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MINI-REVIEW

Protein Phosphorylation Associated with the Stimulation of Neutrophils. Modulation of Superoxide Production by Protein Kinase C and Calcium

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Abstract

Neutrophils and other phagocytic cells of the immune system possess a superoxide-generating oxidase system which is essential for the efficient killing of microbes. The system is activated by a wide variety of stimuli, some of which operate through pathways involving protein kinase C (PKC), while others appear not to. The PKC-dependent pathway is probably the major signal transduction route for most of the stimuli. Alterations in cellular Ca^{2+} and diglyceride levels can have a pronounced stimulatory effect on this pathway by their ability to synergistically activate PKC. This review discusses PKC, the different interactions of this kinase with the plasmalemma that are important in superoxide production, the synergy between Ca^{2+} and diglyceride, and the nature of the phosphoproteins involved. Evidence supporting the existence of the PKC-independent pathway is also reviewed.

Key Words: Phagocyte; neutrophil; chronic granulomatous disease; protein kinase; protein phosphorylation; superoxide; NADPH oxidase; phorbol ester; chemotactic peptide; synergy.

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Introduction

Phagocytic white blood cells (e.g., neutrophils) produce substantial amounts of superoxide (O_2^-)[4] during phagocytosis, or upon perturbation of their plasmalemma by a variety of agents. Superoxide is a major component of the oxygen-dependent antimicrobial and cytocidal arsenal of these cells (for review, see Badwey and Karnovsky, 1980; Curnutte and Babior, 1987). The enzyme system responsible for generating O_2^- is present in the plasmalemma and phagocytic vacuoles of stimulated neutrophils. It produces O_2^- according to the following stoichiometry:

$$NADPH + 2O_2 \longrightarrow NADP^+ + 2O_2^- + H^+$$

This system is complex and contains several components, including a low-potential b-type cytochrome, a flavoprotein, a 67-kDa protein, and a 47-kDa phosphoprotein. In unstimulated cells the oxidase system is dormant and dissociated, with components residing in both the cytosol and the membrane. Upon stimulation there is a translocation of the soluble components to the plasmalemma where the active oxidase is assembled (McPhail *et al.*, 1985; Clark *et al.*, 1987; Ligeti *et al.*, 1988; Tanaka *et al.*, 1988; Babior *et al.*, 1988). Phosphorylation of the 47-kDa protein is closely associated with this process (e.g., Segal *et al.*, 1985; Heyworth *et al.*, 1989b).

Stimuli that elicit NADPH-oxidase activity include substances that bind to specific receptors, such as chemotactic peptides and phorbol esters, and those that intercalate with, and disorganize the plasma membrane (e.g., retinoids) (for review, see Badwey and Karnovsky, 1986). Until the early 1980s very little was known about the biochemical mechanism(s) responsible for initiating O_2^- release. However, Schneider *et al.* (1981) reported that treatment of neutrophils with PMA or the chemotactic peptide fMLP resulted in the enhanced phosphorylation of several proteins. In particular, two proteins with molecular masses of 47 and 49 kDa were intensely labelled, with kinetics compatible with their involvement in O_2^- production. Perhaps the seminal observation that opened the way for a clearer understanding of this signal transduction pathway was the discovery by Castagna *et al.* (1982) that the tumor-promoting phorbol esters bind to and activate PKC. They act by substituting for the endogenous activator of this kinase, *sn*-1,2-diglyceride

[4]The abbreviations used are: O_2^-, superoxide; PMA, 4β-phorbol 12-myristate 13-acetate; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; PKC, protein kinase C: DG, diglyceride; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; CGD, chronic granulomatous disease; DiC8, *sn*-1,2-dioctanoylglycerol; PDGF, platelet-derived growth factor; GM-CSF, granulocyte-macrophage colony stimulating factor; Ins(1,4,5)P_3, inositol(1,4,5)-trisphosphate; PDBu, 4β-phorbol 12,13-dibutyrate; HA1004, *N*-(2-guanidinoethyl)-5-isoquinoline sulfonamide; SDS PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

(DG) (e.g., Nishizuka, 1984). Since PMA had been recognized as a potent stimulator of the oxidative metabolism of neutrophils for nearly a decade (Repine *et al.*, 1974), links between lipid metabolism, activation of PKC, and stimulation of the NADPH-oxidase system were immediately recognized and explored.

Recent mini-reviews in this series have covered aspects of this signalling pathway: the chemotactic receptor (Jesaitis and Allen, 1988); the generation of second messengers (Lambeth, 1988); and components of the oxidase system (Borregaard, 1988; Parkinson and Gabig, 1988). Our contribution will focus on recent discoveries concerning PKC that are relevant to O_2^- generation, the phosphoproteins involved, and synergistic interactions between PKC and Ca^{2+} which may regulate this reponse. Evidence for a stimulatory pathway that is independent of PKC is also discussed.

Properties of Protein Kinase C and Evidence Supporting Its Role in Superoxide Production

Protein kinase C catalyzes the phosphorylation of its substrate proteins on serine and threonine residues that are in the vicinity of basic groups. The most potent peptide substrates contain basic residues on both sides of the phosphorylation site, although this is not essential (House *et al.*, 1987). Phospholipid, Ca^{2+}, and DG are required by the enzyme for optimal catalytic activity with histone III-S as the substrate, which is generally used for assaying PKC (Castagna *et al.*, 1982). In the absence of these activators the kinase is maintained in an inactive state by a pseudosubstrate sequence in its primary structure that is thought to interact with and inhibit the active site (House and Kemp, 1987). The inactive enzyme is recovered in the soluble fraction of unstimulated neutrophils when the medium employed during fractionation contains chelators of divalent cations ($> 2.0\,mM$) (Nishihira and O'Flaherty, 1985; Wolfson *et al.*, 1985). The addition of phospholipid and high levels of Ca^{2+} ($K_a = 70\,\mu M$) partially activate PKC, while the subsequent addition of DG activates the enzyme maximally and reduces this requirement for Ca^{2+} to physiological levels ($< 1.0\,\mu M$). Certain tumor promoters (e.g., PMA, mezerein) can substitute for DG in this role (Castagna *et al.*, 1982). It must be emphasized, however, that the kinetic properties of this enzyme are highly dependent upon the protein substrate utilized, and results with histone III-S may not be applicable to other target proteins (Wolf *et al.*, 1984; Bazzi and Nelsestuen, 1987; Dell *et al.*, 1988).

The levels of activity of PKC in neutrophils are very high, and are comparable to those found in the richest sources of this enzyme (i.e., brain, spleen) (Helfman *et al.*, 1983). The levels in neutrophils exceed those of the

cyclic nucleotide-dependent protein kinases (Helfman *et al.*, 1983). There is strong evidence that PKC plays a role in the stimulation by tumor promoters of O_2^- release from neutrophils. Synthetic diglycerides and tumor promoters that activate PKC stimulate rates of O_2^- release from neutrophils that are similar to those observed with the most effective physiological stimuli, such as opsonized zymosan and fMLP (Curnutte *et al.*, 1984; Robinson *et al.*, 1985; Badwey and Karnovsky, 1986; Cox *et al.*, 1986). This is not generally true for most cells that can be activated by PMA. For example, when platelets and glomerulosa cells are treated with this phorbol ester they release serotonin and aldosterone respectively, but at rates considerably slower than those observed with their physiological agonists (Naka *et al.*, 1983; Kojima *et al.*, 1985). The high rates of O_2^- release from neutrophils instigated by tumor promoters make these cells an excellent paradigm for studying PKC-mediated stimulatory responses.

