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Okadaic acid produces changes in phosphorylation and translocation of proteins and in intracellular calcium in human neutrophils

Relationship with the activation of the NADPH oxidase by different stimuli

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Okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A, profoundly influenced the activity of the NADPH oxidase of human neutrophils. It strongly inhibited stimulation of superoxide generation by phorbol 12-myristate 13-acetate (PMA) and impaired translocation of protein kinase activity and of the two cytosolic components p47-*phox* and p67-*phox* to the plasma membrane. The increase in the phosphorylation of the cytochrome b_{-245} subunits p22-*phox* and gp91-*phox* after stimulation was also blocked. Inhibition of activity was associated with a decrease in cytosolic free Ca^{2+} and was reversed by the Ca^{2+} ionophore A23187, which also restored protein translocation and phosphorylation of the cytochrome. This effect of A23187 was itself blocked by preincubation with cyclosporin A, suggesting that calcineurin might be involved in the re-activation process. In contrast with PMA, the response to the bacterial peptide fMet-Leu-Phe was greatly prolonged after an initial decrease in the rate of onset of NADPH oxidase activity.

INTRODUCTION

The phagocytic cells of the immune system, neutrophils, monocytes, macrophages and eosinophils, contain an unusual electron-transport chain, the NADPH oxidase. Upon cell stimulation, this oxidase system is responsible for a respiratory burst that generates superoxide and is important for the killing and digestion of engulfed microbes (Selvaraj & Sbarra, 1966). Stimulation of phagocytes results in extensive protein phosphorylation (Schneider *et al.*, 1981; Okamura *et al.*, 1984). The proteins phosphorylated include components of the NADPH oxidase: both subunits (p22- and gp91-*phox*) of cytochrome b_{-245}, the terminal component of the electron-transport chain (Garcia & Segal, 1988), and the cytosolic protein p47-*phox*. The latter is clearly involved in the activation of the NADPH oxidase, because its absence in the most frequent type of chronic granulomatous disease with an autosomal recessive inheritance results in failure to generate superoxide (Segal *et al.*, 1985; Nunoi *et al.*, 1988; Casimir *et al.*, 1991). Phosphorylation and dephosphorylation of p47-*phox* correlates well with the kinetics of the respiratory burst (Heyworth & Segal, 1986; Okamura *et al.*, 1988*a*,*b*; Ohtsuka *et al.*, 1990). Besides, cell stimulation results in the translocation of the p47-*phox* from the cytosol to the membrane, where it appears to become associated with the cytochrome b_{-245} (Heyworth *et al.*, 1989), and of p67-*phox*, another protein in whose absence there is a failure to generate superoxide (Nunoi *et al.*, 1988; Clark *et al.*, 1990).

The activity of the NADPH oxidase is transient when phagocytes are triggered with physiological stimuli. It has been postulated that the mechanism of termination of the respiratory burst could be due to autoxidation by its toxic end products (Whitin & Cohen, 1988). Another mechanism, proposed by Grinstein *et al.* (1989), could involve the dephosphorylation of phosphoproteins.

The aim of the present studies was to examine the relationship between protein phosphorylation and the mechanism of the NADPH oxidase by investigating the effect of okadaic acid, a potent protein phosphatase inhibitor. Okadaic acid is a fatty acid derivative that enhances protein phosphorylation in a variety of intact cells by inhibiting protein phosphatases-1 and -2A (Bialojan & Takai, 1988; Cohen, 1991; Hardie *et al.*, 1991), which are active on a wide range of phosphoproteins (Haystead *et al.*, 1989). It seemed possible that okadaic acid might activate the respiratory burst in the absence of any other stimulus or enhance the response to suboptimal concentrations of stimuli. To examine the latter possibility, stimuli whose mechanisms of activation appear to differ were used: *N*-formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe), phorbol myristate acetate (PMA), diacylglycerol and opsonized latex particles (McPhail *et al.*, 1981; Cooke & Hallett, 1985; Wolfson *et al.*, 1985; Gerard *et al.*, 1986; Berkow *et al.*, 1987; Grinstein & Furuya, 1988).

