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STUDIES OF CELLULAR MECHANISMS FOR THE GENERATION OF SUPEROXIDE BY GUINEA-PIG EOSINOPHILS AND ITS DISSOCIATION FROM GRANULE PEROXIDASE RELEASE

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Abstract—Guinea-pig peritoneal eosinophils generated superoxide anions in response to opsonized zymosan, platelet activating factor, sodium fluoride, digitonin, phorbol ester and calcium ionophore, but were refractory to fMLP. These agonists did not stimulate release of eosinophil peroxidase. The phospholipase inhibitor, mepacrine, and the protein kinase inhibitor, trifluoperazine, were effective inhibitors of superoxide production. Activators of protein kinase C, such as exogenously added phorbol ester and endogenously derived diacylglycerol, stimulate superoxide production, which is therefore proposed to be via pathways dependent on phospholipase and protein kinase activity.

Eosinophilia is characteristic of many allergic and hypersensitivity diseases. In recent years, eosinophils and their mediators have been implicated in the development of late asthmatic reactions and increased airways responsiveness following allergen inhalation by human atopic subjects [1, 2] and in our guinea-pig model of allergic airways disease [3, 4]. Studies in asthmatic patients have shown that bronchial hyperresponsiveness is associated with the presence of eosinophil-derived granule products including eosinophil peroxidase (EPO) [5, 6] which have also been suggested to contribute to the pathology of fatal bronchial asthma [7]. In addition, a role for superoxide in the mediation of bronchial hyperreactivity has been demonstrated [8]. Although superoxide anions are the least damaging of the toxic products of oxygen metabolism, spontaneous or enzyme-catalysed dismutation to hydrogen peroxide generates an extracellular source of the highly reactive, membrane-perturbing hydroxyl radical and, in the presence of EPO, of the powerfully oxidizing hypohalous acids. Concomitant release of EPO in response to agonists of superoxide production therefore establishes conditions for the formation of potentially harmful, tissue damaging products of oxygen metabolism.

The enzyme responsible for superoxide production in neutrophils and eosinophils is the respiratory burst oxidase [9]. In view of the accumulation of eosinophils in allergic diseases and in our guinea-pig model, it may be pertinent that the oxidase is more active in stimulated human [10, 11] and guinea-pig [12] eosinophils than in neutrophils.

In many cell types signal–response coupling is via the ubiquitous pathway which links receptor occupancy, via the guanine nucleotide regulatory protein, Gp, to polyphosphoinositide hydrolysis and activation of protein kinase C (Fig. 1). The mechanism of exocytotic secretion of granule contents in many cells is also regulated by G-proteins [13]. In some cell types secretion is regulated both through the G-protein, Gp, and protein kinase C, and through a separate G-protein (Ge) whose action is not mimicked by activators of protein kinase C [14, 15].

In this study we aim to examine the hypothesis that generation of oxygen metabolites by respiratory burst oxidase and secretion of eosinophil peroxidase is dependent on sequential G-protein activation, phospholipid metabolism and protein kinase C activation.

MATERIALS AND METHODS

Chemical and biochemical reagents

Calcium-ionophore A23187 (free acid), formyl-methionyl-leucyl-phenylalanine (fMLP), platelet activating factor L-α-phosphatidylcholine, β-acetyl-γ-*O*-hexadecyl (PAF), phorbol 12-myristate 13-acetate (PMA), zymosan A, trifluoperazine dihydrochloride (TFP), quinacrine dihydrochloride (mepacrine), digitonin, sodium fluoride (NaF), superoxide dismutase (SOD) from bovine erythrocytes, cytochrome *c* (Type VI, from horse heart), 3,4,5-trimethoxybenzoic acid 8-(diethylamino)-octyl ester (TMB-8), *n*-ethylmaleimide (NEM), *p*-phenylenediamine dihydrochloride and dimethylsulphoxide (DMSO) were purchased from the Sigma Chemical Co. (Poole, U.K.).

Compound R59022 (6-[2-[4-[(4-fluorophenyl)-phenymethylene)-1-piperidinyl]ethyl]-7-methyl-5H-thiazolo[3,2-α]pyrinidin-5-one) was purchased from Janssen Life Sciences (Beerse, Belgium).

Dialysed horse serum was from Gibco Ltd (Uxbridge, U.K.) and sterile Percoll from Pharmacia LKB (Uppsala, Sweden). Sterile saline was from Travenol Laboratories Ltd (Norfolk, U.K.).