The association of PKC with membrane fulfills the phospholipid requirement of the enzyme. Treatment of neutrophils with tumor promoters results in a redistribution of the activity of PKC from a soluble to a particulate fraction which is stable in the presence of Ca^{2+} chelators (Fig. 1; Wolfson *et al.*, 1985; Nishihira and O'Flaherty, 1985). This translocation precedes the activation of the oxidase and exhibits a dose–response curve for

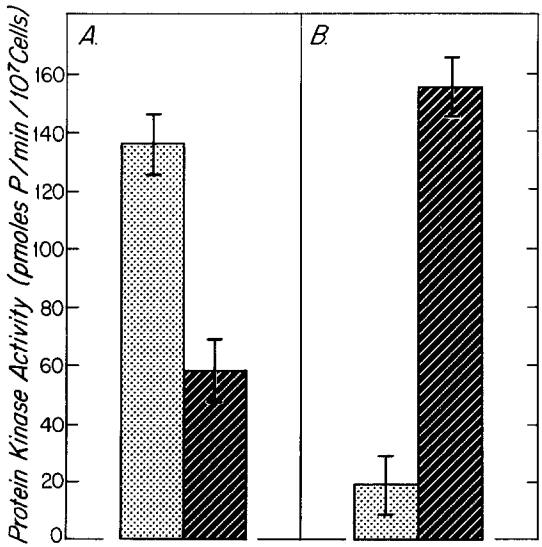

Fig. 1. Effect of the tumor promoter mezerein on the distribution of PKC activity in neutrophils. Results depict the levels of PKC activity in the soluble (stippled bars) and membrane (hatched bars) fractions of unstimulated (A) and stimulated (B) guinea pig neutrophils. Cells were stimulated with an optimal amount of mezerein (38 nM) for 3 min (for details, see Badwey *et al.*, 1988).

PMA similar to that observed for activation of the oxidase system (Wolfson *et al.*, 1985). Neutrophils treated with tumor promoters exhibit an intense phosphorylation of a 47-kDa protein (Schneider *et al.*, 1981) that has been linked to O_2^- release (see next section). This protein is a substrate for PKC *in vitro* (White *et al.*, 1984; Ohtsuka *et al.*, 1986; Kramer *et al.*, 1988b). Finally, a number of inhibitors of PKC (e.g., H-7, sphingosine) block O_2^- release from neutrophils stimulated with tumor promoters (e.g., Wilson *et al.*, 1986; Badwey *et al.*, 1988; Nath *et al.*, 1989; Kramer *et al.*, 1989), and inhibit the phosphorylation of the 47-kDa protein (Ohtsuka *et al.*, 1986; Kramer *et al.*, 1989; Badwey *et al.*, 1989b; Heyworth *et al.*, 1989a).

There is also evidence that PKC plays some role in the initiation of O_2^- production with more physiological stimuli. Stimulation of neutrophils with opsonised zymosan or fMLP results in increases in the levels of *sn*-1,2-diacylglycerols and 1-O-alkyl-2-acylglycerols (Dougherty *et al.*, 1989; Tyagi *et al.*, 1989). Both of these types of diglycerides activate PKC, but with different requirements for Ca^{2+} (Ford *et al.*, 1989). Neutrophils stimulated with opsonised bacteria or fMLP exhibit translocation of PKC to the membrane, which is "tight" with the former and "loose" with the latter stimulus (Christiansen *et al.*, 1987; Horn and Karnovsky, 1986), along with phosphorylation of the 47-kDa protein (Schneider *et al.*, 1981; Heyworth and Segal, 1986). As with PMA, inhibitors of PKC also block O_2^- release from neutrophils stimulated with fMLP (Wilson *et al.*, 1986; Nath *et al.*, 1989; Kramer *et al.*, 1989; Badwey *et al.*, 1989b).

While these data are suggestive of a role for PKC in the activation process with these stimuli, they are by no means conclusive. There is a question as to whether DG is produced fast enough to have a role in initiating O_2^- production in fMLP-stimulated neutrophils (Rider and Niedel, 1987). Moreover, a protein kinase other than PKC is activated during the stimulation of these cells by fMLP (Huang and Laramee, 1988), and it is possible that this kinase also phosphorylates the 47-kDa protein. Finally, the inhibitors of PKC generally used are nonspecific and effect protein kinases other than PKC (Hidaka *et al.*, 1984; Kase *et al.*, 1987; Jefferson and Schulman, 1988). Recent studies indicate that the instigation of O_2^- release by fMLP requires a signal, as yet uncharacterized, in addition to the activation of PKC (Korchak *et al.*, 1988; Dewald *et al.*, 1988; Kramer *et al.*, 1988a).

The 47-kDa Phosphoprotein

Several proteins are phosphorylated when the respiratory burst is activated (for reviews, see Tauber, 1987; Babior, 1988) but for the majority their role in O_2^- generation is purely conjectural. The only phosphoproteins that

are known to be *definitely* involved are the 91- and 22-kDa subunits of cytochrome b_{-245} (Garcia and Segal, 1988) and a protein with an apparent molecular mass of 47 kDa.

Although there was indirect evidence from both kinetic and inhibitor studies implicating protein phosphorylation in the activation of the respiratory burst (e.g., Schneider *et al.*, 1981; White *et al.*, 1984; Okamura *et al.*, 1984), the first conclusive evidence came from studies using the neutrophils of patients with chronic granulomatous disease (CGD) (Segal *et al.*, 1985). CGD is an inherited syndrome characterized by a failure of the patients phagocytic leukocytes to generate O_2^-. There are several forms of the disease, which can be X chromosome-linked or autosomal recessive, depending upon the component of the NADPH oxidase affected by the genetic defect (for review, see Curnutte, 1988). The pattern of protein phosphorylation in normal neutrophils was compared with that in neutrophils from patients with CGD, using PMA as the stimulus. On one-dimensional SDS PAGE, cells from four patients with an autosomal recessive mode of inheritance consistently failed to phosphorylate a protein with an apparent molecular mass of ≈ 44 kDa (Segal *et al.*, 1985). (Depending on the percentage of acrylamide in the gel and the marker proteins used, the apparent molecular mass of the protein varies from 44 to 48 kDa. It is most commonly referred to, and will be here, as the 47-kDa protein.) Neutrophils from X-linked, cytochrome b_{-245}-negative patients revealed an apparently normal pattern of phosphorylation (slight differences are discerned by two-dimensional analysis; see below), indicating that the total absence of phosphorylation of this protein was not secondary to a general failure of the oxidase system. Based on these findings, it was proposed that this molecule is essential for activation of the respiratory burst, either directly as a component of the electron transport chain, or in the regulation of the system. These results have now been confirmed by other laboratories (Kramer *et al.*, 1988b; Nunoi *et al.*, 1988; Okamura *et al.*, 1988b).