EXPERIMENTAL

Cell preparation

Neutrophils were purified from 50 ml of freshly drawn blood containing heparin (5 i.u./ml), as previously described (Segal *et al.*, 1980). The cells were washed once with phosphate-buffered saline (PBS) containing 5 mM-glucose, 1 mM-$MgCl_2$ and 0.5 mM-

Abbreviations used: PMNs, polymorphonuclear leucocytes; PMA, phorbol 12-myristate 13-acetate; fMet-Leu-Phe, *N*-formylmethionyl-leucyl-phenylalanine; DMSO, dimethyl sulphoxide; PBS, phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8.1 mM-Na_2HPO_4/1.5 mM-KH_2PO_4, pH 7.0); PBS-Glc-Mg-Ca, PBS containing 5 mM-glucose, 1 mM-$MgCl_2$ and 0.5 mM-$CaCl_2$; PKC, protein kinase C; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration.

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$CaCl_2$ (PBS-Glc-Mg-Ca) and resuspended to 5×10^7/ml with the same medium.

Treatment with okadaic acid and cyclosporin A

Neutrophil suspensions were preincubated at 37 °C for 30 min with 1 μM-okadaic acid (Moana BioProducts, Hawaii, U.S.A.), taken from a 0.1 mM stock solution in dimethylformamide. Control incubations contained instead only 0.01% (v/v) dimethylformamide. Preincubated cells were used for assays of superoxide generation, translocation experiments and measurement of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), as described below. In experiments of protein phosphorylation, cells were first labelled with $[^{32}P]P_i$ as described below, then treated with okadaic acid and finally stimulated.

In some experiments, cyclosporin A (2 μg/ml; Sandoz, Basel, Switzerland) was added for the last 10 min of incubation with okadaic acid.

Stimulation and measurement of superoxide production

Samples (0.02 ml) from cell suspensions preincubated with or without okadaic acid, containing 10^6 cells each, were used for the kinetic determination of superoxide generation as the superoxide dismutase-inhibitable reduction of cytochrome *c* (Garcia *et al.*, 1986) after cell stimulation. The reactions were carried out at 37 °C in a total volume of 1 ml, in PBS-Glc-Mg-Ca. The soluble stimuli used were fMet-Leu-Phe, PMA and 1,2-dioctanoylglycerol, which were added from stock solutions in dimethyl sulphoxide [DMSO; final concn. in the incubation medium 0.01–0.02% (v/v) in all cases]. The particulate stimuli were latex particles (12 μm in diameter), coated with IgG as previously described (Segal & Coade, 1978) and added to the reactions at a particle/cell ratio of 1:1.

Protein phosphorylation

Freshly purified neutrophils (7×10^7–10^8/ml) were incubated at 37 °C for 60 min in phosphate-free medium (138 mM-NaCl, 10 mM-Hepes, 2.7 mM-KCl, 1 mM-$MgCl_2$, 0.5 mM-$CaCl_2$ and 7.5 mM-glucose, pH 7.4) containing $[^{32}P]P_i$ (25–35 MBq/ml). Cell suspensions were adjusted to 5×10^7/ml with phosphate-free medium, incubated in the absence or presence of okadaic acid as described above and then used, after stimulation, for the analysis of (*a*) total protein phosphorylated or (*b*) purification and quantification of ^{32}P-cytochrome b_{-245}, as follows.

(*a*) Neutrophil suspensions labelled with $[^{32}P]P_i$ and treated or not with okadaic acid were incubated at 37 °C with (final concns.) 50 nM-PMA, 0.1 μM-fMet-Leu-Phe, 0.1 μM-A23187 or A23187 plus PMA. Portions containing 2×10^6 cells were taken from the incubation mixtures at various times up to 8 min, precipitated with trichloroacetic acid (8%, w/v) at 0 °C and analysed by SDS/PAGE and autoradiography.

