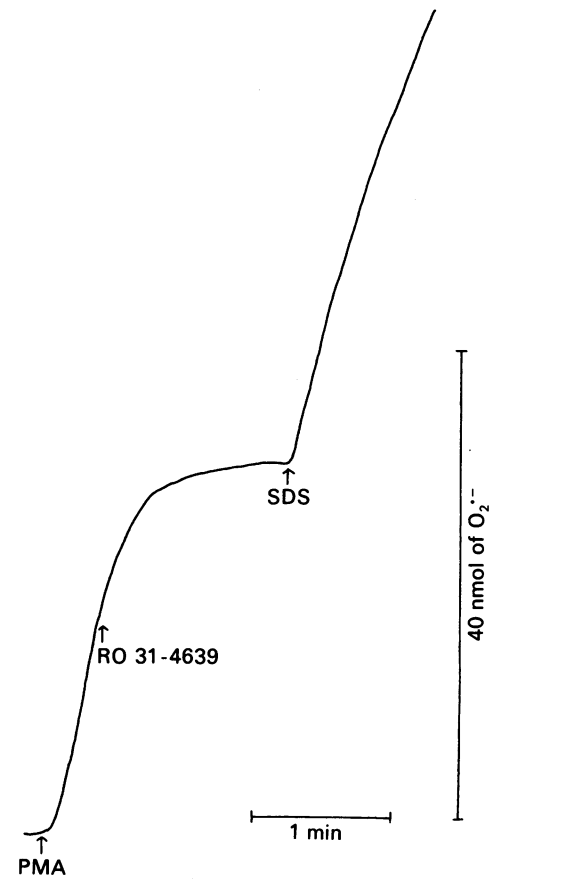


Fig. 5. Restoration of $O_2^{\cdot-}$ generation by the addition of SDS

PMA, RO 31-4639 and SDS were added at final concentrations of 33 nM, 6.7 μM and 50 μM respectively where indicated in the Figure. The generation of $O_2^{\cdot-}$ was recorded as described in the Materials and methods section.

$O_2^{\cdot-}$ generation by cytoplasts was totally inhibited (Figs. 4*a* and 4*b*). Both compounds also inhibited the PMA-stimulated $O_2^{\cdot-}$ generation by neutrophils (Figs. 4*c* and 4*d*). The neutrophils were those used to prepare the cytoplasts in Figs. 4(*a*) and 4(*b*), and therefore the inhibition of the $O_2^{\cdot-}$ generation by the PLA_2 inhibitors was not the result of the preparation used.

In the presence of either inhibitor (compound II or RO 31-4639), the subsequent addition of 50 μM-AA, to both cytoplasts and neutrophils, resulted in $O_2^{\cdot-}$ generation with an initial rate of 90% of the PMA control rate (results not shown), and which was sustained (Fig. 4). The addition of either the detergent SDS (Fig. 5) or AA (not shown), subsequent to the addition of PMA and RO 31-4639, also resulted in the restoration of $O_2^{\cdot-}$ generation by cytoplasts. Similar results were obtained when the PMA-stimulated $O_2^{\cdot-}$ generation was inhibited by each of the other compounds tested. These observations suggest that the requirement for the continuous presence of PLA_2 activity may be necessary to maintain a supply of AA, and that AA is required both to activate the oxidase and to maintain it in the active state.

DISCUSSION

PLA_2 activity has been found in all cell types so far investigated, including neutrophils and alveolar macrophages [33]. Quinacrine hydrochloride and BPB, both inhibitors of PLA_2 activity, have previously been reported to inhibit the $O_2^{\cdot-}$ generation stimulated by a wide range of stimuli [18–20]. Using these and some recently developed inhibitors of PLA_2, we have shown that the activity of this enzyme is necessary for the activation and maintenance of $O_2^{\cdot-}$ generation by the NADPH oxidase. The ability of AA to overcome the inhibitory effects of all the compounds tested strongly suggests that PLA_2 ac-

tivity may be required to release AA, which in turn appears to be required to maintain an active oxidase.