The 47-kDa protein is phosphorylated when neutrophils are stimulated by a wide range of agents. The kinetics of activation correspond well with the kinetics of phosphorylation of the protein (Schneider *et al.*, 1981; Heyworth and Segal, 1986; Kramer *et al.*, 1988b). Figure 2 shows the pattern of protein phosphorylation in neutrophils stimulated with IgG-coated latex particles after varying times. Under these conditions the 47-kDa protein is rapidly and heavily phosphorylated and remains so for between 1 and 2 min, before becoming dephosphorylated. Activation of oxygen consumption by opsonised latex follows a similar time course (Heyworth and Segal, 1986). The 47-kDa protein is a substrate for PKC (Kramer *et al.*, 1988b) and is also phosphorylated in neutrophil cytoplasts and cytoplast lysates upon addition of dibutyryl cyclic AMP, suggesting that the 47-kDa protein is also a substrate

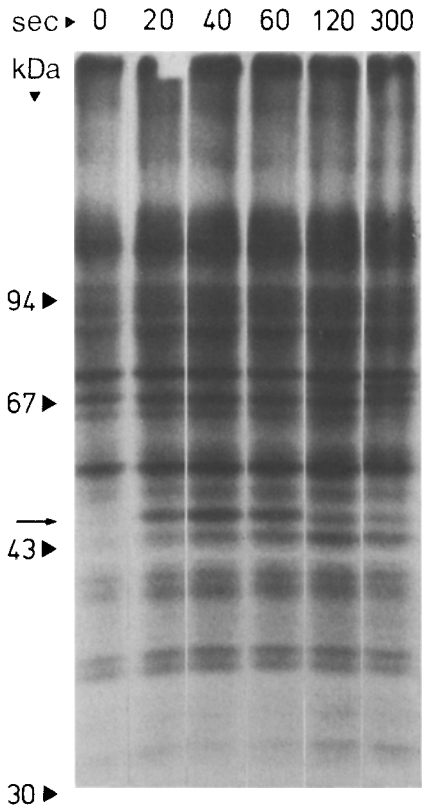

Fig. 2. Time course of protein phosphorylation in neutrophils stimulated with opsonised latex particles. Neutrophils loaded with $^{32}P_i$ were stirred with opsonised latex particles at 37°C. At the indicated times (sec) samples were withdrawn, mixed with trichloroacetic acid, and the precipitated proteins separated by SDS polyacrylamide gel electrophoresis. An autoradiograph of the dried gel is shown, with the positions and size of the marker proteins on the left and the 47-kDa phosphoprotein indicated by the arrow. For full experimental details see Heyworth and Segal (1986).

for cyclic AMP-dependent protein kinase (Kramer *et al.*, 1988a). Dibutyryl cyclic AMP does not stimulate O_2^- production, indicating that the induced phosphorylation alone is not a sufficient signal to activate the NADPH oxidase system.

The subcellular localization of the 47-kDa phosphoprotein was studied in human neutrophils stimulated with PMA (Heyworth *et al.*, 1989b). In most cases of autosomal recessive CGD a failure to phosphorylate this protein is associated with a failure to transport electrons from NADPH to cytochrome b_{-245} (Segal and Jones, 1980). It was therefore hypothesized that in the normal neutrophil the 47-kDa phosphoprotein might become associated

with the electron-transporting components of the NADPH oxidase system. In the most common form of X-linked CGD the genetic deletion results in the absence of the 91-kDa subunit of cytochrome b_{-245} (Royer-Pokora *et al.*, 1986; Dinauer *et al.*, 1987; Teahan *et al.*, 1987). The 22-kDa subunit, although encoded for by a different gene, is also missing in this form of the disease (Parkos *et al.*, 1987; Segal, 1987). The possibility that the absence of these molecules effects the distribution of the 47-kDa phosphoprotein was investigated.

In normal neutrophils labelled with ^{32}P and stimulated with PMA for 2 min there was a dual distribution of the 47-kDa phosphoprotein in the cytosol and membrane fractions. One-dimensional peptide mapping of the protein from the two fractions indicated that they were indeed the same. In neutrophils from X-linked cytochrome b-negative CGD patients the 47-kDa phosphoprotein was only present in the cytosol. In the neutrophils from their heterozygote mothers, which contain intermediate levels of cytochrome b_{-245}, only intermediate levels of this phosphoprotein were translocated to the membrane (Heyworth *et al.*, 1989b). Cytochrome b_{-245}, or a closely linked factor, therefore appears to be essential for membrane-association of the 47-kDa protein. Rotrosen *et al.* (1989) have reported similar findings and in a cell-free system were able to inhibit phosphorylation of the 47-kDa protein with synthetic peptides corresponding to the cytoplasmic carboxy terminal of the 91-kDa subunit of the cytochrome. The reason for this inhibition is unclear. Translocation of the 47-kDa protein, together with that of a 67-kDa protein which is absent in a rarer autosomal recessive form of CGD, has been confirmed by immunoblotting experiments using a polyclonal antiserum which recognizes both proteins (Clark *et al.*, 1989). The 47-kDa protein is one of the cytosolic components required for activation of O_2^- production in the cell-free system. It is missing from the cytosol of neutrophils from the majority of patients with autosomal recessive cytochrome b-positive CGD (Nauseef *et al.*, 1988; Bolscher *et al.*, 1989; Curnutte *et al.*, 1989).

Using two-dimensional gel electrophoresis, Okamura *et al.* (1988a,b) demonstrated a chain of six related phosphoproteins with a molecular mass of ≈ 48 kDa and pI values ranging from ≈ 10 to 7 in normal, stimulated neutrophils (Fig. 3). The two most acidic spots were missing in the X-linked and autosomal cytochrome b-negative CGD patients. In contrast, the complete spectrum of phosphoproteins was missing in the autosomal recessive cytochrome b-positive form of CGD (Fig. 3). If the chain of spots represents the sequential addition of phosphate groups to the protein, accounting for the steady decrease in the pI of the molecule, the absence of the two most acidic spots suggests that the final phosphorylation steps occur after translocation of the protein to the membrane. In two patients with the very rare

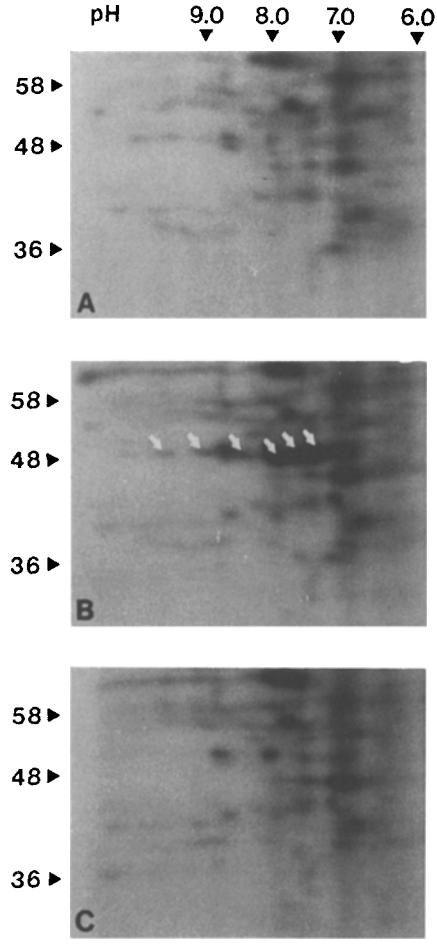

Fig. 3. Two-dimensional autoradiographs showing protein phosphorylation in neutrophils from a normal subject and a patient with autosomal recessive CGD. The white arrows on the autoradiograph in panel B indicate the chain of spots resulting from increasing levels of phosphorylation of the 47-kDa protein in normal, PMA-stimulated neutrophils prelabelled with $^{32}P_i$. The addition of each negatively charged phosphate group lowers the pI of the protein, so the most heavily phosphorylated form, possibly labelled at six or more sites, will be at the acidic end of the chain, with the unphosphorylated precursor protein at the basic end. Compare with panel A from unstimulated normal cells, where only the most basic spots are visible, and panel C from the neutrophils of a patient with autosomal recessive cytochrome b-positive CGD, where all these phosphoprotein spots are totally absent. A series of spots in the 49-kDa region of the gel also exhibited an enhanced incorporation of ^{32}P upon stimulation, and this pattern was not altered in the patient. For full experimental details see Okamura *et al.* (1988a). The positions of molecular mass markers (kDa) are shown on the left, and the pH gradient along the top of the figure. (Data of J. T. Curnutte, N. Okamura, and B. M. Babior.)