(*b*) $[^{32}P]P_i$-labelled cell suspensions, treated or untreated with okadaic acid, were adjusted to 3×10^7/ml with phosphate-free medium and incubated for 3 min at 37 °C with (final concns.) 0.2% DMSO (control), 10 nM-PMA, 0.1 μM-A23187, or A23187 plus PMA, in a total volume of 4 ml. Incubations were ended by dilution with 1 vol. of ice-cold 0.15 M-NaCl. Cell pellets were resuspended with 0.5 vol. of 6% (w/v) sucrose containing 1 mM-EDTA, 1 mM-EGTA, 50 μM-leupeptin and 1 mM-di-isopropyl fluorophosphate, left on ice for 20 min and then sonicated for 20 s at an amplitude of 8 μm. The suspensions of disrupted cells were centrifuged at 1000 *g* for 5 min at 4 °C, and the organelle-containing post-nuclear supernatants were then centrifuged at 100000 *g* for 30 min, all at 3 °C. Cytochrome b_{-245} was partially purified from each organelle pellet (Harper *et al.*, 1984; Segal, 1987) and then subjected to SDS/PAGE. The gels were scanned to quantify the bands of p22-*phox* and gp91-*phox* and autoradiographed to locate phosphorylated protein. The bands corresponding to gp91-*phox* and p22-*phox* were excised from the gels and counted for radioactivity by liquid scintillation.

Translocation of proteins

Neutrophil suspensions were preincubated with or without okadaic acid, as described above, then adjusted to 2.5×10^6 cells/ml with PBS-Glc-Mg-Ca and incubated at 37 °C for 3 min with (final concns.) 0.1% DMSO (controls), 10 nM-PMA, 0.1 μM-A23187, or PMA plus A23187, in a total volume of 5 ml. Incubations were terminated by adding 2 vol. of ice-cold PBS. Cell pellets were resuspended with 0.2 vol. of 6% sucrose containing 1 mM-di-isopropyl fluorophosphate and 50 μM-leupeptin, left on ice for 20 min and sonicated for 15 s at an amplitude of 8 μm. The suspensions of disrupted cells were centrifuged at 1000 *g* for 5 min and the post-nuclear supernatants were re-centrifuged at 100000 *g* for 30 min, at 3 °C. The supernatants therefrom (cell cytosols) were analysed for p47-*phox* and p67-*phox* by SDS/PAGE and Western blotting, as described below, and for protein kinase activity as described by Phillips *et al.* (1989).

Measurement of $[Ca^{2+}]_i$

Neutrophils (5×10^7/ml) were loaded with fura-2/AM (10 μM) for 10 min at 25 °C, then washed with PBS-Glc-Mg-Ca and incubated at 37 °C for 30 min with or without okadaic acid. Portions containing 10^6 cells each were diluted to 1.5×10^6/ml with PBS-Glc-Mg-Ca and used for measurement of $[Ca^{2+}]_i$, before and after stimulation with (final concns.) 50 nM-PMA or 10 nM-fMet-Leu-Phe and after addition of 0.1 μM-4-bromo-A23187. Incubations were carried out in the magnetically stirred cell of a fluorescence spectrophotometer, at 37 °C. Excitation spectra (300–400 nm) were run successively before and after cell lysis with 0.1% (w/v) Triton X-100, after addition of $CaCl_2$ (final concn. 5 mM), and after calcium chelation with EGTA (final concn. 40 mM). Emission at 490 nm was recorded in all cases, and Ca^{2+} concentrations were calculated in accordance with Grynkiewicz *et al.* (1985).

Electrophoresis and Western blots

SDS/PAGE was performed as described by Laemmli (1970), on 10 or 12% (w/v) acrylamide gels. Western blots were carried out in a semi-dry apparatus for 90 min, at 0.8 mA/cm^2. The nitrocellulose membranes were stained with Ponceau Red, destained and probed with affinity-purified antibodies from rabbit polyclonal antisera raised to *C*-terminal peptides of p47-*phox* and p67-*phox* respectively (Harlow & Lane, 1988; Rodaway *et al.*, 1990; Leto *et al.*, 1990). Reactive bands were detected by reaction with an alkaline-phosphatase-conjugated goat anti-rabbit antibody. The bands were quantified by densitometry scanning of the dark-blue product of phosphatase activity.

RESULTS

Protein phosphorylation in neutrophils treated with okadaic acid

Treatment of $[^{32}P]P_i$-labelled neutrophils with okadaic acid produced a marked increase in protein phosphorylation which was maximum after 30 min (Fig. 1). This enhancement occurred in the absence of added stimuli and was not accompanied by superoxide generation.