Digitonin (0.4 mM) and NaF (500 mM) were made fresh each day in distilled water. Stock solutions of PMA (0.8 mM), fMLP (1 mM), A23187 (1 mM) and

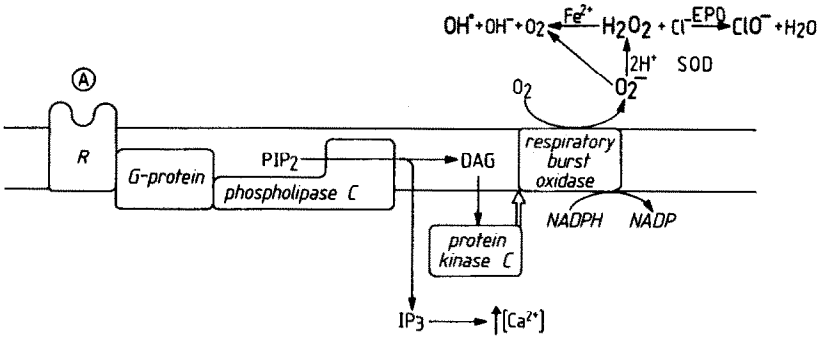

Fig. 1. A model for the activation of eosinophil respiratory burst oxidase.

R59022 (10 mM) were made in DMSO and stored at −20°. DMSO in assays was at a final concentration of <1%, and the same concentration of DMSO was added to controls. Stock solutions of mepacrine (10 mM), TFP (10 mM), TMB-8 (5 mM), SOD (0.4 mg/mL), NEM (20 mM) and cytochrome *c* (1.2 mM) were prepared in phosphate-buffered saline, pH 7.4 (PBS_I; NaCl 137 mM, KCl 2.7 mM, $Na_2HPO_4 \cdot 2H_2O$ (8.1 mM), KH_2PO_4 (1.5 mM) and stored at −20°. A stock solution of PAF (10 mM) was made in ethanol and aliquots stored at −20° were diluted with PBS for use in assays.

Opsonized zymosan (OPZ) was prepared freshly each day by the method of Petreccia *et al.* [10] using either fresh normal guinea-pig serum or serum which was frozen at −20° immediately after preparation and thawed once.

Methods

Cell isolation and purification. Peritoneal eosinophilia was elicited in guinea-pigs (male, Dunkin–Hartley strain) in response to intraperitoneal injections of horse serum and cells recovered by peritoneal lavage using methods based on the procedure described by Litt [16]. Guinea-pigs were injected intraperitoneally with horse serum (0.5 mL) twice a week for 2 weeks, followed thereafter by one injection per week. Animals were lavaged under methoxyfluorane inhalation anaesthesia and allowed to recover. Sterile normal saline (50 mL) was injected i.p. and the lavage fluid drained out through a 16-gauge Teflon cannula with 6–8 drainage holes cut around the walls. Recovery of lavage fluid was routinely 80–90% and contained eosinophils at 30–50% purity which were subsequently purified to >98% on discontinuous gradients of Percoll as described by Gartner [17].

Lavage fluid was filtered through gauze and cells pelleted by centrifugation at 450 *g* for 10 min at 22°. All subsequent procedures were at room temperature. Erythrocytes were lysed with hypotonic 0.2% NaCl and cells finally suspended in HEPES-buffered saline with glucose (HBSG: HEPES 20 mM, NaCl 137 mM, glucose 5.6 mM, pH 7.2). Dilutions of Percoll were made in HBSG with density gradients consisting of 2.0 mL 1.105 g/mL, 2.0 mL 1.085 g/mL and 2.0 mL 1.075 g/mL Percoll. Cells from one guinea-pig in HBSG (2.0 mL) were layered onto a gradient, which was centrifuged at 750 *g* for 30 min at 22°. Eosinophils were harvested from the 1.085/1.105 interface using a glass Pasteur pipette and were washed twice in HBSG before counting in a haemocytometer. Cell viability was determined always to be >97% by Trypan Blue exclusion. Approximately 2–5 × 10^7 eosinophils were isolated from one guinea-pig by this method.

