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MINI-REVIEW

Activation of the Respiratory Burst Oxidase in Neutrophils: On the Role of Membrane-Derived Second Messengers, Ca^{++}, and Protein Kinase C

J. David Lambeth[1]

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Abstract

A major bactericidal mechanism of neutrophils involves activation of the respiratory burst oxidase to generate superoxide (O_2^-). The oxidase is activated rapidly, often within a minute, in response to extracellular signals such as chemoattractants, inflammatory mediators, and invading microorganisms. Increasing evidence indicates that lipases also respond rapidly, releasing potent regulatory molecules from progenitor lipids. Released molecules include potential regulators of protein kinase C—diacylglycerol (DAG), arachidonate, and sphingosine—and levels of one of these, DAG, frequently correlate with O_2^- production. In this author's view, the available data implicate DAG and protein kinase C as key factors in the regulation of the respiratory burst. Herein, the array of activating agonists, the generation and function of some lipid-derived mediators, and evidence pertaining to the participation of protein kinase C are reviewed.

Key Words: Neutrophil; respiratory burst; NADPH-oxidase; protein kinase C; phospholipases; diacylglycerol; second messengers; superoxide; sphingosine; arachidonate.

Agonists of the Respiratory Burst and Their Proposed Loci of Action

O_2^- generation and other responses (degranulation, aggregation) are initiated in neutrophils by a variety of agonists that are summarized in Table I, and described in greater detail below. Regardless of the stimulus, the same O_2^--generating oxidase is utilized (McPhail *et al.*, 1984; McPhail and Snyderman, 1983), and activation pathways converge at some point at, or prior to,

[1]Department of Biochemistry, Emory University Medical School, Atlanta, Georgia, 30322.

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Table I. Stimuli which Activate the Respiratory Burst in Neutrophil

Agonist	Characteristics of stimulus	Characteristics of burst
Opsonized zymosan	Serum-coated particle	Acts after a 90-sec lag, sustained response
fMLP	Receptor mediated	Acts after a 15-sec lag; terminates after ~1–2 min
C5a	Receptor mediated	Similar to fMLP
Con A	Lectin	Acts after about a 60-sec lag; sustained response
PMA	Protein kinase C activator	Acts after ~60 sec, sustained response
DAG	Protein kinase C activator	Similar to PMA, but longer lag
A23187	Calcium ionophore	Sustained response
Arachidonate	May act to alter physical properties of plasma membrane	Duration limited by cytotoxicity
Fluoride	Activates phosphoinositide hydrolysis, perhaps via a G protein	Acts slowly, with a lag of ~8 min; weak activation
PAF	1-alkyl,2-acetyl PC	Weak respiratory burst with rapid termination
LTB_4	Arachidonate metabolite	Acts rapidly (~10 sec); with very rapid termination after ~10 sec

Supporting reference citations are provided in the text.

this enzyme. For all but very weak activators such as leukotriene B_4 (LTB_4)[2] and platelet-activating factor (PAF), the maximal rates of O_2^- generation are similar (5–10 nmol/min/10^6 cells), consistent with the participation of a single oxidase. The kinetics of the response differ, however, both in the lag before onset and in the duration. Differences in the responses to various agonists (e.g., kinetics and strength of response), when correlated with second messenger generation, can, in principal, be exploited to investigate a role for a

[2]Abbreviations: LTB_4, leukotriene B_4; PAF, platelet-activating factor; DAG, diacylglycerol; F^-, fluoride; PA, phosphatidic acid; O_2^-, superoxide; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; 5-HETE, 5-hydroxyeicosatetraenoic acid; and fMLP, formyl-methionyl-leucyl-phenylalanine.

putative mediator. The commonly used agonist and some of their activation properties will first be summarized, followed by a discussion of their effects on potential signaling pathways in neutrophil.

Single Agonist Treatments

Formyl-methionyl-leucyl-phenylalanine (fMLP), a tripeptide analog of some bacterial proteins, is chemotactic at low concentrations, while at high levels it initiates a brief (1–2 min) burst of $O_2^- \cdot$ release that terminates after ~90 sec, possibly due to internalization (Niedel *et al.*, 1979). The peptide binds to a plasma membrane receptor (Goetzl *et al.*, 1981; Hoyle and Freer, 1984; Allen *et al.*, 1986) and its effects are mediated via a pertussis toxin-inhibitable guanine nucleotide regulatory protein (G protein) (Verghese *et al.*, 1985b; Lad *et al.*, 1985; Okamura *et al.*, 1985; Snyderman *et al.*, 1986). Another receptor-mediated chemoattractant is the complement component *C5a*. Although producing a weaker response, its actions are similar to fMLP (Bender and Van Epps, 1985).

Serum-treated zymosan, a phagocytic stimulus, initiates sustained O_2^- generation after a lag of ~90 sec. The zymosan alone does not produce a significant response, indicating that serum components must participate. Among the serum factors that adhere to the zymosan, immunoglobulin G (IgG) (acting via Fc receptors) and the complement component C3b appear to be important, since aspects of activation can be reproduced by zymosan pretreated with these isolated components (Murata *et al.*, 1987). IgG and C3b alone, however, do not activate significantly, suggesting that their mode of presentation is important.

Concanavalin A (Con A), a lectin with bivalent binding properties, produces a sustained respiratory burst after a lag of ~1 min. This stimulus may link and immobilize cell surface components by virtue of its bipartite structure, and in this sense may mimic some aspects of activation by opsonized zymosan. Effects are not prevented by pertussis toxin (Verghese *et al.*, 1985b; Okamura *et al.*, 1985), suggesting a lack of G-protein involvement. Activation is rapidly reversible with α-methyl mannoside and can be reinitiated following its removal, providing evidence for the reversibility of oxidase activation.

Leukotriene B_4 and platelet activating factor are weak activators. For both, the onset of O_2^- release is rapid, but terminates much more rapidly than with fMLP. In particular, LTB_4 results in a very short burst (Omann *et al.*, 1987), with a maximal rate and duration only 10% that of formyl peptide. These compounds bind to low-abundance receptors (Becker, 1986; O'Flaherty *et al.*, 1986), and may function via a G protein (Naccache *et al.*, 1986).

Arachidonate and other fatty acids activate the respiratory burst when added to intact neutrophils (Curnutte *et al.*, 1984; Badwey *et al.*, 1984; Kakinuma, a1974; Badwey *et al.*, 1981). The mechanism differs (e.g., see Maridonneau-Parini and Tauber (1986)] from that for cell-free activation by amphiphiles, reviewed elsewhere in this series. The fatty acid must partition into membranes, and activation correlates with the production of physical changes in the membrane. The response is rapid, but may be limited to ~ 5 min due to cytolytic effects (Cohen *et al.*, 1986). At eary times, activation is reversible by fatty acid-free albumin, and can be reinitiated after albumin removal (Badwey *et al.*, 1984).