Effect of okadaic acid on the stimulation of fMet-Leu-Phe

Activation of neutrophils with a sub-optimal concentration of the bacterial peptide fMet-Leu-Phe (10 nM for 10^6 cells/ml, which produced an initial rate 30–50% of that obtained with 100 nM)

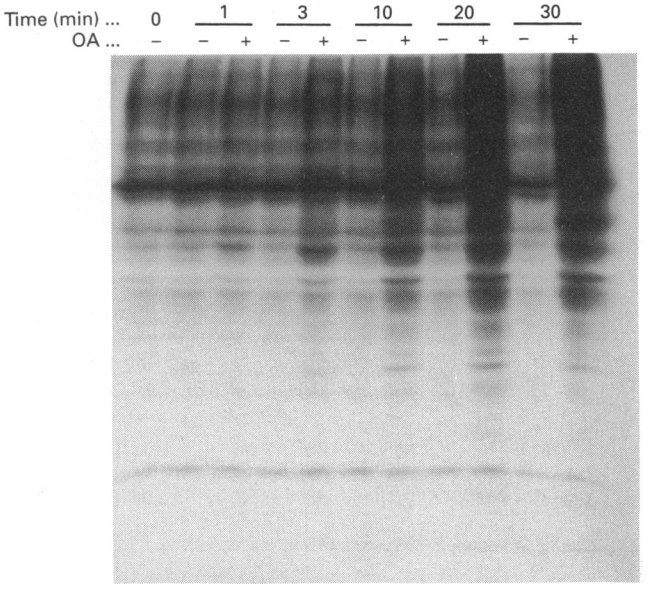

Fig. 1. Protein phosphorylation in neutrophils treated with okadaic acid

Autoradiograph of SDS/polyacrylamide gel of [^{32}P]P_i-labelled neutrophils, incubated in the presence (+) or absence (−) of okadaic acid (OA) for the times indicated. The autoradiograph shown is representative of three separate experiments.

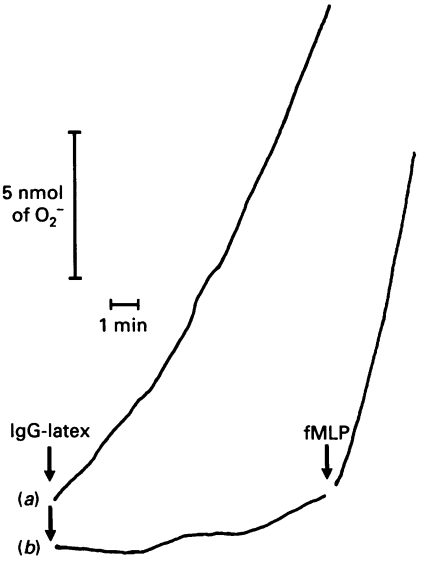

Fig. 3. Effect of pre-treatment with okadaic acid on the stimulation by opsonized latex

Superoxide generation by untreated neutrophils stimulated with non-ingestible opsonized latex particles (*a*) and by okadaic-treated neutrophils first stimulated with latex particles and subsequently with 20 nM-fMet-Leu-Phe (*b*). Two identical experiments gave the same results.

resulted in the very rapid onset of a rather brief NADPH oxidase response (Fig. 2*b*). Incubation with okadaic acid followed by stimulation with fMet-Leu-Phe produced an increased lag phase, followed by a massive enhancement of the duration of activation (Fig. 2*b*). The burst was still active 7 min after stimulation and the total amount of superoxide generated increased by 7-fold, to 350 nmol of O_2^-/10^7 cells, compared with untreated cells, which produced 50 nmol/10^7 cells over a 2 min burst. When stimulation was performed with an optimal dose of fMet-Leu-Phe (100 nM), pre-treatment with okadaic acid produced a lengthening of the burst from 4 min to 7.2 min and an increase in the superoxide generated from 130 to 562 nmol/10^7 cells.

A maximum enhancement of the response to fMet-Leu-Phe was achieved after preincubation with 1 μM-okadaic acid for > 30 min or with 0.2 μM for 60 min, but there was nearly no enhancement after pre-treatment with 0.1 μM-okadaic acid for up to 60 min.