Assays. Superoxide production was measured spectrophotometrically as superoxide dismutase-inhibitable reduction of cytochrome *c* as described by Yamashita *et al.* [12]. Assays, in duplicate, were carried out at 37° in a final volume of 1.0 mL PBS_I and contained 0.5 × 10^6 eosinophils, except when OPZ (1.0 × 10^6 eosinophils/mL) or PAF (2.0 × 10^6 eosinophils/mL) was the agonist. Initial experiments determined the optimum concentration of each agonist for superoxide production, so that the effect of inhibitors, mepacrine and TFP, on activation by 1 mg/mL OPZ, 1 μM PAF, 50 mM NaF, 2 μM digitonin, 1 μM A23187 and 1.6 μM PMA was tested. Superoxide production stimulated by OPZ, PAF, digitonin, fMLP and A23187 was measured in assays which contained 0.9 mM $CaCl_2$ plus 0.5 mM $MgCl_2$. Assays employing PMA or NaF contained no added calcium or magnesium. Reduction of cytochrome *c* was monitored continuously at 550 nm when PMA or PAF was the agonist, so that lag time and initial rate of superoxide production could be measured. Discontinuous assays were carried out for the other agonists, and reactions were stopped by placing the tubes on ice and adding EGTA to a final concentration of 2 mM, or in the case of NaF, NEM to 1 mM. Cells were pelleted by centrifugation at 450 *g* for 10 min at 4° and the optical density of the supernatant at 550 nm measured. The maximum rate of superoxide production per minute was calculated in continuous assays, or in discontinuous assays where there was no lag phase to the response and the response was linear. Total superoxide production per assay was calculated in all other cases.

The reduction of cytochrome *c* was entirely due to superoxide formation in the assays, since inclusion

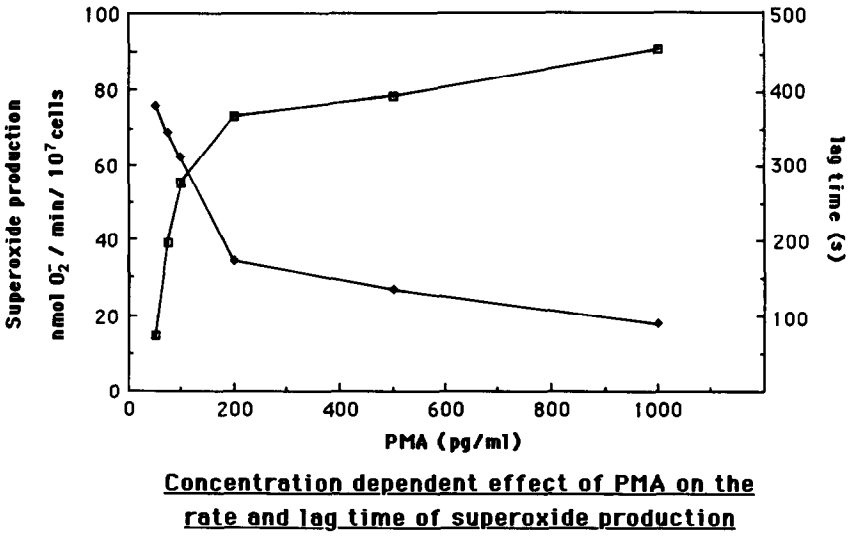

Fig. 2. Concentration-dependent effect of PMA on the rate and lag time of superoxide production. Eosinophils (0.5×10^6) in PBS_I were preincubated at 37° for 3 min prior to addition of PMA. Initial rates (■——■) and lag time (◆——◆) of O_2^- formation were determined in continuous assays. The lag time was calculated as described by Cohen and Chevaniec [47]. The results are from a single experiment, representative of three separate experiments.

of SOD at a final concentration of 0.65 μM completely inhibited the increase in optical density measured in response to the agonists.

Stimulated release of eosinophil peroxidase (EPO) was quantified in assays (150 μL) containing 1×10^5 eosinophils in PBS_{II}, pH 7.0, i.e. 10 mM KH_2PO_4 adjusted to pH 7.0 with 10 mM $Na_2HPO_4 \cdot 2H_2O$, containing 137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 0.6 mM $MgCl_2$ and 5.6 mM glucose. Total EPO content was determined in cells lysed with 2% Triton TX-100. EPO activity was measured spectrophotometrically as described by Khalife *et al.* [18].

A significant value indicates $P < 0.05$ when paired data are analysed by Student's *t*-test.

RESULTS

Superoxide (O_2^-) production

Phorbol 12-myristate 13-acetate. PMA stimulated O_2^- production in a concentration-dependent fashion (Fig. 2). The effect was independent of extracellular Ca^{2+} and Mg^{2+} and PMA was equally effective in the presence of 2 mM EGTA. The onset of O_2^- production occurred after a lag time that was inversely proportional to the initial concentration of added PMA. Near maximal responses, of approximately 90 nmol O_2^-/min/10^7 cells, were obtained with PMA at a concentration of 1.6 nM with a residual lag time of about 90 sec. No O_2^- was produced by cells in the absence of PMA and 80 pM was the lowest concentration of PMA at which O_2^- generation could be reproducibly measured.