X-linked cytochrome b-positive form of CGD, in which a point mutation leads to a nonfunctional cytochrome with a normal heme spectrum (Dinauer *et al.*, 1989), phosphorylation of the 47-kDa group was totally normal (Okamura *et al.*, 1988b). This suggests that the defective cytochrome in these patients still provides a binding site for the 47-kDa protein.

The cDNA that encodes the 47-kDa protein has been cloned using a polyclonal antiserum to screen a cDNA library derived from differentiated HL-60 cells (Lomax *et al.*, 1989; Volpp *et al.*, 1989). Recombinant fusion proteins recognized by the antiserum reconstituted O_2^- production in a cell-free system containing cytosol deficient in the 47-kDa protein. A partial, C-terminal fusion protein containing all the potential sites of phosphorylation (see below) also displayed activity (Volpp *et al.*, 1989).

Northern blot analysis revealed that both normal and autosomal recessive CGD monocytes contain mRNA transcripts for 47-kDa protein, which indicates that the absence of the protein in the patients is not due to a failure of transcription (Lomax *et al.*, 1989). Unfortunately there is a discrepancy between the published nucleotide sequences, resulting in a mismatched region of 54 amino acid residues (247–300). However, the two deduced amino acid sequences have some features in common. There is an N-terminal glycine residue which could be myristoylated, although the aspartate at position 2 and the lack of serine at position 5 may make it an unfavorable substrate for the myristoyltransferase enzyme (Grand, 1989). The C-terminal third of the molecule contains many arginine and serine residues. The former make it very positively charged. As basic residues in the vicinity of serine are a common feature of the sites phosphorylated by serine/threonine protein kinases, this region of the 47-kDa protein contains many such sites. This was not unexpected considering the appearance of this phosphoprotein on two-dimensional gels (Okamura *et al.*, 1989a,b) where it appears as a chain of at least six spots, each representing a more highly phosphorylated form of the protein (Fig. 3). The pI of the protein as predicted by Volpp *et al.* (1989) is 10.4, which as expected is slightly more basic than the first phosphoprotein spot on two-dimensional analysis (see Fig. 3). The deduced amino acid sequences from both groups possess some regions of homology with other proteins, but excessive speculation about these may be premature considering the uncertainty surrounding the sequence (Lomax *et al.*, 1989; Volpp *et al.*, 1989).

Phosphorylation of the Subunits of Cytochrome b_{-245}

In neutrophils and macrophages both the 22- and 91-kDa subunits of cytochrome b_{-245} were phosphorylated when the respiratory burst was stimulated with PMA or IgG-coated latex particles (Garcia and Segal, 1988).

The partially purified proteins were identified by their absence from the cells of patients with X-linked CGD. In patients with autosomal recessive cytochrome b-positive CGD (who lack the 47-kDa phosphoprotein) phosphorylation of the subunits was normal. Neither the stoichiometry of phosphorylation nor the identity of the phosphoamino acid(s) is known. The amino acid sequence of the 22-kDa subunit (Parkos *et al.*, 1988) contains two serine residues (Ser-63 and Ser-168) preceded on the N-terminal side by basic residues (Arg-Lys-Lys-X-Ser) which could be possible sites for phosphorylation by several protein serine/threonine kinases, including cAMP-dependent protein kinase and PKC (Edelman *et al.*, 1987). However, in this sequence arginine is four rather than three residues from the serine. Inspection of the 91-kDa subunit sequence reveals two potential sites (Ser-77 and ser-556) for phosphorylation by PKC, of the form Ser-X-X-Arg (House *et al.*, 1987). In addition, the sequence Arg-Arg-Ser (residues 198–200) occurs at the site of phosphorylation of some substrates of cAMP-dependent protein kinase (Cohen, 1988). The exact identity of the sites phosphorylated on the cytochrome subunits, and the nature of the kinases involved, remain to be determined. The physiological significance of the reaction is also unclear as the time course of phosphorylation of neither subunit paralleled that of increased oxygen consumption. For both subunits it was a later phenomenon (Garcia and Segal, 1988). It could be involved in fully assembling the components of the oxidase system or terminating electron transfer.

Phosphorylation of Other Proteins during Stimulation of the Phagocyte Respiratory Burst

In the first study on protein phosphorylation in human neutrophils two proteins in the 45–50 kDa range were reported to be rapidly phosphorylated in response to fMLP and PMA (Schneider *et al.*, 1981). The 47-kDa protein has been firmly linked to O_2^- production, but the other protein, with a molecular mass of 49 kDa, has received little attention. While studying protein phosphorylation in guinea pig neutrophils, we observed that activation of O_2^- release with PMA was always accompanied by incorporation of ^{32}P into the 49-kDa as well as the 47-kDa protein (Badwey *et al.*, 1989a). In resting cells the intensity of labelling of the 47-kDa protein was very low, while that of the 49-kDa band was usually higher. It was, however, always significantly increased upon stimulation. Phosphorylation of the two proteins only occurred at concentrations of PMA or DiC8 high enough to stimulate O_2^- generation. Maximal phosphorylation of the 49-kDa protein and optimal rates of O_2^- production both required higher concentrations of stimuli than was necessary for maximal phosphorylation of the 47-kDa protein. These studies were all performed with guinea pig neutrophils, but the overall

pattern of phosphorylation and the behavior of the 47- and 49-kDa proteins is very similar to that seen in human neutrophils (Heyworth and Segal, 1986). The 49-kDa protein is phosphorylated normally in patients with CGD (Heyworth, unpublished observation; Fig. 3).