Effect of okadaic acid on the stimulation by activators of protein kinase C (PKC) and opsonized particles

In contrast with the stimulation by fMet-Leu-Phe, the response to PMA, normally a potent activator of the oxidase through PKC, was almost completely blocked by okadaic acid (Fig. 2*c*), even at concentrations of PMA as high as 0.32 μM. Inhibition by okadaic acid was observed at 0.2 and 1.0 μM, but not at 0.1 μM. Because we do not know the local concentrations of okadaic acid within the cytoplasm, inhibition by a relatively low concentration cannot be taken as evidence for the involvement of phosphoprotein phosphatase-2A rather than phosphatase-1 (MacKintosh & Cohen, 1989). The same results were obtained when dioctanoylglycerol, at concentrations between 1 and 10 μM, or opsonized latex particles (Fig. 3) were used as the stimulus. Okadaic-acid-treated cells that had failed to respond to PMA, dioctanoylglycerol or opsonized latex particles did respond to subsequent

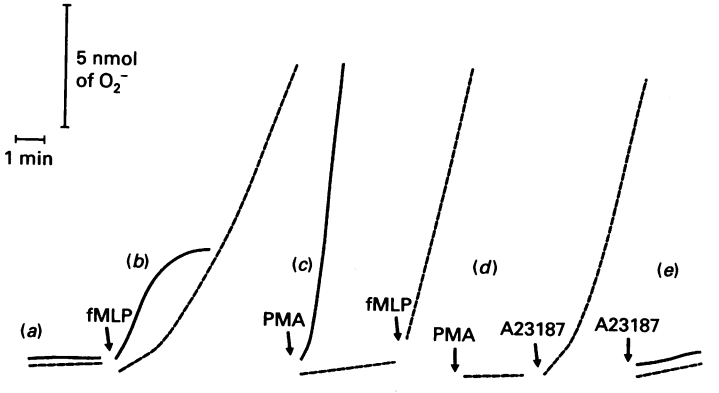

Fig. 2. Superoxide generation by neutrophils stimulated with fMet-Leu-Phe or PMA after pre-treatment with okadaic acid

Superoxide generation by neutrophils untreated (continuous lines) or pre-treated with okadaic acid (broken lines) after the following additions: none (*a*); 10 nM-fMet-Leu-Phe (*b*); 50 nM-PMA, followed by 10 nM-fMet-leu-Phe for okadaic-treated cells only (*c*); 50 nM-PMA, followed by 0.1 μM-A23187 (*d*); 0.1 μM-A23187 (*e*). The curves shown are representative of at least six separate experiments.

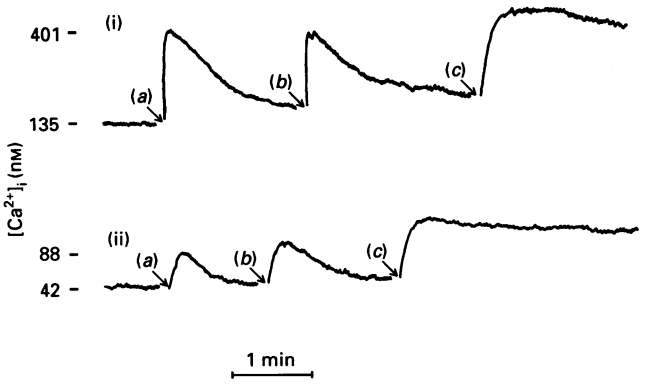

Fig. 4. $[Ca^{2+}]_i$ in untreated or okadaic-acid-treated neutrophils, before and after stimulation with fMet-Leu-Phe

$[Ca^{2+}]_i$ (fura-2 fluorescence) was measured in untreated (i) and okadaic-acid-treated (ii) neutrophils, before and after addition of 20 nM-fMet-Leu-Phe (*a*), 0.2 μM-fMet-Leu-Phe (*b*) and 0.1 μM-Br-A23187 (*c*). Representative results of one out of three similar experiments are shown.