The effect of the phospholipase inhibitor mepacrine and of the protein kinase inhibitor TFP was investigated when O_2^- production was stimulated by excess (1.6 μM) PMA (Figs 3 and 4). Concentration-dependent inhibition of the response to PMA was observed with both inhibitors. TFP, being the more effective, almost completely abolished the response at a concentration of 20 μM. Even in the presence of 1.0 mM mepacrine, PMA continued to stimulate almost 60% of the maximum response.

Digitonin. Digitonin optimally stimulated O_2^- production at a concentration of 2 μM, and concentrations higher than this were inhibitory. Using a continuous spectrophotometric assay a lag time of 2 min was routinely observed and the effects of inhibitors were subsequently investigated using 10 min discontinuous assays. In the absence of any inhibitors, digitonin stimulated the production of 554 ± 44 nmol O_2^-/10 min/10^7 eosinophils (N = 4). The response was completely dependent on the presence of both Ca^{2+} and Mg^{2+} in the buffer and was effectively inhibited by mepacrine (Fig. 3) and TFP (Fig. 4). Mepacrine (1 mM) produced 84% inhibition and TFP (15 μM) 96% inhibition of the response.

Sodium fluoride. Continuous assays showed that NaF (50 mM) stimulated O_2^- production after a lag phase of about 6 min. In discontinuous assays, NaF (50 mM) stimulated the production of 481 ± 40 nmol O_2^-/30 min/10^7 cells (N = 4), in the absence of extracellular Ca^{2+} and Mg^{2+}. Under these conditions inhibition by 1 mM mepacrine (Fig. 3) was 78% and inhibition by 15 μM TFP (Fig. 4) was 97% of the maximum response.

A23187. The calcium ionophore A23187 (1 μM) strongly stimulated O_2^- production without a lag period, an initial rate of 132 ± 16 nmol O_2^-/min/10^7 cells being linear over a 3 min discontinuous assay. The effect was strictly dependent on extracellular $Ca^{2+}$and Mg^{2+}, and was abolished completely by

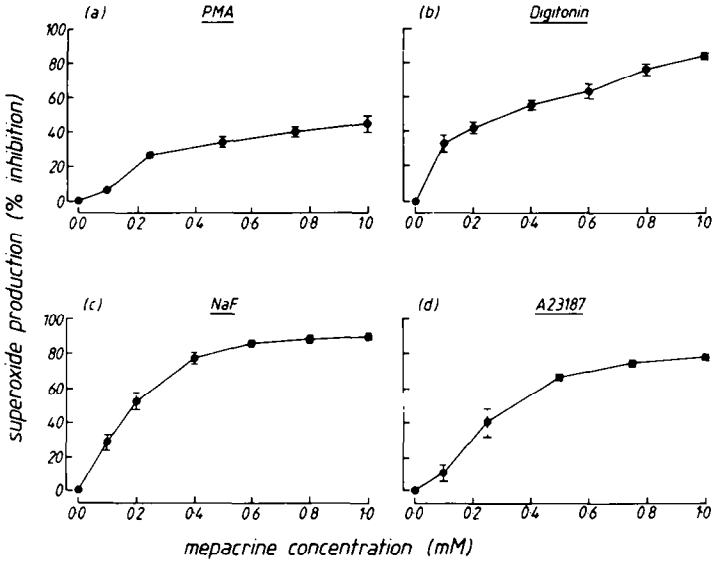

Fig. 3. The effect of mepacrine on superoxide production stimulated by: (a) 1.6 μM PMA, (b) 2 μM digitonin, (c) 50 mM NaF and (d) 1 μM A23187. Eosinophils (0.5×10^6) in PBS_1 were preincubated for 10 min at 37° with mepacrine before addition of the agonist. Continuous or discontinuous assays were followed for 3–10 min before termination of the reaction (see Materials and Methods). Results are expressed as mean ± SE for N = 3 (a) and N = 4 (b–d) experiments.