Many growth factor receptors possess tyrosine phosphorylating protein kinase activity, and the phosphorylation/dephosphorylation of tyrosine residues is clearly an important cellular control mechanism (Hunter and Cooper, 1985). The platelet-derived growth factor receptor is one such molecule, and as PDGF has been shown to activate neutrophils (Tzeng *et al.*, 1984), the inevitable link has been made between tyrosine phosphorylation and neutrophil activation. Human neutrophils and promyelocytic leukemia cells contain a cytosolic tyrosine kinase capable of phosphorylating a tyrosine : glutamate copolymer and a peptide based on the *src* protein. Phosphotyrosine phosphatase activity is also present in these cells (Kraft and Berkow, 1987). Stimulation of neutrophils with fMLP (but not with PMA) rapidly increased the level of tyrosine phosphorylation in two membrane proteins with molecular masses of 62 and 125 kDa. The 62-kDa protein is possibly a tyrosine kinase undergoing autophosphorylation (Huang *et al.*, 1988). The relationship between phosphorylation of these proteins and neutrophil activation was not investigated. Wright *et al.* (1988) have reported the tyrosine phosphorylation of 41- and 43-kDa proteins in neutrophils stimulated with PMA, fMLP, and arachidonate. With all three stimuli, O_2^- production paralleled the kinetics of phosphorylation of the 43-kDa protein. Phosphorylation of a $\approx$40-kDa protein on tyrosine (in addition to proteins of 54, 78, and 92 kDa) was also observed in neutrophils treated with GM-CSF (Gomez-Cambronero *et al.*, 1989). Tyrosine phosphorylation of the 40- and 54-kDa proteins also increased in response to fMLP. The GM-CSF response was blocked by pertussis toxin, suggesting the involvement of a G-protein. The tyrosine kinase inhibitor ST 638 decreased the level of phosphorylation of these proteins and inhibited O_2^- production in neutrophils pretreated with GM-CSF and stimulated with fMLP. Taken together, these data indicate that tyrosine phosphorylation has a role in the regulation of the respiratory burst. It may be significant in this context that tyrosine at residue 97 of the 47-kDa protein is a potential site of phosphorylation, as there is an arginine seven residues away on the N-terminal side and an intervening acidic residue (Glu-96) (Volpp *et al.*, 1989; Hunter and Cooper, 1985).

Synergistic Stimulation of Neutrophils and Its Implications

One of the first chemical reactions observed during the stimulation of neutrophils with physiological agents is a phospholipase C-catalyzed

hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce the second messengers, DG and Ins(1,4,5)P_3 (Ohta *et al.*, 1985; Burnham *et al.*, 1989). As noted above, DG activates protein kinase C (Castagna *et al.*, 1982). Ins(1,4,5)P_3 stimulates the release of calcium from its intracellular storage sites (e.g., Berridge and Irvine, 1984). This production of two second messengers in a single reaction may allow for the tight coordination between the activation of PKC and the elevation of intracellular Ca^{2+}. We have been exploring the advantages of this situation.

Stimulation of PKC and the elevation of intracellular Ca^{2+} can be achieved separately by treating cells with tumor promoters which activate the protein kinase (e.g., mezerein) or low levels of the calcium ionophore A23187, respectively. Neutrophils treated with optimal amounts of tumor promoters generate O_2^- at very high rates, whereas cells treated with the calcium ionophore do not (Pozzan *et al.*, 1983; Robinson *et al.*, 1984; Badwey *et al.*, 1988). However, treatment of neutrophils with suboptimal amounts of both an activator of PKC and a calcium ionophore resulted in a dramatic stimulation of O_2^- release (Robinson *et al.*, 1984; DiVirgilio *et al.*, 1984; Penfield and Dale, 1984; Finkel *et al.*, 1987; Badwey *et al.*, 1988). This synergy is illustrated in Fig. 4 for cells treated with A23187 (0.025 μM) and

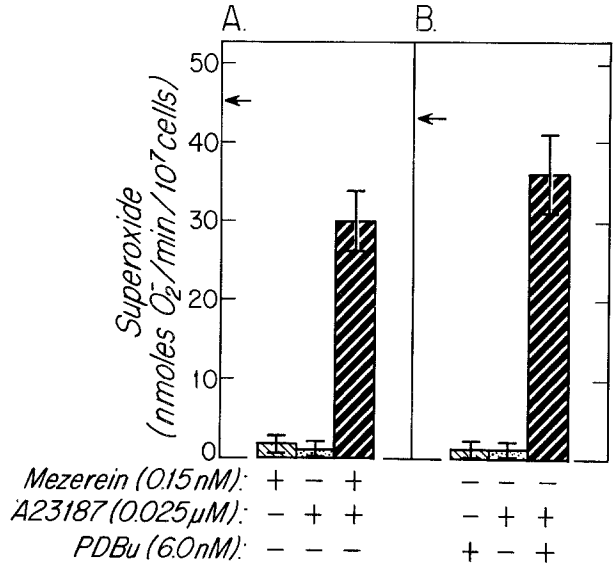

Fig. 4. Synergistic stimulation of superoxide release from neutrophils. Superoxide release was measured from cells treated with suboptimal amounts of the calcium ionophore A23187 (0.025 μM) and either mezerein (0.30 nM) (part A) or phorbol 12,13-dibutyrate (PDBu; 6.0 nM) (part B). At these concentrations, none of the agents alone stimulated significant amounts of O_2^- release.

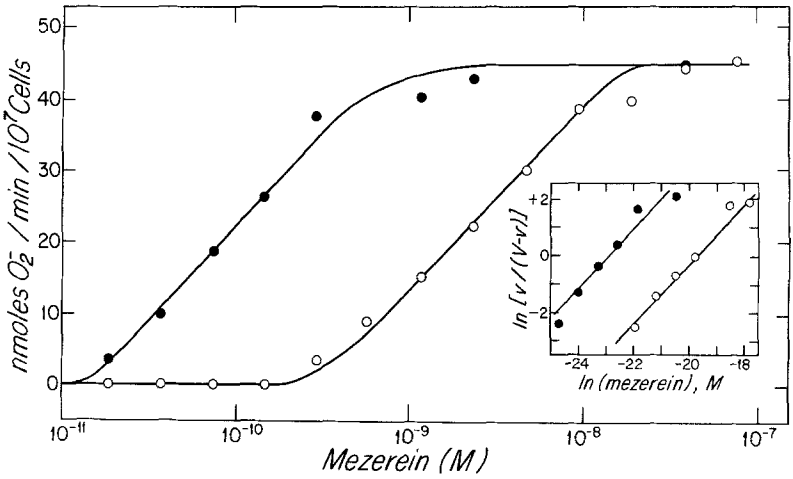

Fig. 5. Effect of the calcium ionophore A23187 on the dose–response curve for mezerein. Superoxide release was measured after stimulation of neutrophils with different concentrations of mezerein in the absence (○) and presence (●) of ionophore A23187 (0.025 μM). The inset shows the Hill plots of these data.

either mezerein (0.15 nM) (part A) or PDBu (6.0 nM) (part B). Other combinations of stimuli can also be effective in promoting synergy (McPhail *et al.*, 1984; O'Flaherty *et al.*, 1985; Bass *et al.*, 1987; Dougherty and Niedel, 1986).

The kinetics of this synergistic response are shown in Fig. 5. Addition of ionophore A23187 to neutrophils dramatically reduced the amounts of the tumor promoter required to stimulate half-maximal O_2^- production, but did not effect the rate of O_2^- production at optimal levels. Thus, elevation of intracellular Ca^{2+} increases the responsiveness of the cell to activators of PKC, providing a rationale for the production of both DG and Ins(1,4,5)P_3 in a single reaction.

As noted above, neutrophils stimulated with tumor promoters exhibit a redistribution of the activity of PKC from a soluble to a particulate fraction (translocation) that is stable in the presence of Ca^{2+} chelators (i.e., "chelator-resistant" or "membrane inserted" PKC) (Fig. 5; Wolfson *et al.*, 1985; Nishihira and O'Flaherty, 1985). To our surprise, neutrophils stimulated synergistically did not exhibit a similar chelator-resistant redistribution of PKC activity, even though the cells released O_2^- at rates comparable to that of cells treated with optimal amounts of tumor promoters (Badwey *et al.*, 1988).