Calcium ionophores: A23187 and ionomycin produce a rapid-onset, prolonged respiratory burst (Becker *et al.*, 1979). *Protein kinase C activators* such as phorbol esters (Karnovsky *et al.*, 1985) and synthetic diacyglycerols (DAGs) (Cox *et al.*, 1986) activate after a lag of ~ 1 min, and produce a sustained production of O_2^- beyond 15 min. *Fluoride* (F^-) is a weak activator with an unusually long lag (almost 10 min). F^- activates G proteins, and may function at this level in neutrophils (Strnad *et al.*, 1986; English *et al.*, 1987; Curnutte *et al.*, 1979), although the observed rapid reversibility is atypical of F^- effects on G proteins (English *et al.*, 1987).

Priming and Synergy

The use of two agonists added either together or in sequence (referred to as "priming") often leads to enhanced neutrophil responses. Virtually all combinations of activators have been tested (McPhail *et al.*, 1984; Bender *et al.*, 1983; Robinson *et al.*, 1984; Dale and Penfield, 1984; Bass *et al.*, 1987; Bender and Van Epps, 1985; McCall *et al.*, 1979). In addition to agonists, some compounds that do not produce an observable respiratory burst alone [e.g., 5-HETE (O'Flaherty and Nishihira, 1987; Badwey *et al.*, 1988), GM-CSF (Weisbart *et al.*, 1987), and cytochalasin B (Lehmeyer *et al.*, 1979)] enhance the response to agonists. Typically, the concentration of an agonist required to prime is about tenfold lower than that required for same agonist to activate (McPhail *et al.*, 1984). Responses are often characterized by a lower concentration requirement for the second agonist (Dougherty and Niedel, 1986), a shortening of the lag period (Wymann *et al.*, 1987; English *et al.*, 1981), and/or an increase in the maximal rate of O_2^- generation with the second agonist (Bender and Van Epps, 1985).

While a systematic review of the priming/synergism literature is beyond the scope of this review, two "classes" of priming require mention, and will be elaborated in later sections. First, a low dose (nonactivating) of a protein kinase C agonist primes to a variety of second agonists. PMA or synthetic DAG followed by fMLP is particularly effective in augmenting O_2^- generation (McPhail *et al.*, 1984; Bass *et al.*, 1987). Second, several "Ca^{++}-linked"

stimuli (fMLP, A23187, ionomycin) added prior to or simultaneously with PMA result in enhanced O_2^- generation (Robinson *et al.*, 1984; Dale and Penfield, 1984; Finkel *et al.*, 1987; McPhail *et al.*, *et al.*, 1984; Bender *et al.*, 1983; English *et al.*, 1981).

Second Messenger-Generating Enzymes That Are Activated by Respiratory Burst Agonists

Rather than effecting a simple metabolic sequence, agonist binding orchestrates a cacophony of enzymatic processes, with activation of cyclases, lipases, etc., resulting in the generation of many potential second messengers. Some of these enzymes, along with the lipid substrates on which they act, are shown in Fig. 1. In this section, the activation by agonists of these enzymes will be described. In the next section, data relating to the possible participation of two of the more auspicious second messengers—DAG and Ca^{++}—will be summarized, and the fourth section will examine data relating to the participation of protein kinase C.

Adenylate Cyclase and Guanylate cyclase

Although fMLP and A23187 induce a transient rise in cyclic AMP (Smolen and Weissmann, 1981; Ignarro *et al.*, 1988; Nishizuka, 1986; Simchowitz *et al.*, 1980), pharmacologically induced increases in cyclic nucleotides inhibit rather than activate the respiratory burst (Lehmeyer and Johnston, 1978; Takenawa *et al.*, 1986; Simchowitz *et al.*, 1980). The increase in cyclic AMP may be secondary to the Ca^{++} elevation that occurs with some agonists (Verghese *et al.*, 1985a). Thus, although cyclic nucleotides do not mediate activation, they may participate in a feedback regulatory mechanism.

Phospholipase C and Phosphoinositide Hydrolysis

Figure 1 shows the major lipase-linked pathways to be considered. The preferred substrate for several isolated phospholipases C is phosphatidylinositol 4,5-bisphosphate (PIP_2) (Rebecchi and Rosen, 1987; Ryu *et al.*, 1987). With PIP_2, the products are the protein kinase C activator DAG and inositol-1,4,5-trisphosphate (IP_3), which is subsequently degraded to inositol di- and monophosphate via the sequential action of phosphatases. IP_3 functions as a signal for the elevation of cytosolic Ca^{++} (Prentki *et al.*, 1984).

Many of the receptor-linked agonists in Table I activate the phosphoinositide hydrolytic pathway. The most thoroughly studied [reviewed in Snyderman *et al.* (1986)] is fMLP, which induces phosphoinositide turnover