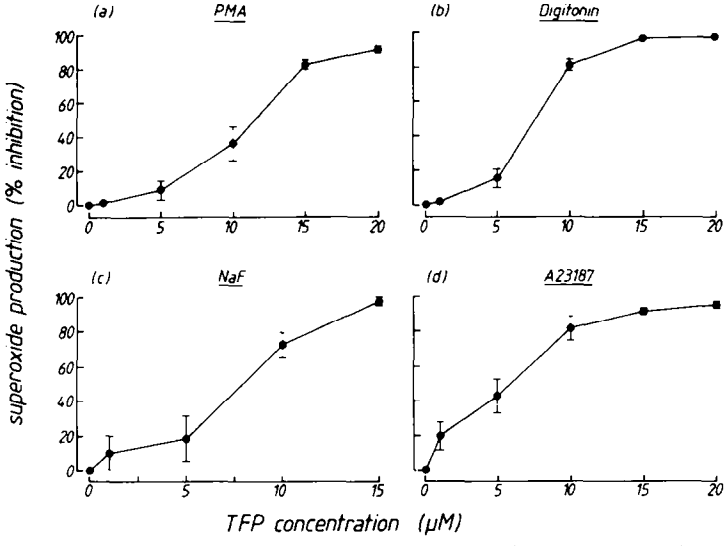

Fig. 4. The effect of TFP on superoxide production stimulated by: (a) 1.6 μM PMA, (b) 2 μM digitonin, (c) 50 mM NaF and (d) 1 μM A23187. Eosinophils (0.5×10^6) in PBS_1 were incubated for 10 min at 37° with TFP before addition of the agonist. Continuous or discontinuous assays were followed for 3–10 min before termination of the reaction (see Materials and Methods). Results are expressed as mean ± SE for N = 4 (a, c, d) and N = 3 (b) experiments.

addition of 2 mM EGTA to the buffer. Mepacrine (Fig. 3) and TFP (Fig. 4) inhibited the response over the same concentration ranges as observed for the other agonists.

Receptor-directed agonists. Opsonized zymosan was a relatively weak inducer of O_2^- production and at 1.0 mg/mL stimulated the formation of 107.5 ± 11.8 nmol O_2^-/10 min/10^7 cells (N = 4). A lag phase of 1.0 min was routinely observed in experiments using a continuous assay. The effect of OPZ was highly sensitive to inhibition by mepacrine (Fig. 5a) and TFP (Fig. 5b). Inhibition by mepacrine was 100% at 0.25 mM and inhibition was 93 ± 3% at 8 μM TFP. Production of O_2^- in response to OPZ was completely abolished by the removal of extracellular Ca^{2+} and Mg^{2+} with 2 mM EGTA.

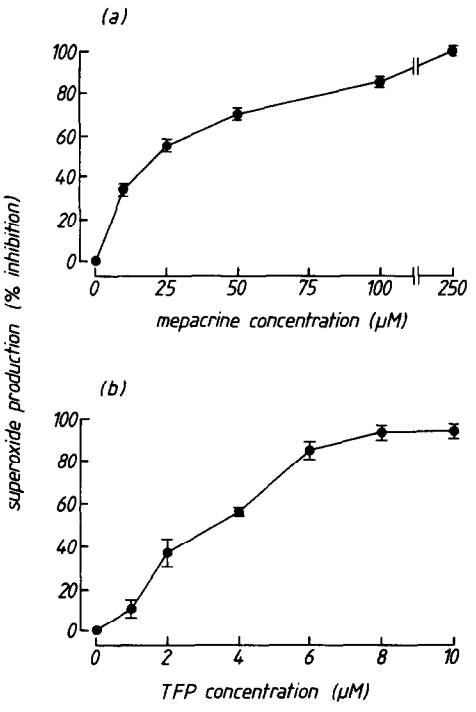

Fig. 5. (a) The effect of mepacrine on superoxide production stimulated by opsonized zymosan (1 mg/mL). (b) The effect of TFP on superoxide production stimulated by opsonized zymosan (1 mg/mL). Eosinophils (1×10^6) in PBS_I were preincubated for 10 min with mepacrine and for 10 min with TFP prior to addition of OPZ (1 mg/mL). Assays were for 10 min and were terminated by cooling on ice and addition of 2 mM EGTA. The results are expressed as the mean ± SE of four (a) and three (b) independent experiments.