Protein kinase C is a single polypeptide chain that consists of a catalytic and a regulatory domain. The regulatory domain contains the pseudosubstrate sequence, and perhaps the sites which bind DG, Ca^{2+}, and phospholipid,

although these have not yet been identified with certainty. Membrane-bound PKC can be cleaved into its regulatory and catalytic fragments by calpain, a Ca^{2+}-requiring protease. The catalytic fragment is released from the membrane and is active in the absence of Ca^{2+}, phospholipid, and DG (Inoue *et al.*, 1977; Melloni *et al.*, 1985, 1986). We considered the possibility that the increase in cellular Ca^{2+} during synergy may promote this reaction, which could possibly account for the absence of translocation under these conditions. However, the PKC in the soluble fraction of cells stimulated synergistically was dependent upon Ca^{2+} and phospholipid for activity, in an identical manner to the enzyme from unstimulated cells (Badwey *et al.*, 1989).

Protein kinase C can associate with phospholipid vesicles in two steps; reversible binding that requires Ca^{2+} followed by insertion of the enzyme into the membrane to form a complex that is not reversed by Ca^{2+} chelation (Bazzi and Nelsestuen, 1988). To obtain evidence that the reversible complex is active in neutrophils stimulated synergistically, we used inhibitors (H-7, sphingosine) which block PKC by different mechanisms. The isoquinolinesulfonamide H-7 inhibits PKC and the cyclic nucleotide-dependent protein kinases with similar affinities, but is not active against the calmodulin-dependent kinase. HA1004, an analog of H-7, inhibits the cyclic nucleotide-dependent protein kinases but does not affect PKC, and is therefore useful as a control. H-7 is a competitive inhibitor of the substrate ATP and inhibits both the intact PKC molecule and its catalytic fragment that is active in the absence of Ca^{2+} and DG (Hidaka *et al.*, 1984). In contrast, sphingosine inhibits only the intact kinase and competes for the enzyme activator sites (Hannun *et al.*, 1986b; Wilson *et al.*, 1986; Lambeth *et al.*, 1988).

H-7 and sphingosine inhibited O_2^- release from neutrophils stimulated synergistically, and with optimal amounts of tumor promoters. HA1004 was ineffective in cells stimulated in either fashion (Badwey *et al.*, 1988). These data are consistent with a role for PKC in cells stimulated synergistically and provide additional evidence that the catalytic fragment of the kinase is not involved in this reponse. It must be reemphasized here that these compounds can affect protein kinases other than PKC, and it is not certain that cells are equally permeable to H-7 and HA1004.

One possible explanation for the synergy in O_2^- release is that the elevation of cellular Ca^{2+} may enable neutrophils to bind much larger quantities of tumor promoters at suboptimal concentrations. The amount bound during synergistic stimulation may then be comparable to the amount bound by cells treated with optimal amounts of the activators. This hypothesis is examined in Fig. 6, which compares O_2^- release and the specific binding to neutrophils of [^{3}H]PDBu at 100 nM (part A) and 6.0 nM (part B). The higher concentration stimulates a maximal rate of O_2^- production, whereas the lower amount is virtually inactive. If neutrophils treated with 6.0 nM PDBu are also

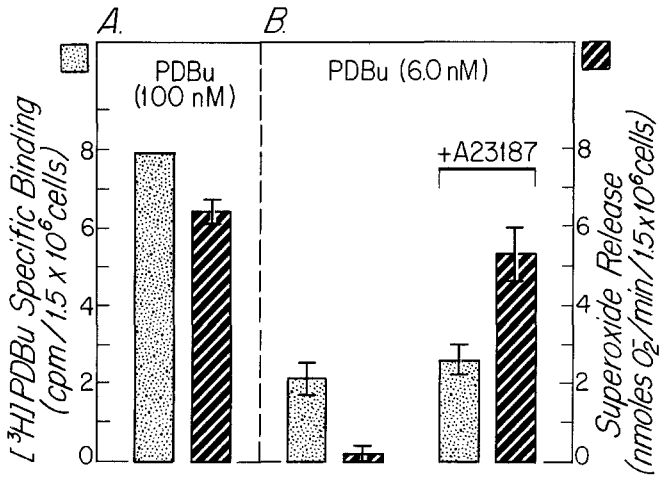

Fig. 6. Specific binding of [^{3}H]PDBu to neutrophils. The specific binding of [^{3}H]PDBu to neutrophils (solid bars) was measured at an optimal concentration (100 nM) (part A), and a suboptimal concentration (6.0 nM) with and without ionophore A23187 (0.025 μM) (part B). The rates of O_2^- release are shown for comparative purposes (striped bars). Data are expressed as the mean $\pm$ S.D. for several preparations of cells. Optimal conditions for binding and O_2^- release were employed. For full details see Badwey *et al.* (1988).

exposed to A23187, the rate of O_2^- release approaches the rate observed for cells stimulated with 100 nM PDBu. While A23187 does increase the specific binding of [^{3}H]PDBu to neutrophils under these conditions by $\approx 25\%$ ($p < 0.01$), the amount specifically bound is still considerably less than that observed at 100 nM PDBu. These data indicate that Ca^{2+} does not cause comparable binding of the PKC activator to cells, but instead is involved in some type of compensating mechanism with the activator. Several caveats are warranted here. By necessity, [^{3}H]PDBu binding measurements involve a washing step to remove unbound PDBu. This procedure is likely to promote dissociation of any loosely bound PKC–phospholipid–PDBu complexes which may be active (Bazzi and Nelsestuen, 1989). Moreover, increases in [^{3}H]PDBu binding do not necessarily correlate with PKC-membrane binding, which in turn does not necessarily correlate with kinase activity (Bazzi and Nelsestuen, 1987).

A general model that can explain the synergy in O_2^- release by neutrophils has been proposed (May *et al.*, 1985; Wolf *et al.*, 1985b). In this model, increased levels of Ca^{2+} lead to increased binding of PKC to the membrane, and this interaction enables the enzyme to respond to suboptimal concentrations of tumor promoters, resulting in an increase in activity. Under these conditions the association of the kinase with the membrane is weak and is readily reversed upon removal of Ca^{2+} (Wolf *et al.*, 1985a). The concentrations

of Ca^{2+} that are achieved in neutrophils by the generation of Ins(1,4,5)P_3 can result in a significant fraction of PKC becoming associated with the plasmalemma (Phillips *et al.*, 1989). Translocation of PKC to the membrane is observed in neutrophils stimulated synergistically when Ca^{2+} chelators are omitted from the medium used to disrupt the cells (O'Flaherty and Nishihira, 1987). This observation and our data with inhibitors of PKC are consistent with the model described above.