It has previously been shown that both AA and linoleate stimulated $O_2^{\cdot-}$ generation by whole neutrophils, and that the activity was terminated following the addition of bovine serum albumin [28]. The subsequent addition of excess AA was able to restore the oxidase activity. As bovine serum albumin binds free fatty acids, these observations would appear to support our proposal that the continuous presence of AA is required to maintain the activity of the oxidase.

The activation of the oxidase has recently been described in a cell-free system consisting of a combination of both membrane and cytosolic fractions [30,34,35]. In this system, the activity of the oxidase is reported to be stimulated by the addition of AA, *cis*-unsaturated fatty acids or SDS. There appears to be a high degree of specificity as a number of other detergents are unable to stimulate the oxidase in this system [36]. As the stimulation of the oxidase by AA has been found to be unaffected by the presence of cyclo-oxygenase and lipoxygenase inhibitors, the activator cannot be a product of AA metabolism by either pathway [18,19,30]. The stimulation of $O_2^{\cdot-}$ generation by AA in a cell-free system is also unaffected by the presence of H-7, a PKC inhibitor, which suggests that this mechanism of activation does not involve PKC [36]. Therefore the reported ability of both SDS and AA to stimulate the oxidase in a cell-free system agrees with our proposal that the active oxidase requires AA.

The involvement of PKC in the activation of the oxidase has been well documented [1,7,8]. However, H-7 and other PKC inhibitors are unable to inhibit totally the activation of the oxidase [37] and PKC/PMA has been reported to stimulate $O_2^{\cdot-}$ generation only weakly in a cell-free system [38]. This suggests that PKC alone may not be solely responsible for the activation of the oxidase. We consider that AA released by PLA_2, in association with PKC activity, is necessary to activate and maintain $O_2^{\cdot-}$ generation.

The level of free AA is low in unstimulated cells [21] but rises following the addition of a wide range of activators of the oxidase. In human neutrophils 80% of the molecules of phosphatidylinositol contain AA esterified at the *sn*-2 position [39]. Therefore the turnover of phosphatidylinositol to generate $InsP_3$ and diacylglycerol may also provide the substrate for AA release by PLA_2. In the cell there are many pathways for the metabolism of AA, e.g. the synthesis of prostaglandins and leukotrienes. Therefore the requirement for continuous PLA_2 activity may be due to the need to maintain a supply of AA for the oxidase which is in competition with the synthetic pathways.

The exact mode of action of the externally added SDS or AA on the oxidase is at present unknown. The ability of both SDS and AA to restore $O_2^{\cdot-}$ generation may be due to their ability to interact directly with the oxidase, substituting for the AA lost in the presence of the PLA_2 inhibitors.

Lipocortins are a family of glycoproteins which are considered to reversibly regulate the activity of PLA_2 *in vivo* [40,41]. In agreement with this proposed role for lipocortin, synthetic oligopeptides from lipocortin I have been shown to inhibit the activity of porcine pancreatic PLA_2 [42]. The phosphorylated lipocortin is unable to inhibit PLA_2 [21,43]. The addition of recombinant lipocortin has been reported to inhibit the fMetLeuPhe-stimulated H_2O_2 generation by neutrophils in agreement with the observations reported in this paper [47]. The addition of glucocorticoids to neutrophils is considered to stimulate the synthesis of lipocortin with an associated inhibition of chemotaxis, degranulation and AA release [44,45]. If PLA_2 activity is necessary for $O_2^{\cdot-}$ generation, then the anti-inflammatory effects of a number of steroids may be due to their ability to increase the levels of the natural PLA_2 inhibitor and hence decrease the ability of neutrophils to generate $O_2^{\cdot-}$.

The addition of fMetLeuPhe to neutrophils initiates a number of responses in addition to the stimulation of the activity of the oxidase. As mentioned above, both degranulation and the chemotactic response are inhibited following the addition of glucocorticoids. This suggests that PLA_2 activity and AA release may have a role in the overall triggering of the physiological responses of the neutrophil including secretion.

An increase in the release of AA has also been reported to occur following the addition of thrombin to platelets [32]. Like the activation of the NADPH oxidase, this process was considered to be associated with the stimulation of phosphatidylinositol turnover and the activation of PKC [46]. As PLA_2 is a relatively universal enzyme, it is possible that AA release may play a role in systems other than the activation of NADPH oxidase.

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