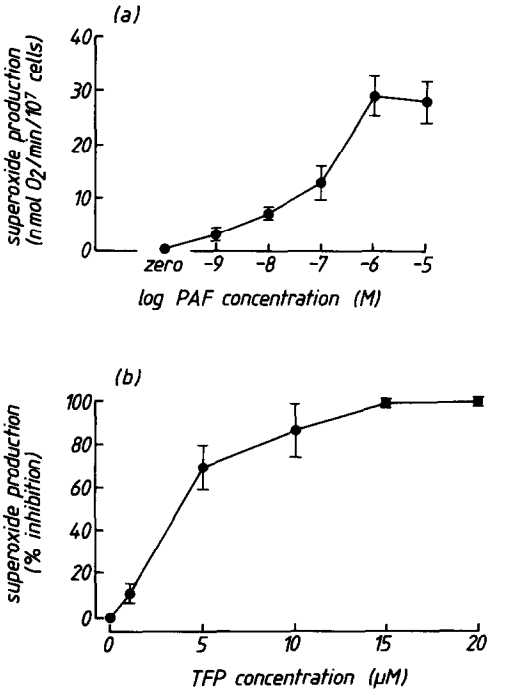

Fig. 6. (a) Concentration-dependent effect of PAF on the rate of superoxide production in the presence of R59022 (10 μM). (b) The effect of TFP on superoxide production stimulated by PAF (1 μM) and R59022 (10 μM). Eosinophils (2×10^6) were incubated in PBS_I containing 0.9 mM $CaCl_2$ plus 0.5 mM $MgCl_2$ and glucose (1 g/L). Cells were preincubated at 37° with R59022 for 2 min and with TFP for 3 min prior to addition of PAF. Initial rates of superoxide formation were determined in continuous assays. Results are expressed as the mean ± SE of five (a) and three (b) independent experiments.

PAF alone was a weak agonist, stimulating a low rate of O_2^- production at the optimal concentration of 0.1 μM in some cell preparations (results not shown) whilst other preparations remained refractory. However, in the presence of R59022, a specific inhibitor of DAG kinase [19], at a concentration of 10 μM, the response to PAF was enhanced and the concentration–response curve was highly reproducible between cell preparations (Fig. 6a). Under these conditions O_2^- production was optimal at 1 μM PAF, which gave a rate of 29.1 ± 3.5 nmol O_2^-/min/10^7 cells (N = 5). The response to 1 μM PAF was completely abolished by 15 μM TFP (Fig. 6b).

The effect of calcium on PAF-induced O_2^- production was investigated by using the extracellular calcium chelator EGTA and the intracellular calcium antagonist TMB-8. The results shown in Table 1 show that there was no significant difference in O_2^- generation in the presence or absence of extracellular Ca^{2+} and Mg^{2+}. However, TMB-8 significantly reduced the rate of O_2^- production when incubations contained Ca^{2+}/Mg^{2+} and this was further significantly decreased by the removal of extracellular Ca^{2+}/Mg^{2+} by 2 mM EGTA.

Eosinophils did not generate O_2^- in response to fMLP in the concentration range 1 nM to 10 μM, indicating that receptors for this peptide may be absent on guinea-pig eosinophils.

EPO release

No release of EPO was observed when eosinophils were treated with PMA (1.6–64 μM), digitonin (0.4–16 μM), NaF (20–80 mM), A23187 (0.25–20 μM), OPZ (0.02–5 mg/mL) and PAF (0.1 nM–1 μM) in the presence or absence of R59022 (10 μM) or fMLP (1 nM–10 μM). Concentrations of PAF greater than 10 μM were increasingly cytotoxic, as demonstrated by Trypan Blue exclusion, and 100 μM PAF released the total cellular content of EPO and lactic dehydrogenase measured by the method of Cabard and Wroblewski [20].

DISCUSSION

The protein kinases C are a ubiquitous family of enzymes which phosphorylate a wide range of proteins including the respiratory burst oxidase [21]. Diacylglycerol, which may be generated in plasma membranes via the action of phospholipases C, A_2 or D [22], initiates activation of protein kinase C.

Table 1. The effect of Ca^{2+} on superoxide production stimulated by PAF (1 μM) and R59022 (10 μM)

Condition	Superoxide production (nmol/min/10^7 cells)	
(1) + 0.9 mM $CaCl_2$/0.5 mM $MgCl_2$	34.4 ± 2.9	
(2) + 2 mM EGTA	28.7 ± 4.4	(1)–(2) NS
(3) + 0.5 mM TMB-8 + 0.9 mM $CaCl_2$/0.5 mM $MgCl_2$	7.6 ± 0.5	(1)–(3) $P < 0.01$
(4) + 0.5 mM TMB-8 + 2 mM EGTA	5.2 ± 0.4	(3)–(4) $P < 0.001$

Eosinophils were incubated as described in the legend to Fig. 6a. Ca^{2+}/Mg^{2+} or EGTA were added to the buffers at the concentrations shown. Cells were preincubated with TMB-8 for 5 min prior to addition of PAF. Discontinuous assays were stopped by the addition of SOD (0.65 μM) after 3 min. Results are the mean ± SE of four independent experiments.
NS, not significantly different.