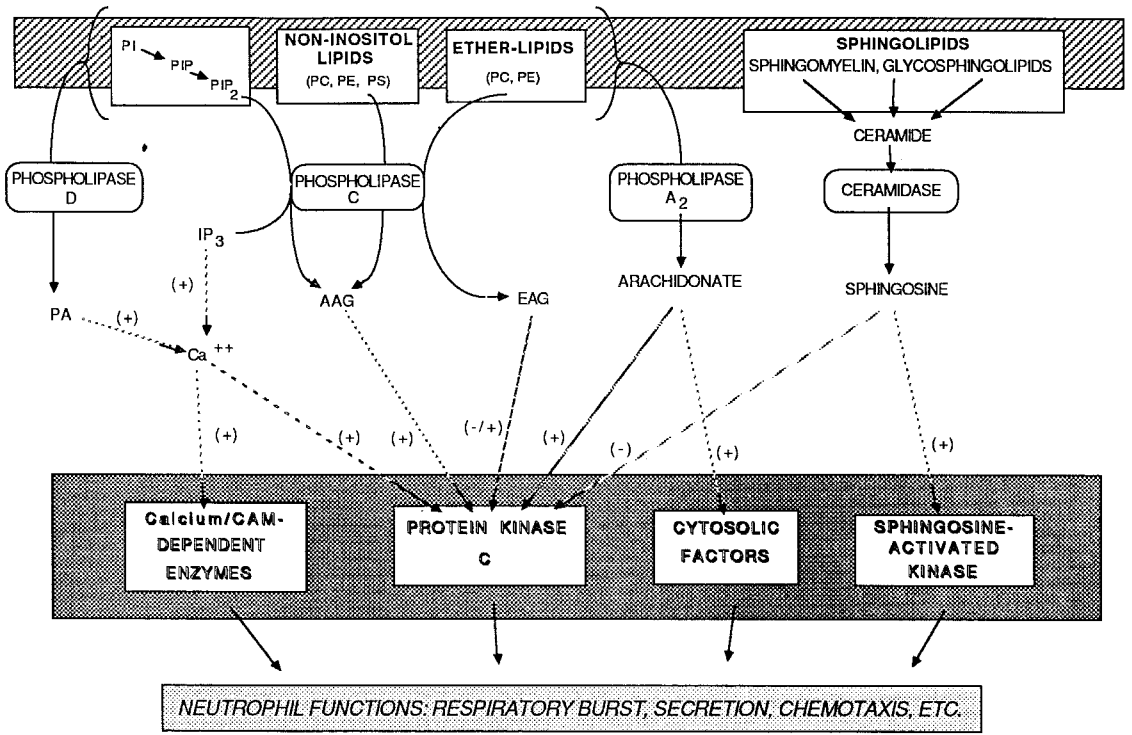

Fig. 1. Potential lipid-linked signaling pathways in the neutrophil. Some relevant lipases are shown in the round-edged boxes, with the lipid substrates on which they act above and their bioactive products below. Abbreviations are as in the text, except for AAG (diacylglycerol) and EAG (1-O-alkyl-2-acyl glycerol). Also shown are effector enzymes upon which the mediator molecules act.

and phosphatidic acid (PA) generation in intact (Bradford and Rubin, 1985; Dougherty *et al.*, 1984; Serhan *et al.*, 1983; Takenawa *et al.*, 1985; Volpi *et al.*, 1983) and permeabilized (Bradford and Rubin, 1986) cells and in isolated plasma membrane preparations (Anthes *et al.*, 1987; Smith *et al.*, 1986). Phosphoinositide hydrolysis is initiated rapidly in response of fMLP, and is sustained for roughly the duration of the respiratory burst. Both O_2^- generation and phosphoinositide turnover are blocked by pretreatment of cells with pertussis toxin (Brandt *et al.*, 1985; Krause *et al.*, 1985; Ohta *et al.*, 1985; Smith *et al.*, 1985). Thus, data are consistent with the participation of a phosphatidylinositol (PI)-derived product as an activation signal.

Phagocytosis of opsonized zymosan stimulates incorporation of [^{32}P]phosphate and [^{3}H]inositol into cellular lipid (Sbarra and Karnovsky, 1960), primarily PI, with increased [^{32}P]phosphate also seen in PA (Karnovsky and Wallach, 1961). The latter is thought to arise from phosphorylation of DAG by the action of diglyceride kinase (Sastry and Hokin, 1966), as shown in the upper reaction in Fig. 2. In pulse-chase experiments, phagocytosis reportedly did not cause a decrease in labeling of the phosphoinositides (Tou and Stjernholm, 1974) and it was concluded that the increased labeling of this pool was due to increased synthesis rather than enhanced turnover. Our recent study (Tyagi, unpublished) as well as another (Meshulam *et al.*, 1988) contradict this view. In our study using [^{3}H]inositol-prelabeled cells, opsonized zymosan caused a large release of inositol phosphates, a decrease in phosphoinositides, and a sustained production of DAG, consistent with enhanced phosphoinositide hydrolysis.

F^- likewise causes phosphoinositide hydrolysis, along with increases in inositol phosphates and cytoplasmic Ca^{++}, and generation of PA (English *et al.*, 1987; Strnad *et al.*, 1986). Of interest, PIP_2 hydrolysis, increased cytosolic Ca^{++}, and O_2^- generation are kinetically dissociated. Phosphoinositide hydrolysis occurs most rapidly, followed by a slower increase in

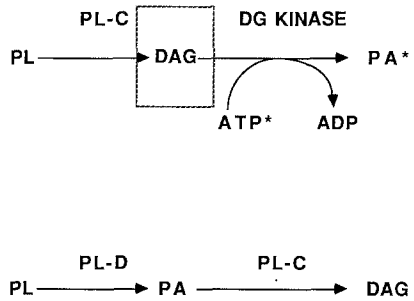

Fig. 2. Possible pathways for diacylglycerol generation. Shown are phospholipase C- and D-linked pathways for the generation of phosphatidic acid (PA) and diacylglycerol (DAG) from parent phospholipids (PL).

cytoplasmic Ca^{++} (maximal at ~ 7 min), and O_2^- generation correlates with a second "wave" of PA that might reflect DAG generation.

Other agonists listed in Table I have been implicated in phosphoinositide hydrolysis. In [^{32}P]phosphate-prelabeled cells, PAF causes a rapid, pertussis toxin-inhibitable decrease in cellular PIP_2 and PIP, with a parallel increase in [^{32}P]PA (Naccache *et al.*, 1985a, 1986).[3] The period during which lipid changes occur corresponds to that of the respiratory burst. LTB_4 produces a similar, very brief increase in both labeled PA and cytosolic Ca^{++} (Omann *et al.*, 1987), as well as an increase in inositol phosphates (Bradford and Rubin, 1985; Andersson *et al.*, 1985), consistent with phosphoinositide hydrolysis. Con A also causes a decrease in the levels of PIP_2 and PIP, but, unlike fMLP, this response is not affected by pertussis toxin (Verghese *et al.*, 1985b). Ca^{++} inophores similarly induce an increase in phosphoinositide hydrolysis (Cockcroft, 1986), probably due to Ca^{++} activation of the phosphoinositide-specific phospholipase C (Bradford and Rubin, 1986). However, accumulation of IP_3 was not noted with either ionomycin (Bradford and Rubin, 1985) or A23187 (Ohta *et al.*, 1985). Although high calcium can activate phospholipase C, it seems clear that the rapid increase in Ca^{++} seen with surface receptor-linked stimuli is a consequence rather than a cause of phosphoinositide hydrolysis (Bradford and Rubin, 1985; Cockcroft, 1986; Di Virgilio *et al.*, 1985; Krause *et a.*, 1985). Agonists that activates protein kinase C directly do not cause substantial breakdown of phosphoinositides or accumulation of PA (Serhan *et al.*, 1983).