The catalytically active form of PKC is a quaternary complex of enzyme protein, phospholipid (provided by the membrane), Ca^{2+}, and the activator (e.g., DG, PMA, PDBu) (Kikkawa *et al.*, 1983). A striking interdependence exists among these cofactors, i.e., changing the concentration of one can markedly alter the affinity of the enzyme for the other two (Wise *et al.*, 1982; Hannun *et al.*, 1986a; Bazzi and Nelsestuen, 1987). In the intact cell, the concentrations of enzyme and membrane may be considered constant. Therefore a high concentration of cellular Ca^{2+}, produced by ionophore A23187, should at least partially compensate for a low level of the activator in forming the active complex of PKC. Such a situation may explain how small changes in [^{3}H]PDBu binding can accompany a large increase in O_2^- release (Fig. 6). Thus, the synergy between Ca^{2+} and PDBu in activating purified PKC (e.g., Bazzi and Nelsestuen, 1987) may be reflected in neutrophils in the synergistic release of O_2^-.

To test this model further, we examined protein phosphorylation in neutrophils stimulated synergistically (Fig. 7; Heyworth *et al.*, 1989a). Treatment of neutrophils with the ionophore alone had little, if any, effect (lane b), while treatment with a low concentration of mezerein (0.3 nM) alone resulted in a small, variable increase in the labelling of the 47-kDa protein (lane c). This increase was always less than that observed during synergy. Treatment of neutrophils with a combination of these agents resulted in a significant incorporation of ^{32}P into the 47-kDa protein together with a smaller but reproducible increase in phosphorylation of the 49-kDa protein. H-7 (200 μM) blocked this phosphorylation of the 47-kDa protein, whereas HA1004 did not (Heyworth *et al.*, 1989a). Thus, this protein, a known substrate for PKC, was phosphorylated during synergy, and this reaction was blocked by H-7, an antagonist of PKC.

It is noteworthy that in neutrophils stimulated synergistically there was a lower level of phosphorylation of both the 47- and particularly the 49-kDa proteins than in cells stimulated with an optimal amount of mezerein, even though the values for O_2^- release were comparable. Neutrophils stimulated synergistically exhibit a number of biochemical phenomena distinct from cells treated with optimal amounts of tumor promoters alone. These include increases in cellular Ca^{2+} (Finkel *et al.*, 1987), phospholipase A_2 activity (Volpi *et al.*, 1985; McColl *et al.*, 1986; Billah and Siegel, 1987), production of certain

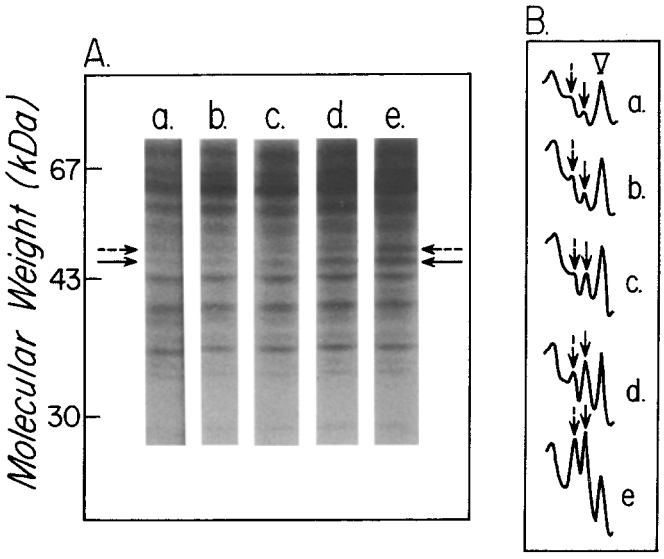

Fig. 7. Protein phosphorylation during synergistic stimulation of neutrophils with low amounts of mezerein and calcium ionophore A23187. Part A compares portions of autoradiographs from neutrophils treated for 3 min with: (a) Me_2SO (0.25% v/v); (b) ionophore A23187 (0.025 μM): (c) low mezerein (0.30 nM); (d) low mezerein (0.30 nM) plus ionophore A23187 (0.025 μM); (e) an optimal amount of mezerein (38 nM). The 47- and the 49-kDa proteins are indicated by the unbroken and the broken arrows, respectively. Part B presents the densitometric scans of these bands, along with peak V which is shown for orientation purposes. For details see Heyworth *et al.* (1989a).

mediators (McColl *et al.*, 1987; McIntyre *et al.*, 1987), and alterations in inositol phospholipid metabolism (Volpi *et al.*, 1985; Tyagi *et al.*, 1988). Some of these phenomena may interact with, bypass, and/or cooperate with these proteins and lower the level of phosphorylation needed to trigger O_2^- release.

In this section we have demonstrated that Ca^{2+} and PKC (via DG) can interact synergistically to dramatically potentiate the neutrophil respiratory burst. Under these conditions, slight changes in either the levels of DG or Ca^{2+} may have pronounced effects on the cellular response. Thus, major control of the overall process may be expected at any enzyme that affects the levels of these second messengers [e.g., Ins(1,4,5)P_3-phosphatase]. Furthermore, we provide evidence that the loosely bound, membrane-associated form of PKC is involved in this response. Activity for this form of PKC has not previously been demonstrated in whole cells and has only recently been demonstrated in a cell-free system (Bazzi and Nelsestuen, personal communication). While the membrane inserted form of PKC is chronically stimulated (i.e., active in the absence of Ca^{2+} and DG), the membrane-associated form is not and requires the continuous generation of DG and/or elevation of Ca^{2+} for

activity (Bazzi and Nelsestuen, 1989). This may allow for the transient stimulation of neutrophils, as observed with fMLP. Finally, these studies demonstrate that the extent of phosphorylation of the 47-kDa protein (and the 49 kDa protein) need not always correlate with the rate of O_2^- release.

Evidence for a Stimulatory Pathway for O_2^- Production That Does Not Involve Protein Kinase C

Compounds that intercalate into membranes and increase the liquid-crystalline fraction of the lipid bilayer (e.g., retinoids) can stimulate high rates of O_2^- release by neutrophils (Badwey *et al.*, 1984, 1986). The NADPH-oxidase system appeared to be the source of the O_2^- in retinal-stimulated cells as neutrophils from patients with CGD failed to respond to this stimulus (Badwey *et al.*, 1986). Retinal generally inhibits, rather than mimics, the stimulatory effects of phorbol esters in a variety of cells (Verma *et al.*, 1979; Kensler and Mueller, 1978). Indeed, a high concentration of retinal (100 μM) has been shown to block O_2^- release and the phosphorylation of the 47-kDa protein in neutrophils stimulated with PMA (Witz *et al.*, 1980; Kensler and Trush, 1981; Pontremoli *et al.*, 1986a,b,c).

A lower amount of retinal (25 μM) stimulates phosphoinositide-specific phospholipase C in neutrophils (Lochner *et al.*, 1986) and promotes a redistribution of the activity of PKC from a soluble to a particulate fraction in these cells (Badwey *et al.*, 1989b). Surprisingly, O_2^- release instigated by retinal is largely resistant to inhibitors of PKC, under conditions where PMA and fMLP stimulation were inhibited. Moreover, in cells stimulated with retinal there was no enhanced phosphorylation of the 47- and the 49-kDa proteins, although evidence of increased protein denaturation with this stimulus may have obscured these events (Badwey *et al.*, 1989b). These data suggest that a stimulatory pathway for O_2^- release may exist that is independent of PKC.