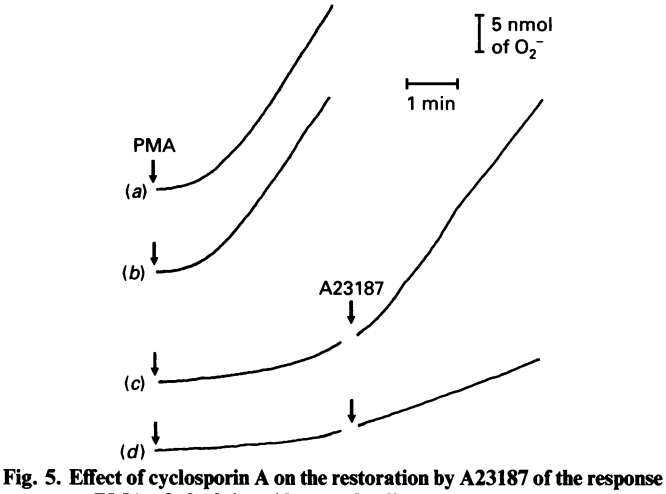

Fig. 5. Effect of cyclosporin A on the restoration by A23187 of the response to PMA of okadaic-acid-treated cells

Superoxide generated in response to 32 nM-PMA was measured. Neutrophils were preincubated in the absence (*a*) or presence (*b*) of cyclosporin A, or pre-treated with okadaic acid (*c*) or with okadaic acid plus cyclosporin A (*d*), then stimulated and 0.1 μM-A23187 was subsequently added. Similar results were obtained in two other experiments.

stimulation with fMet-Leu-Phe in the same way as when fMet-Leu-Phe was the only stimulus (Figs. 2*c* and 3*b*).

Ca^{2+}

The Ca^{2+} ionophore A23187 completely reversed the inhibition by okadaic acid of PMA activation at concentrations well below those that result in stimulation by the ionophore alone (Figs. 2*d* and 2*e*). This suggested that the inhibition might be related to changes in $[Ca^{2+}]_i$. There was a substantial decrease in $[Ca^{2+}]_i$ to 40–50 % of the normal value in okadaic-acid-treated cells (Fig. 4, baselines before stimulation). fMet-Leu-Phe induced a transient rise in $[Ca^{2+}]_i$ immediately after stimulation of control cells, and this was much decreased, but not abolished, by pre-treatment with okadaic acid (Fig. 4). Addition of 4-bromo-A23187 after Ca^{2+} had returned to basal concentrations produced a more permanent increase in $[Ca^{2+}]_i$.

Cyclosporin A completely inhibited the restoration of the

Table 1. Phosphorylation of cytochrome b_{-245} subunits in neutrophils pre-treated with okadaic acid

Conditions of incubation and isolation of the cytochrome b_{-245} subunits are described in the Experimental section. The phosphoprotein/total protein ratio is arbitrarily considered as 1 for non-stimulated neutrophils. Total protein = area of p22- or gp91-*phox* peaks obtained by scanning Coomassie-Blue-stained gels. Phosphoprotein = c.p.m. in p22- or gp91-*phox* bands excised from Coomassie-Blue-stained gels after having been located by autoradiography.

	Ratio phosphoprotein/total protein	
Stimuli	p22-*phox*	gp91-*phox*
None	1	1
A23187	0.80	0.91
PMA	0.73	1.15
A23187 + PMA	1.58	3.03

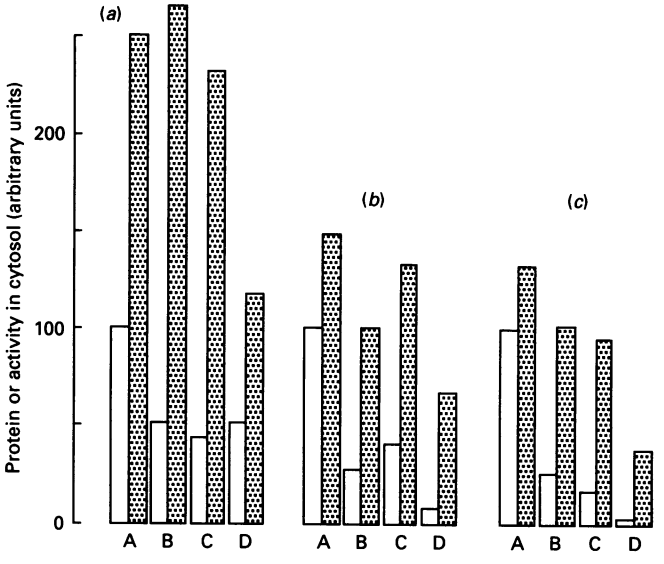

Fig. 6. Translocation of protein kinase activity and of p47-*phox* and p67-*phox* after pre-treatment with okadaic acid