The studies reviewed above support a role for phosphoinositide hydrolysis in the activation of the respiratory burst. Nevertheless, there are some conditions under which there is a dissociation between phosphoinositide turnover and activation. For example, in cells primed with low-dose phorbol myristate acetate (PMA) and then activated with fMLP, there is an enhanced respiratory burst, but a decrease in both inositol phosphate generation and the Ca^{++} flux, suggesting a decrease in phosphoinositide hydrolysis (Della Bianca *et al.*, 1986) that is probably due to inhibition of phospholipase C (Smith *et al.*, 1987; Kikuchi *et al.*, 1987).[4] The effect is further accentuated in Ca^{++}-depleted cells (Grzeskowiak *et al.*, 1986) wherein both fMLP-induced inositol phosphate and O_2^- generation are virtually eliminated. Low-dose PMA restores the fMLP-activated respiratory burst, but not inositol phosphate generation. Dissociation of phosphoinositide metabolism and O_2^- generation is also seen in Ca^{++}-depleted cells using other agonist combinations

[3] Unexpectedly, much of the calcium flux induced by platelet-activating factor was pertussis toxin independent, suggesting a nonreceptor-linked mechanism as well as the pertussis-inhibitable IP_3 generation (Naccache *et al.*, 1985a).

[4] The effect of PMA on inositol phosphate levels may also be due to enhanced breakdown of released inositol phosphates, as is seen in platelets (Connolly *et al.*, 1986).

such as Con A plus fMLP (Rossi *et al.*, 1986) or F^- (Della Bianca *et al.*, 1988). Thus, under some conditions, activation need not be linked to phosphoinositide hydrolysis. Although these studies document a dissociation between neutrophil activation and Ca^{++} fluxes, another potential mediator, DAG, was not quantified, and might arise from sources other than phosphoinositides. As will be described later, DAG generation continues to occur in the PMA-primed cell.

Phospholipase C Action on Non-Inositol-Containing Phospholipids

One explanation for DAG generation in the face of diminished phosphoinositide hydrolysis is that the activated phospholipase(s) C may use alternative phospholipid substrates, as depicted in Fig. 1. Phospholipases that do not act on PIs have been isolated (Wolf and Gross, 1985). Breakdown of phosphatidylcholine (PC) to generate DAG has been noted in several tissues in response to phorbol esters and protein kinase C-linked agonists (Irving and Exton, 1987; Besterman *et al.*, 1986; Muir and Murray, 1987; Mufson *et al.*, 1981; Daniel *et al.*, 1986) and non-phosphoinositide-linked DAG generation has been noted in Ha-*ras*-transformed cells (Lacal *et al.*, 1987). In platelets, not only PI, but also phosphatidylserine (PS), PC, (diacyl)phosphatidylethanolamine (PE), and 1-alkeneyl-2-acyl PE were depleted in response to agonists (Takamura *et al.*, 1987). For neutrophil, activation of lipases acting on noninositol lipids also occurs under some conditions. Radiolabeled 1-O-alkyl PC, formed from added labeled 1-O-alkyl-lyso PC, is degraded to diglyceride and PA upon stimulation with fMLP (Kakinuma *et al.*, 1987; Pai *et al.*, 1988) and phorbol ester (Badwey *et al.*, 1988).

These studies suggest that *endogenous* 1-O-alkyl or 1-O-alk-1′-enyl lipids may also serve as phospholipase substrates. Neutrophils contain extraordinarily high contents of alkyl lipids (Tence *et al.*, 1985; Mueller *et al.*, 1982, 1984). About 50% of PC in neutrophils contains the 1-O-alkyl linkage. Almost two-thirds of PE is alkyl-linked, mostly in the alkenyl form. Thus, in the neutrophil, alkyl-containing glycerolipids should be considered as potential substrates for phospholipase C.

Phospholipase D Action in Neutrophils

The first evidence for a role for phospholipase D in neutrophil was that, in cells preincubated with [^{32}P]phosphate, PA formed in response to fMLP had only 10% of the specific activity of cellular ATP (Cockcroft, 1984). The rationale for this experiment is depicted in Fig. 2. If PA is derived exclusively from phospholipase C-generated DAG via phosphorylation by diglyceride kinase (upper reaction), then it should have the same specific activity as ATP.

If it is derived directly via phospholipase D (lower reaction), then it will have the same specific activity as the parent lipid, which should be low after the short (90 min) labeling period. PA generation (using cytochalasin B-pretreated cells) was maximal at ~ 15 sec, while its specific activity increased only gradually, and approached that of ATP at ~ 2 min. As detailed below, little DAG (mass) is generated at 15 sec, but increases significantly at later times (Honeycutt and Niedel, 1986), which may account for the increase in specific activity of PA. Subsequent studies (Cockcroft and Allan, 1987) showed that the fatty acid composition of both DAG and PA were similar, and data were consistent with at least some of the DAG being generated from PA rather than *vice versa*, as is shown in the lower reaction in Fig. 2. Apparently, the principal pathway for PI hydrolysis is phospholipase D, but sufficient PI is converted to PIP and PIP_2 to supply the needs of the cell for IP_3 for regulation of cellular Ca^{++} (Cockroft *et al.*, 1985). FMLP-activated phospholipase D action has also been demonstrated (Pai *et al.*, 1988) in studies in which [^{3}H]alkyl-PC was converted directly to PA without intermediate diglyceride formation. Thus, as for phospholipase C, PIs need not be the exclusive substrate. The relative contribution of phospholipase D *versus* phospholipase C pathways to DAG generation is not clear.

PA itself has recently been implicated as a regulator of Ca^{++} in some systems (Salmon and Honeyman, 1980; Moolenaar *et al.*, 1986; Putney *et al.*, 1980). There is little information as to whether PA functions as a second messenger in neutrophil (e.g., for secretion). However, the occurrence of PA does not correlate well with O_2^-, suggesting that PA does not mediate this response.

Phospholipase A_2 Action in Neutrophils

Figure 3 shows some phospholipase A_2-associated pathways in neutrophils. Phospholipase A_2 acts on a variety of diacyl- and 1-O-alkyl-2-acyl-containing glycerolipids. When the substrate is alkyl-PC, the products are not only arachidonate, but also alkyl lyso-alkyl-PC, which can be converted by an acetyltransferase into PAF (1-O-alkyl-2-acetyl PC) (Chilton *et al.*, 1984; Jouvin-Marche *et al.*, 1984). Arachidonate is metabolized via cyclooxygenase to form prostaglandins (e.g., PGE_2) or via lipoxygenase to generate 5-HETE and LTB_4. The flux through the latter pathways is determined in part by the activity of phospholipase A_2 that limits the availability of arachidonate (Jouvin-Marche *et al.*, 1984; McIntyre *et al.*, 1987; Sun and McGuire, 1984), but additional regulation at the level of the lipoxygenase (Clancy *et al.*, 1983) and acetyltransferase (Alonso *et al.*, 1982) occurs and may dictate the distribution of products. Recent evidence suggests that phospholipase A_2 is regulated by the combined effects of protein kinase C and Ca^{++} (McIntyre