Additional evidence for such a pathway was obtained with a different stimulus, the synthetic diglyceride DiC8 (Badwey *et al.*, 1989c). This agent stimulates high rates of O_2^- and H_2O_2 release by neutrophils (Cox *et al.*, 1986; Wilson *et al.*, 1986; Bass *et al.*, 1988). We observed that O_2^- generation stimulated with a near optimal concentration of DiC8 (2 μM) was markedly inhibited ($> 70\%$) by antagonists of PKC (i.e., 150 nM staurosporine, 200 μM H-7), while at an optimal level of DiC8 (7.8 μM) there was very little inhibition ($\approx 25\%$). However, 150 nM staurosporine reduced the level of phosphorylation of the 47- and the 49-kDa proteins to that observed in unstimulated cells *when either concentration* of DiC8 was used. Neutrophils stimulated with 7.8 μM DiC8 in the presence of staurosporine can therefore

generate large quantities of O_2^- without an enhanced phosphorylation of the 47-kDa protein.

In cell-free systems derived from neutrophils it is possible to activate superoxide generation. These systems usually require membrane, cytosolic protein factors, Mg^{2+}, and an anionic lipid (e.g., SDS, fatty acids, phosphatidate) (Curnutte, 1985; McPhail *et al.*, 1985; Bellavite *et al.*, 1988). Superoxide production is potentiated by GTP or its nonhydrolyzable analog GTP-γ-S (Gabig *et al.*, 1987; Ligeti *et al.*, 1988, 1989). Phosphatidate is more effective at low concentrations than arachidonate in triggering this system (Bellavite *et al.*, 1988). Some of the cell-free systems do not appear to require PKC, as there is no requirement for ATP (Curnutte *et al.*, 1987; Ligeti *et al.*, 1988) and they are insensitive to H-7 (Cox *et al.*, 1987; Bellavite *et al.*, 1988). The 47-kDa protein is an essential cytosolic component of the cell-free system (Caldwell *et al.*, 1988; Volpp *et al.*, 1988; Curnutte *et al.*, 1989; Lomax *et al.*, 1989). It therefore appears that this protein can be modified for its role in O_2^- production either by phosphorylation, or by the presence of an anionic lipid. According to the predicted amino acid sequence of the 47-kDa protein (Lomax *et al.*, 1989; Volpp *et al.*, 1989) the majority of potential phosphorylation sites are in the very basic C-terminal region. The result of phosphorylation would be to neutralize the strong positive charge in this region, possibly subtlely changing the conformation and/or promoting interaction with other components of the system (such as cytochrome b_{-245}—see above). Binding of anionic lipid to this region would also reduce the positive charge, conceivably with the same consequences.

Neither retinal or DiC8 can substitute for the anionic lipid in the cell-free system (Traynor *et al.*, 1989; unpublished data), but phosphorylation of DiC8 to phosphatidate can be catalyzed in cells by diglyceride kinase (May *et al.*, 1986). Retinal stimulates phospholipase C in neutrophils, which generates diglyceride as a product (Lochner *et al.*, 1986). It is therefore possible that phosphatidate generated in this fashion may reduce or obviate the need for phosphorylation of the 47- (and perhaps the 49-) kDa protein during stimulation of whole cells, under certain circumstances. These points are summarised in Fig. 8. This scheme also provides a partial explanation for the existence of a phospholipase D in neutrophils that is rapidly activated upon cell stimulation (e.g., Pai *et al.*, 1988a,b). Interestingly, this phospholipase also appears to be stimulated by both PKC-dependent and independent pathways (Tetterhorn and Mueller, 1988). Finally, it should be noted that bone marrow-derived macrophages not exposed to cytokines produce O_2^- in response to opsonized zymosan particles but not PMA. Upon exposure to cytokines they become responsive to PMA (Phillips and Hamilton, 1989). In mouse peritoneal macrophages interferon gamma increases PKC activity (Hamilton *et al.*, 1985).

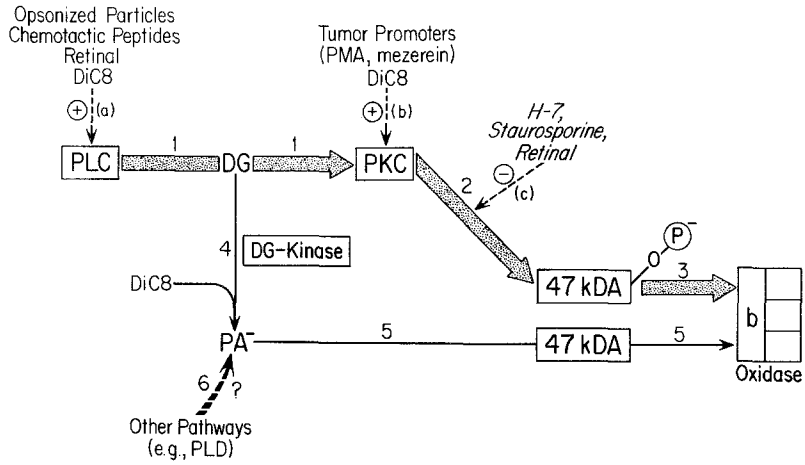

Fig. 8. Stimulatory pathways of superoxide production by neutrophils. Binding of physiological stimuli to neutrophils (point a; opsonised particles, chemotactic peptides) results in the activation of different types of phospholipase C (PLC), which produce diglyceride (DG) as a product (reaction 1). Retinal and *sn*-1,2-dioctanoylglycerol (DiC8) also stimulate the phosphoinositide-specific phospholipase C in these cells (point a; Lochner *et al.*, 1986; Restrepo *et al.*, 1989). Diglyceride activates protein kinase C (PKC), which catalyzes the phosphorylation of the 47-kDa protein (47 kDa) (reaction 2). This phosphoprotein undergoes a translocation from the cytosol to the membrane, where it may bind to the cytochrome b component of the oxidase system (reaction 3; Heyworth *et al.*, 1989b). The exact function of the 47-kDa protein is not known. Diglyceride can be phosphorylated to phosphatidate (PA^-) in a reaction catalyzed by diglyceride kinase (reaction 4). Phosphatidate may also be a second messenger that stimulates O_2^- release by a mechanism that involves the 47-kDa protein but is independent of PKC (reaction 5; Bellavite *et al.*, 1989). Phosphatidate can also be formed via pathways that do not involve the diglyceride kinase reaction. Retinal both stimulates PLC (point a) and inhibits PKC (point c). Thus, DG generated in reaction 1 with this stimulus may only function by the PKC-independent pathway. On the other hand, DiC8 can directly activate PKC (point b) and is phosphorylated to PA^- (reaction 4) and should therefore be able to operate through both pathways. + indicates stimulation; − indicates inhibition.

The question as to whether the cell-free oxidase system represents the PKC-independent pathway that operates in the whole cell remains to be answered. Establishing a role for phosphatidate in this system, and its possible interactions with the 47- and 49-kDa phosphoproteins, would greatly increase our understanding of the mechanisms which activate the NADPH oxidase system. It would also provide a deeper understanding of the mechanisms which modulate the response and may provide novel strategies for intervention.

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Note Added in Proof

Volpp *et al.* (1989, *Proc. Natl. Acad. Sci.* USA **86**, 9563) and Lomax *et al.* (1989, *Science* **246**, 987) have published corrections to their reported nucleotide sequences for the cDNA encoding the 47 kDa protein (see text). The resulting consensus sequence encodes a protein of 390 amino acid residues with a predicted molecular weight of 44, 632. The general properties of the protein and the regions of homology with other proteins are largely unchanged from these originally reported.

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