In addition, some synthetic diacylglycerols and tumour-promoting phorbol esters, such as PMA, are potent activators of protein kinase C [23]. PMA intercalates into cell membranes and mimics the effect of endogenous DAG, i.e. it induces Ca^{2+}dependent translocation of inactive protein kinase C from the cytosol to the plasma membrane. DAG activates protein kinase C in the membrane by decreasing the calcium requirement of the enzyme, thereby rendering it active at physiological intracellular calcium concentrations. The concentration-dependent effect of PMA on the rate and lag time of O_2^- production in eosinophils (Fig. 2) may reflect the extent and temporal relationship between translocation of protein kinase C [24, 25], or components of the oxidase [26], to the plasma membrane and subsequent activation of the respiratory burst.

Mepacrine is an inhibitor of both phospholipase C and A_2 [27], whilst besides having effects on calmodulin TFP inhibits protein kinase C activity and O_2^- production in neutrophils stimulated with PMA [28]. As activation of O_2^- production by PMA is independent of phospholipase activity it was expected that mepacrine would be an ineffective inhibitor. The partial inhibition that was observed may be the result of a direct interaction of mepacrine with membrane phospholipid, proposed to be responsible for the inhibitory effect of mepacrine on cell responses [29]. The marked inhibition by TFP of PMA-induced O_2^- production strongly indicates that in eosinophils activation of the oxidase occurs via protein kinase C-mediated phosphorylation of a component of the oxidase, as has been demonstrated in neutrophils [28, 30]. Further support for this suggestion was rendered by the observation that the specific diacylglycerol kinase inhibitor, R59022, was most effective in enhancing O_2^- production in response to PAF (Fig. 6a).

DAG generated in cell membranes is normally rapidly metabolized by either DAG kinase or DAG lipase and is therefore present only transiently following receptor activation. Our results indicate that metabolism of DAG in PAF-activated eosinophils is normally so rapid that little or no protein kinase C activation occurs. However, when a prominent route of DAG metabolism is inhibited then DAG accumulation occurs and, concomitantly, activation of protein kinase C leading to the generation of O_2^- anions. The presence of the proposed pathway is confirmed by inhibition of the response to PAF, in the presence of R59022, by TFP (Fig. 6b). Our results obtained in the study using PMA reflect these conclusions. PMA is a DAG-mimic only slowly metabolized by cells and therefore able to cause sustained activation of protein kinase C and rates of O_2^- production nearly an order of magnitude higher than those achieved with OPZ or PAF.

The results in Table 1 indicate that the effect of PAF in the eosinophil is largely independent of extracellular Ca^{2+}, whilst release of Ca^{2+} from intracellular stores is an important part of the stimulus–response transduction mechanism. This is in contrast to other cell types, such as macrophages [31], platelets [32] and neutrophils [33], in which PAF causes an increase in intracellular calcium derived principally from extracellular Ca^{2+}. TMB-8 is reported [34] to reduce availability of intracellular Ca^{2+} by either inhibiting the influx of extracellular Ca^{2+} or by blocking release of Ca^{2+} from intracellular storage sites. Chelation and removal of extracellular Ca^{2+} by EGTA was without significant effect on the response to PAF, except in the presence of TMB-8. The results therefore indicate a small contribution of extracellular Ca^{2+} influx to the rise in intracellular Ca^{2+} concentration which appears to be part of the mechanism of eosinophil activation by PAF. In addition, O_2^- is generated via Ca^{2+}-independent processes, i.e. in the presence of both TMB-8 and EGTA. In this respect, PAF and OPZ demonstrate different mechanisms since O_2^- production in response to OPZ was completely inhibited by 2 mM EGTA. Two types of PAF receptor have been described [33] on rabbit neutrophils, high affinity receptors dependent on extracellular Ca^{2+} and

receptors which are independent of external Ca^{2+} and through which concentrations of PAF > 1 nM stimulate polyphosphoinositide-specific phospholipase C and subsequent biochemical events. It appears the latter type of PAF receptor are responsible for the stimulation of superoxide generation in eosinophils.