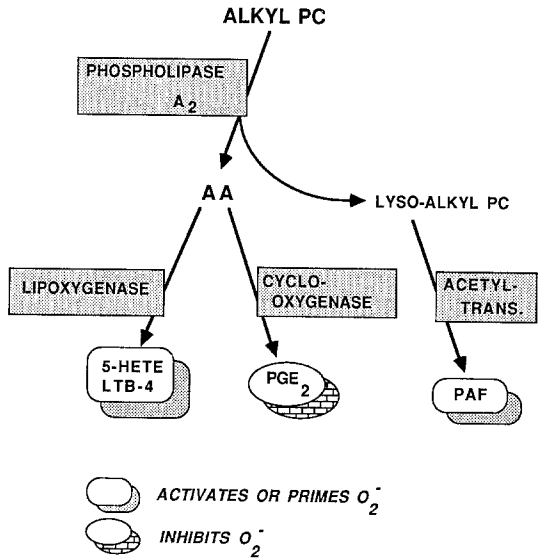

Fig. 3. Phospholipase A_2-related pathways. Shown are some pathways related to the action of phospholipase A_2 and generation of arachidonic acid (AA). Abbreviations are as in the text.

et al., 1987), as depicted in Fig. 4.[5] Thus, phosphoinositide hydrolysis by phospholipase C may contribute to the regulation of phospholipase A_2-associated pathways via calcium and DAG, consistent with the finding that activation of phospholipase C preceds that of phospholipase A_2 (Takenawa *et al.*, 1983).

The most potent stimulus for activation of the phospholipase A_2-linked pathways is A23187, which results in the production not only of arachidonate, but also of PAF, LTB_4, and HETEs (Borgeat and Samuelsson, 1979; Palmer and Salmon, 1982). This pertussis toxin-resistant (Ohta *et al.*, 1985) stimulus not only elevates Ca^{++}, but also activates PI hydrolysis and protein kinase C translocation (Nishihira *et al.*, 1986; Rider and Niedel, 1987), consistent with regulation of the phospholipase A_2 by the dual effects of Ca^{++} and protein kinase C. Opsonized zymosan and aggregated IgG effect a variable but generally smaller arachidonate release, with less metabolism to other products. In most studies,[6] fMLP and C5a produce little or no

[5] It seems clear that in neutrophil (Walsh *et al.*, 1981), activation of protein kinase C alone (with phorbol esters) is not a strong stimulus of this pathway, although some activation of arachidonate "turnover" (i.e., incorporation into phospholipids) has been noted (Hirata, 1981; Kramer *et al.*, 1984) and might reflect a low level of phospholipase A_2 activation.

[6] In contrast, there appears to be more activation of these pathways in neutrophils from other species such guinea pig and rabbit (Ohta *et al.*, 1985; Bokoch and Gillman, 1984. Since these neutrophil preparations are elicited in peritoneum using inflammatory stimuli, it is possible that they have achieved a "primed" state, and therefore respond differently than human neutrophils that are derived from peripheral blood.

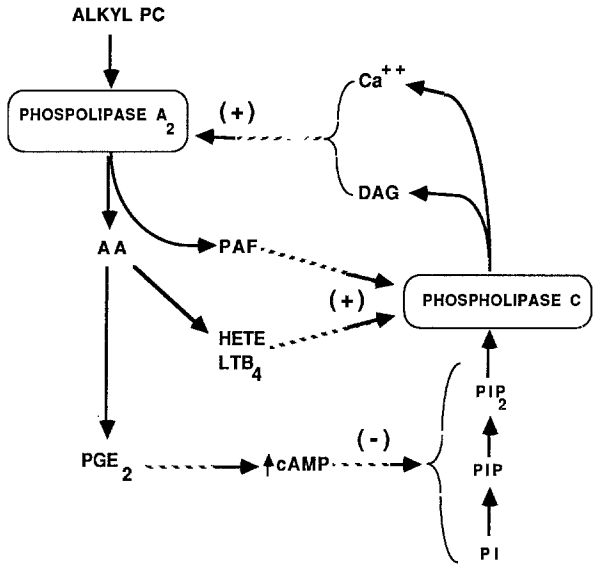

Fig. 4. Possible mutual regulatory interactions between phospholipase C- and phospholipase A_2-linked pathways. Shown are some possible points of regulation by which one pathway may affect the function of the other, as detailed in the text.

activation of these pathways (Walsh *et al.*, 1981; Godfrey *et al.*, 1987; Jouvin-Marche *et al.*, 1984; Alonso *et al.*, 1982; Haines *et al.*, 1987; Palmer and Salmon, 1982). However, with fMLP and other weak stimuli, if a high concentration of exogenous arachidonate is also provided, significant further metabolism occurs, suggesting that arachidonate availability may limit activity under these conditions.

The possible participation of phospholipase A_2-linked pathways in the activation of the respiratory burst has been a recent area of study. Although LTB_4 and PAF are themselves very weak activators (see above), they can, in combination, provide significantly more activation, and can prime the cells for an increased response to other stimuli such as fMLP (O'Flaherty, 1985; Dewald and Baggiolini, 1985). 5-HETE also primes cells towards phorbol ester and DAG (O'Flaherty and Nishihira, 1987). In general, however, the activity of these pathways correlates poorly with the respiratory burst. For example, A23187, while a good stimulus for activation of phospholipase A_2 and related pathways, is a poor stimulus for activation of the burst (Walsh *et al.*, 1981); fMLP, a good stimulus for the burst, does not significantly activate phospholipase A_2 pathways. In a recent study (Godfrey *et al.*, 1987) using A23187, aggregated IgG, and opsonized zymosan as activators, no correlation was seen between O_2^-

generation and phospholipase A_2-mediated arachidonate generation or its subsequent metabolism.

Nevertheless, various inhibitors of the pathways in Fig. 3 affect neutrophil activation. Mepacrine, a phospholipase A_2 inhibitor, produced a parallel dose response for inhibition of both arachidonate release and O_2^- generation (Maridonneau-Parini *et al.*, 1986), and *p*-bromophenacyl bromide blocks the response to fMLP (Takenawa *et al.*, 1986); Smolen and Weissmann, 1980; Bromberg and Pick, 1983). However, effects on phosphoinositide/protein kinase C pathways have not been reported, and the latter inhibitor suppresses glucose transport in macrophages and may therefore inhibit nonspecifically (Tsunawaki and Nathan, 1986). In the same study, several other agonists initiated arachidonate release without O_2^- generation, and arachidonate release could be suppressed without affecting the respiratory burst. The authors concluded that release of arachidonate and cell activation were frequently coincidental, but were not obligatorily linked.