Protein kinase activities (*a*) and amounts of p47-*phox* (*b*) and p67-*phox* (*c*) were measured in cytosols from untreated (□) and okadaic-acid-treated (▨) neutrophils, incubated without any additions (A) or with 0.1 μM-A23187 (B), 10 nM-PMA (C) or A23187 plus PMA (D). Results were normalized against the values for untreated, non-stimulated, neutrophils (A, □), which were arbitrarily taken as 100. Two separate experiments showed identical results.

response of okadaic-acid-treated cells to PMA by A23187 (Figs. 5*c* and 5*d*), whereas it did not itself affect the response of untreated cells to PMA (Figs. 5*a* and 5*b*). This suggested the possible involvement of calcineurin, a Ca^{2+}/calmodulin-dependent serine/threonine phosphatase which is inhibited by cyclosporin A (Liu *et al.*, 1991).

Cytochrome b_{-245} phosphorylation in neutrophils treated with okadaic acid

Stimulation of neutrophils with PMA induces the phosphorylation of both subunits of the cytochrome b_{-245}, p22-*phox* and gp91-*phox* (Garcia & Segal, 1988). This phosphorylation did not increase above basal levels when okadaic-acid-treated neutrophils were incubated with PMA or with sub-optimal concentrations of A23187 (Table 1), in keeping with the near absence of superoxide

generation in those cases (Fig. 2). Incubation with the combination of A23187 and PMA, in which case superoxide generation is restored (Fig. 2*d*), produced a marked enhancement of the phosphorylation of both cytochrome subunits (Table 1).

Translocation of proteins from cytosol to membranes

The proteins p47-*phox* and p67-*phox*, essential for the activity of the NADPH oxidase, translocate to the membranes upon stimulation of neutrophils with PMA (Clark *et al.*, 1990). Protein kinases have also been shown to translocate from the cytosol to the membranes in a Ca^{2+}-dependent manner (Wolfson *et al.*, 1985; Phillips *et al.*, 1989). We observed that treatment with okadaic acid before cell stimulation inhibited all these translocations (Fig. 6), suggesting that this could be the reason for the inhibition of superoxide generation in response to PMA (Fig. 2). Moreover, the Ca^{2+} ionophore A23187 restored the translocation of these proteins (Fig. 6), while at the same time restoring superoxide generation.

DISCUSSION

The NADPH oxidase of neutrophils is activated to produce superoxide by a variety of agents, with a lag phase between addition of the stimulus and initiation of the response. This lag phase is thought to be caused by a sequence of intermediate steps which take place after triggering, before the onset of the burst of oxygen consumption. After the maximum rate of superoxide generation has been achieved, deactivation occurs, a process which is agonist-dependent. Instead, the duration of the lag is the same for surface agonists such as C5a, leukotriene B_4, fMet-Leu-Phe and platelet-activating factor (Wymann *et al.*, 1987). When the stimulus is the bacterial peptide fMet-Leu-Phe, the duration of the response is 2–3 min. In contrast, when PMA or permeant diacylglycerols elicit the respiratory burst through a direct effect on PKC, the lag phase is longer (about 25 s) and deactivation does not seem to take place.

Phosphorylation of the cytosolic protein p47-*phox*, which is part of the NADPH oxidase system, appears to be necessary for activation of the burst. The kinetics of activation of the burst and phosphorylation of p47-*phox* correspond to each other (Heyworth & Segal, 1986), although differences were observed in the degree of phosphorylation in response to different stimuli, for example PMA elicited a higher level of phosphorylation than fMet-Leu-Phe. The direct correlation between activation and phosphorylation prompted us to study the effect of the potent inhibitor of protein phosphatases okadaic acid (Bialojan & Takai, 1988) on the NADPH oxidase activity of neutrophils. It was thought that an enhancement of steady-state protein phosphorylation might trigger activity or prolong the respiratory burst. Although no direct activation was observed, okadaic acid greatly prolonged oxidase activity after stimulation with fMet-Leu-Phe. This indicated that dephosphorylation might be involved in the deactivation of the NADPH oxidase.