Diacylglycerol is generated in neutrophils by phospholipase C-mediated hydrolysis of polyphosphoinositides [35] and via phospholipase D-mediated hydrolysis of phosphatidylcholine [36]. Induction of phospholipid metabolism in neutrophils is stimulus-specific [37] and there may be activation pathways which are dependent on phospholipase A_2-mediated lipid hydrolysis and independent of protein kinase C [38, 39]. Receptor occupancy is linked to phospholipid metabolism via guanine nucleotide binding proteins [22], of which sodium fluoride is a ubiquitous activator [40]. Our results indicate that in eosinophils, as in neutrophils, sodium fluoride stimulates O_2^- production through the classical signal transduction pathways. Fluoride elicits the respiratory burst by persistent activation of a G-protein, and the subsequent events mediated by phospholipase(s) and protein kinase C are inhibited by mepacrine and TFP respectively (Figs 3c and 4c). We have also shown (unpublished results) that in the presence of lithium chloride, 50 mM NaF stimulates polyphosphoinositide hydrolysis and accumulation of inositol phosphates in eosinophils prelabelled to equilibrium with tritiated inositol. The polyphosphoinositide-specific phospholipase C [41] is therefore involved in stimulus–response coupling in the eosinophil (Fig. 1). However, a fundamental difference between neutrophils and eosinophils exists. Our results show that in eosinophils, O_2^- production in response to sodium fluoride is independent of extracellular Ca^{2+}, whilst the effect of fluoride in neutrophils is dependent on influx of extracellular Ca^{2+} [42, 43].

Calcium ionophore A23187, by increasing the intracellular Ca^{2+} concentration, activates phospholipase A_2 in the cell membrane, an enzyme having an absolute requirement for Ca^{2+} [44]. Mepacrine inhibition of O_2^- production in response to A23187 may reflect a phospholipase A_2-dependent pathway [27]. In support of this hypothesis unsaturated fatty acids, including arachidonate released by the activation of phospholipase A_2 [37, 45], activate protein kinase C in a calcium-dependent fashion [46]. Inhibition by TFP (Fig. 4d) indicates a role for protein kinase C in the response to A23187 in eosinophils, as has previously been shown in neutrophils [24].

The kinetics of O_2^- production in guinea-pig eosinophils in response to digitonin are similar to those previously observed for guinea-pig neutrophils [47]. Digitonin is a surface active agent which has been shown to stimulate oxidative metabolism and phosphoinositide turnover in guinea-pig neutrophils [48]. Since both mepacrine and TFP are effective inhibitors (Figs 3b and 4b), digitonin may stimulate phospholipid metabolism in a non-receptor-mediated way, leading to O_2^- production via the proposed pathway of protein kinase C activation.

The mechanism(s) by which the respiratory burst oxidase of eosinophils is activated is stimulus-specific, as reflected by the Ca^{2+} dependency of the agonists used in this study. It appears though, that O_2^- production is dependent on sequential phospholipase and protein kinase activation. Phospholipase C, A_2 or D activity may be part of the signal transduction mechanism; indeed, all three pathways have been demonstrated in neutrophils. It may be pertinent that eosinophils possess a high level of phospholipase D activity [49]. Although we have preliminary evidence for the phosphoinositide cycle, in depth studies of phospholipase activation in eosinophils remain to be performed.

Release of EPO

Only cytolytic concentrations of PAF (>10 μM) caused release of EPO and such a mechanism is unlikely to be of physiological significance. We were unable to demonstrate the cytolytic mechanism for release of EPO in response to A23187 previously described by Fukuda *et al.* [50]. The possibility that EPO remained bound to the cell surface following degranulation in response to the agonists, as previously suggested [50], was excluded by histochemical staining of stimulated cell preparations which failed to show an accumulation of EPO at the cell surface.

The observation by Barnes and co-workers [51] that PAF (10 pM–1 μM) induces EPO release from guinea-pig eosinophils may reflect a difference in the metabolic capacity of peritoneal eosinophils raised in response to polymyxin B and to horse serum. Polymyxin B is an inhibitor of protein kinase C [52] and chronic stimulation of peritoneal eosinophils with this antibiotic may alter membrane receptor-mediated biochemical events. Certainly, polymyxin B has been shown [53] to affect the oxidative metabolism of eosinophils stimulated by both OPZ and PMA.

In eosinophils raised in response to horse serum, the biochemical pathways leading to the generation of O_2^- anions do not result in release of EPO. It is conceivable that activation of the same pathways which lead to O_2^- production inhibit the release of EPO. This would provide a mechanism for protection against the concomitant release of O_2^- and EPO, and the subsequent formation of the highly reactive hypohalous acids in normal tissues. Protein kinase C mediates inhibitory as well as stimulatory mechanisms in many cell types [21] and EPO may only be released when protein kinase C is inhibited. We are currently investigating this possibility.

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