Some inhibitors of the lipoxygenase pathway also inhibit the burst (Bromberg and Pic, 1983; Smolen and Weissmann, 1980), but these may also have nonspecific effects on glucose transport, on receptor-linked processes, and on the NADPH-oxidase itself (Walsh *et al.*, 1981; Ozaki *et al.*, 1986). In a recent study using a series of lipoxygenase inhibitors, no correlation was observed between LTB_4 synthesis and inhibition of the respiratory burst (Ozaki *et al.*, 1986).

Cyclooxygenase inhibitors also affect neutrophil function. Indomethacin stimulates fMLP-induced O_2^- generation (Takenawa *et al.*, 1986; Bromberg and Pick, 1983). Stimulation may relate to prevention of the formation of PGE_2, an inhibitor of the respiratory burst: In one study (Takenawa *et al.*, 1986), PGE_2 and other cAMP-linked stimuli inhibited the synthesis of both DAG and PA in response to fMLP, although there was no effect on the early breakdown of PIP_2 or on rapid Ca^{++} fluxes (De Togni *et al.*, 1984). Data suggested inhibition of phosphorylation of inositol lipids by PI kinase(s), as depicted in Fig. 4. Thus, it may be that, by preventing PGE_2 formation, indomethacin allows continued flux through the phosphoinositide pathways, with sustained generation of second messengers.

Metabolism of Sphingolipids to Generate Biologically Active Compounds

Long-chain bases such as sphingosine and sphinganine are potent inhibitors of protein kinase C, both in isolated enzyme systems and in whole cells (see below), and may also affect other cellular sites including a sphingosine-activable kinase (Faucher *et al.*, 1988), as depicted in Fig. 1. A sensitive new assay (Merrill *et al.*, 1988) has allowed quantitation of endogenous sphingosine; neutrophils contain significant quantities of the lipid, the level of which changes with agonist treatments (Wilson *et al.*, 1988). The basal

level is ~ 10% of that which, when added exogenously, inhibits the respiratory burst, but high local concentrations generated endogenously may affect function. Levels increase spontaneously to ~ 150% of basal during a 1-h incubation. After a similar incubation, the capacity to mount a respiratory burst declines (Mandell *et al.*, 1987), although a cause-and-effect relationship with sphingosine level has not been established. A variety of agonists either slow the time-dependent increase or actually decrease the quantity of free sphingosine: phorbol ester was particularly effective, resulting in a decline to ~ 1/3 of basal after an hour.

Since *de novo* synthesis was too slow to account for the observed appearance, the time-dependent rise in sphingosine apparently resulted from breakdown of sphingolipid precursors (as in Fig. 1) in reactions analogous to those for glycerolipid hydrolysis. In highly purified liver plasma membranes, a neutral pH-optimum ceramidase rapidly hydrolyzes added or endogenously generated ceramide to sphingosine (Slife *et al.*, 1988). Thus, the level of sphingosine may be regulated by enzymes that hydrolyze the sphingolipids to ceramide, which is then rapidly converted to sphingosine. Controlled sphingosine generation thus represents a potential regulatory pathway.

Diacylglycerol *versus* Calcium as Signals for Activation of the Respiratory Burst

The picture that emerges from studies described above is that, for many (but not all) cases, activation correlates with phosphoinositide turnover. Hydrolysis of PIP_2 via phospholipase C results in the generation of two potential second messengers: DAG and Ca^{++} (via IP_3). Because these are generally coordinately generated, it is often difficult to sort out whether one or both mediates a given response. This section therefore summarizes evidence pertaining to each as potential messengers for O_2^- generation.

Diacylglycerol

Attention is directed to DAG, since added synthetic DAGs activate O_2^- generation (Fujita *et al.*, 1984; Cox *et al.*, 1986). Several methods are used to monitor endogenous DAG generation.[7] First, as depicted in Fig. 2, when

[7] It is not clear whether these methods distinguish between 1-O-alkyl-2-acyl glycerol and diacylglycerol, since the migration of these and their phosphatidic acid products is similar or identical on commonly used TLC systems. Although there appears to be little evidence for generation of alkyl glycerols form endogenous lipids, this possibility should not be discounted given the high content of alkyl lipids in neutrophil. This difference may have interesting implications, since diacylglycerol is a protein kinase C activator, whereas alkyl-acyl glycerol can act either as a weak activator (Heymans *et al.*, 1987) or as inhibitor (Agwu *et al.*, 1986) or protein kinase C, and is known to prime neutrophil responses (O'Flaherty *et al.*, 1984).

cells are preincubated with [^{32}P]phosphate to label ATP pools, the appearance of [^{32}P]labeled PA reflects the prior appearance of DAG, which has been phosphorylated by the action of diglyceride kinase. Although the latter enzyme is a major pathway for DAG metabolism in platelets (Bishop and Bell, 1986), other enzymes such as diglyceride lipase may also participate, thus complicating interpretations. In addition, the appearance of labeled PA does not accurately reflect the rate of diglyceride generation. Thus, although the PA data (see *Phospholipase C and Phosphoinositide Hydrolysis*) point to the formation of DAG in response to agonists, actual levels and kinetics of formation cannot be ascertained with this approach. Two other methods have also been used. The first involves prelabeling cells with [^{3}H]phospholipid precursors (glycerol or a fatty acid such as arachidonate), and measuring released [^{3}H]DAG. This has the advantage of high sensitivity, but is biased by selective incorporation and release of label in distinct phospholipid pools. The second involves direct measurement of diglyceride mass by chemical or enzymatic methods (Kennerly *et al.*, 1979; Preiss *et al.*, 1986).

Using mass measurement, a rapid appearance of DAG is detected following fMLP (Honeycutt and Niedel, 1986; Rider and Niedel, 1987), consistent with a signaling role. Cytochalasin B, which augments the rate and extent of fMLP-activated O_2^- release, caused a corresponding increase in the magnitude and duration of fMLP-stimulated DAG generation. The onset, duration, and dose dependence for the DAG response paralleled O_2^- generation. Similar results were also seen using other agonists (ionomycin and Con A). Also consistent with a mediator role for DAG, an inhibitor of diglyceride kinase (R59022) that should elevate cellular DAG, enhances O_2^- generation by fMLP and other agonists (Muid *et al.*, 1987; Gomez-Cambronero *et al.*, 1987).