In contrast, we observed an almost total inhibition of the response of neutrophils to PMA after treatment with okadaic acid. However, the PMA-unresponsive neutrophils did respond normally to subsequent stimulation with fMet-Leu-Phe. This was not altogether surprising, as PKC-activating agents have been found to employ different mechanisms of activation. In particular it is known that, unlike PMA, fMet-Leu-Phe stimulation is insensitive to or even up-regulated by staurosporine and therefore is unlikely to involve PKC (Gerard *et al.*, 1986; Berkow *et al.*, 1987; Combadiere *et al.*, 1990). The okadaic-acid-induced inhibition of stimulation by PMA was overcome by the addition of the Ca^{2+} ionophore A23187, thus suggesting a role for Ca^{2+} in the restoration of activity. Incubation with okadaic acid decreased $[Ca^{2+}]_i$ in unstimulated neutrophils by 50–60% and decreased the transient increase in Ca^{2+} after stimulation with fMet-Leu-Phe. Although it has been shown that PMA can activate the oxidase at 'vanishingly low concentrations of calcium' (Di Virgilio *et al.*, 1984), okadaic acid clearly alters this state of affairs, possibly by its influence on the intracellular location of kinases.

The response of neutrophils to fMet-Leu-Phe also appeared to have been affected, in that the lag phase after triggering was markedly increased, although this effect was probably masked by the massive increase in the duration of the stimulation. That the enhancement of the burst was not prevented by a diminished $[Ca^{2+}]_i$ could be due to the fact that the major cause of the rise in $[Ca^{2+}]_i$ after stimulation is an influx from the extracellular space (Lew *et al.*, 1984), and this influx probably causes a local Ca^{2+} concentration at the sub-membrane level which is sufficient for the respiratory burst to occur even if the overall $[Ca^{2+}]_i$ is low. Indeed, the existence of regional changes in $[Ca^{2+}]_i$ in response to fMet-Leu-Phe has been documented (Sawyer *et al.*, 1986). In contrast, stimulation by PMA does not produce any increase in $[Ca^{2+}]_i$.

The reversal of the effect of okadaic acid on $[Ca^{2+}]_i$ by ionophore A23187, together with the restoration of the agonist action of PMA, indicate that a Ca^{2+}-dependent protein might be involved in the process. A potential candidate is the phosphoprotein phosphatase type 2B calcineurin, the only class of phosphatase that requires Ca^{2+} and calmodulin for activity (Cyert *et al.*, 1991). We therefore determined the effect of cyclosporin A, an inhibitor of calcineurin (Liu *et al.*, 1991), on the ability of A23187 to reverse the inhibition of PMA stimulation in okadaic-acid-treated cells. It was found that cyclosporin A inhibited almost completely the stimulatory action of A23187. It is possible that the activity of some component of the NADPH oxidase is inhibited by phosphorylation, under the conditions created by okadaic acid, and this block is overcome by the enhanced activity of calcineurin as a result of the A23187-mediated influx of Ca^{2+} into the cells.

The diminished translocation of p47-*phox*, p67-*phox* and protein kinase activity from cytosol to membranes that we observed after treatment with okadaic acid seems to be the cause of the inability to produce superoxide on stimulation by PKC activators. Incubation with the Ca^{2+} ionophore A23187 restored both the translocation of those proteins and the superoxide-generating activity upon stimulation with PMA, probably owing to the observed increase in $[Ca^{2+}]_i$. It is worth noting that translocation seems to be necessary, but not sufficient, for the expression of NADPH oxidase activity, as demonstrated by the increased translocation produced by the ionophore A23187 at a concentration which does not elicit respiratory-burst activity (Figs. 2 and 6).

The dramatically different effect of pre-treatment with okadaic acid on the subsequent stimulation by fMet-Leu-Phe or PMA/diacylglycerol provides further evidence of a substantial heterogeneity in the activation of the respiratory burst. Okadaic acid should prove very useful for the evaluation of the mechanisms involved in this process.

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