As described earlier (see *Phospholipase C and Phosphoinositide Hydrolysis*), certain cell treatments (e.g., Ca^{++} depletion and PMA priming) dissociate phosphoinositide hydrolysis/IP_3 generation from activation (i.e., activation occurs despite an absence or decrement in phosphoinositide hydrolysis); these data have been interpreted as precluding a signaling role for DAG/protein kinase C, based on the assumption that diglyceride is derived exclusively from inositol-containing lipids. For one of these conditions, priming by low-dose PMA, we have recently shown (Tyagi *et al.*, 1988) that, despite a decrease in the hydrolysis of PIP_2 and PIP, priming resulted in a significant *increase* in DAG and in early [^{32}P]PA generation (the latter in [^{32}P]phosphate-prelabeled cells). Presumably, DAG was released from lipids other than phosphoinositides. Under several conditions, DAG mass showed an excellent correlation with the initial rate of O_2^- generation, and the duration of the respiratory burst was similar to that of diglyceride elevation. Under priming conditions, activation did not correlate with

cellular Ca^{++}, since the elevation of Ca^{++} by fMLP was blunted, as was IP_3 generation. These studies provide precedent for enhanced DAG generation in the face of inhibited phosphoinositide turnover, and may account for activation of the respiratory burst under such condition.

Calcium

A rapid rise in cytosolic Ca^{++} is perhaps the earliest evidence of cell activation by formyl peptide (Korchak *et al.*, 1984; Lazzari, 1986) and other surface receptor-linked stimuli (Gennaro *et al.*, 1984; Naccache *et al.*, 1984). The Ca^{++} is released from intracellular nonmitochondrial stores (Hamachi *et al.*, 1985; Sklar and Oades, 1985). The rapid changes in Ca^{++}, plus the finding that high concentrations of Ca^{++} inophores activate O_2^- generation, have provoked considerable interest in this ion as a potential messenger. However, interpretations are complicated by pervasive enzymatic requirements for Ca^{++}, as well as artifactual activation of some enzymes (e.g., phospholipases C and A_2, and protein kinase C) at high divalent ion concentration. As an example, chelation of intracellular Ca^{++} with the fluorescent Ca^{++} indicator Quin 2 both lowers basal Ca^{++} and diminishes the fMLP-activated Ca^{++} flux, with parallel inhibition of fMLP-induced O_2^- generation (Lew *et al.*, 1984; Sklar and Oades, 1985). It is thus unclear whether the inhibited burst relates to a permissive requirement for basal Ca^{++} or to a requirement for the Ca^{++} flux.

It is generally agreed, however, that Ca^{++} alone is not a sufficient signal for activation of the respiratory burst. Ca^{++} can be elevated to levels similar to those achieved by fMLP using ionophores (Sklar and Oades, 1985; Korchak *et al.*, 1984b; Pozzan *et al.*, 1983; Lew *et al.*, 1984) or a monoclonal antibody to a cell surface component (Naccache *et al.*, 1984), yet these treatments do not result in significant O_2^- generation. A series of Ca^{++}-linked activators (fMLP, C5a, LTB_4, and PAF) effected similar kinetics and extents of cytosolic Ca^{++} elevation, but resulted in widely different rates of O_2^- production. In electropermeabilized cells wherein cytosolic Ca^{++} was maintained at the physiological basal level (100 nM), fMLP elicited a respiratory burst in the absence of a Ca^{++} flux (Grinstein and Furuya, 1988). Thus, a Ca^{++} flux does not seem to be required.

Nevertheless, some evidence suggests that Ca^{++}, although not crucial, may accelerate or augment responses, perhaps via effects on protein kinase C, as has been inferred from model studies (Wolf *et al.*, 1985). Subthreshold doses of Ca^{++} ionophore and phorbol ester synergize to activate the respiratory burst (Di Virgilio *et al.*, 1984; Dale and Penfield, 1984). Increased intracellular Ca^{++} also shortens the lag prior to activation by some stimuli (Hallet and Campbell, 1984). Low concentrations of the Ca^{++}-linked

agonists fMLP, 5-HETE as well as Ca^{++} ionophore prime neutrophils for activation by tumor promoters and DAG (Badwey *et al.*, 1988; Finkel *et al.*, 1987; O'Flaherty and Nishihira, 1987). Priming by Ca^{++}-linked stimuli is sometimes correlated with translocation of protein kinase C to a membrane fraction, and also results in an increased affinity of [^{3}H]phorbol dibutyrate to its cellular "receptor" (i.e., protein kinase C) (Dougherty and Niedel, 1986). One interpretation is that the agonist-induced rapid elevation in Ca^{++} promotes the translocation of protein kinase C, thus sensitizing the cell to other signals (e.g., DAG) that utilize the protein kinase C pathway.

On a Role for Protein Kinase C

Activation of the respiratory burst by phorbol esters and DAGs implicates protein kinase C in at least one mode of activation. Although other cellular sites of action for these molecules are sometimes reported, evidence supports protein kinase C as the target in neutrophil: For a series of phorbol esters, O_2^- generation paralleled activation of isolated kinase (Karnovsky *et al.*, 1985). Also, activation by phorbol dibutyrate showed the same concentration dependence as that for binding of [^{3}H]phorbol dibutyrate to its cellular receptor (protein kinase C) (Tauber *et al.*, 1982). Furthermore, when isolated plasma membranes from resting neutrophils were supplemented with either cytosol or purified protein kinase C plus factors required to activate protein kinase C (including PS, Ca^{++}, and PMA), O_2^- generation was activated, albeit at a lower rate than is seen in membranes from activated cells (Cox *et al.*, 1985).

Although there seems to be little doubt that protein kinase C mediates the respiratory burst in response to phorbol esters, there is debate with regards to its role when other agonists are used. Because the assay for protein kinase C requires inclusion of activating factors, it is not yet possible to determine directly the activation state of the enzyme in agonist-treated cells. Phosphorylation of specific proteins can bear witness to prior activation of protein kinase C enzyme in some cells (e.g., the 40-kD phosphorylation in platelets), but, despite of an abundance of agonist-induced phosphorylations, no such unique informants are known in the neutrophil. With activating stimuli, however, protein kinase C is often noted to "translocate" from the cytosol to a membrane fraction.[8] This migration, assayed following

[8] It is not clear whether this "translocation" actually represents a physical movement of the kinase, or an enhanced ability of membrane-bound kinase to persist in its membrane association during isolation.

subcellular fractionation (either as activity or phorbol dibutyrate binding sites), can be taken as indirect evidence for protein kinase C activation.

Following addition of phorbol esters to neutrophils or their cytoplasts, protein kinase C translocates within seconds to a membrane fraction, and migration correlates with O_2^- generation (Christiansen *et al.*, 1986; Wolfson *et al.*, 1985; Gennaro *et al.*, 1986). DAG, mezerein (another protein kinase C activator), Ca^{++} ionophores, and PAF also cause translocation (Nishihira *et al.*, 1986). Likewise, F^- induces translocation (Strnad *et al.*, 1986), the delayed time course of which parallels the delayed activation of O_2^- generation. Phagocytic stimuli such as bacteria stimulate translocation (Christiansen *et al.*, 1987). In a recent immunocytochemical study of the effects of phagocytosis on protein kinase C, a high density of protein kinase C was visualized associated with phagocytic vesicles (Deli *et al.*, 1987).

Translocation data are less clear for receptor-linked stimuli. Although fMLP and C5a produce translocation in cytoichalasin B-pretreated cells (Pike *et al.*, 1986; Nishihira *et al.*, 1986), none was detected in cells that were not pretreated. In addition, no increase in membrane protein kinase C was seen for LTB_4 or PAF, regardless of whether cells were cytochalasin pretreated. However, these latter stimuli provide a very brief respiratory burst, as does fMLP in the absence of cytochalasin, and it may have been difficult to observe an ephemeral appearance of the membrane-associated kinase. In other studies (Pontremoli *et al.*, 1986), whereas PMA produced a stably membrane-bound kinase, the membrane association following fMLP (cytochalasin-pretreated cells) was more labile, and was detected only when cells were lysed in the presence of Ca^{++}. FMLP-induced translocation in non-cytochalasin-treated cells has recently been observed, but only when the incubation was terminated within the first 30 sec after fMLP (i.e., a time during which O_2^- production is maximal), and then only if Ca^{++} was present during cell lysis (Horn and Karnovsky, 1986). In 5-HETE-primed cells, enhanced translocation was observed in response to activators of protein kinase C (O'Flaherty and Nishihira, 1987; Badwey *et al.*, 1988), while in diglyceride-primed cells (Bass *et al.*, 1987) and in Ca^{++}-depleted cells activated by F^- (Della Bianca *et al.*, 1988), translocation was not seen. Thus, most but not all activation paradigms produce detectable translocation.

Inhibitors have also been used to try to define a role for protein kinase C. Some compounds (chloropromazine, trifluoperazine, and verapamil), which were originally characterized as inhibitors of Ca^{++}-linked pathways, but which also inhibit protein kinase C, block the fMLP- and PMA-activated respiratory burst (Della Bianca *et al.*, 1985; Karnovsky *et al.*, 1985; Korchak *et al.*, 1984a). We have recently used long-chain sphingoid bases (sphinganine and sphingosine) to investigate the role of protein kinase C in activation of the burst (Wilson *et al.*, 1986; Lambeth *et al.*, 1988). These inhibitors bind

competitively with respect to DAG, phorbol ester, and Ca^{++}, and do not inhibit the activity of the proteolytically formed catalytic domain of the enzyme (Hannun *et al.*, 1986), indicating an effect on the regulatory domain. Micromolar concentrations of long-chain bases blocked activation of the respiratory burst by all agonists tested (PMA, DAG, fMLP, opsonized zymosan, arachidonate, and A23187). The same EC_{50} was observed regardless of which agonist was used, indicating a common inhibited site. Inhibition of fMLP activation was also seen when neutrophils had been primed with a low dose of PMA (Tyagi *et al.*, 1988). Consistent with protein kinase C as the affected site, the inhibitor blocked PMA-induced phosphorylations, did not affect the fMLP-stimulated Ca^{++} flux or generation of DAG (Lambeth *et al.*, 1988), and did not directly inhibit the respiratory burst oxidase. Long-chain base also displaced [^{3}H]phorbol dibutyrate from its cellular binding site, and displacement paralleled inhibition of O_2^- generation, providing support for protein kinase C as the inhibited site. In structure–function studies using a series of sphingosine analogs (S. R. Tyagi, unpublished observations), the order of effectiveness was the same for inhibition of both protein kinase C and the respiratory burst. Thus, studies using sphingoid long-chain bases support a role for protein kinase C in the activation of the respiratory burst by a variety of agonists.

Nevertheless, results using some other protein kinase C inhibitors (polymyxin, retinal, H-7) appear to contradict those using sphingolipids. Although some bock neutrophil activation under some circumstances (Cooke and Hallett, 1985; Naccache *et al.*, 1985b), interpretation of the results is complicated. For example, polymyxin, rather than inhibiting protein kinase C-related processes in HL-60 cells, produces translocation of protein kinase C and induces a phosphorylation pattern similar to that seen with phorbol ester (Kiss *et al.*, 1987). Retinal, also used as a protein kinase C inhibitor, can activate rather than inhibit the respiratory burst, and stimulates phosphoinositide hydrolysis (Lochner *et al.*, 1986). A widely used class of inhibitors is the isoquinolinesulfonamides such as H-7 and C-I (Gerard *et al.*, 1986; Salzer *et al.*, 1987; Sha'afi *et al.*, 1986). These are competitive with respect to ATP (thus acting on the catalytic domain in both the holoenzyme and the proteolytic form), and also affect kinases other than protein kinase C, including cyclic AMP-dependent protein kinase. In general, these inhibitors block leukocyte activation by PMA and DAG, but not by receptor-Linked stimuli such as fMLP and C5a. These data have lead to the suggestion that fMLP may activate by additional pathways that do not involve protein kinase C. The discrepancy between results with isoquinolinesulfonamides and sphingoid long-chain bases is not presently understood, and further information regarding the inhibitory properties and specificities of both inhibitors may illuminate new aspects of signaling mechanisms in neutrophil.

Summary and Conclusions

From the available evidence, a plausible model for signaling mechanisms in neutrophil can be assembled. According to this model, protein kinase C plays a central role, although some apparently contradictory data (i.e., some inhibitor studies) remain to be reconciled. The substrate protein(s) for protein kinase C that is responsible for activation may not be the NADPH-oxidase itself as discussed in other reviews of this series, but it is not yet clear how specific phosphorylations are coupled to activation of the oxidase, nor whether kinases other than protein kinase C might also participate, perhaps in a phosphorylation cascade. Several mediators (DAG, arachidonate or its metabolites, sphingosine) that are generated or removed in response to binding of agonists to cell surface receptors may affect the function of protein kinase C. To date, a good correlation with activation is seen only with DAG, but it seems likely that other effectors modulate protein kinase C-mediated responses under some conditions. DAG can be derived from phosphoinositide breakdown, but recent evidence also suggests that, under some conditions (e.g., PMA priming), it is derived from other sources. Interconnections between phospholipase C- and phospholipase A_2-mediated pathways, as well as possible links to sphingolipid pathways, provide at least theoretical opportunities for interdigitating effects of a complex network of regulatory machinery that may affect the respiratory burst. Delineation of the relative contributions and interactions among these pathways provides a challenge for future studies, not only in neutrophil but in a variety of cell types in which protein kinase C functions.

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