

# Fluoride activates diradylglycerol and superoxide generation in human neutrophils via PLD/PA phosphohydrolase-dependent and -independent pathways

Susan C. Olson, Shiv Raj Tyagi and J. David Lambeth

*Department of Biochemistry, Emory University Medical School, Atlanta, GA 30322, USA*

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In contrast to the rapid, ethanol-inhibited superoxide generation by the receptor-linked agonist formyl-methionyl-leucyl-phenylalanine (fMLP), fluoride-activated superoxide generation occurs after a prolonged lag, and as shown herein is relatively ethanol-insensitive. We have investigated fluoride-activation of diradylglycerol generation and phospholipase D activity. Fluoride induces a very large increase in diradylglycerol mass (both 1,2-diacylglycerol (DAG) and 1-*O*-alkyl,2-acylglycerol (EAG)), with kinetics similar to superoxide generation. Unlike fMLP-activated diglyceride generation which is completely inhibited by ethanol, that produced by fluoride is only partially (30%) blocked. When the phosphatidylcholine pool is  $^3\text{H}$ -prelabeled, fluoride activates both  $^3\text{H}$ ]phosphatidic acid (PA) and  $^3\text{H}$ ]diglyceride generation with similar kinetics. Partial inhibition of the production of these species by ethanol was seen, coincident with the appearance of  $^3\text{H}$ ]phosphatidylethanol, indicating phospholipase D-dependent transphosphatidylation had occurred. The data are consistent with the fluoride activation of PA and diglyceride generation by both phospholipase D-dependent and -independent (presumably phospholipase C) mechanisms.

Superoxide generation; Diacylglycerol; Diradylglycerol; Phospholipase D; Fluoride; Neutrophil

## 1. INTRODUCTION

A major microbicidal mechanism in polymorphonuclear leukocytes (neutrophil) is the respiratory burst, which generates cytotoxic species such as superoxide and other species which are generated secondarily from the superoxide [1]. The burst is catalyzed by the respiratory burst oxidase (NADPH-oxidase), an enzyme which is dormant until cells become activated following a variety of stimuli, including the receptor-linked agonist fMLP [2], fluoride [3,4], protein kinase C activators such as phorbol 12-myristate 13-acetate (PMA) [5] and DAG [6,7], the calcium ionophore A23187 [8], and particulate matter such as opsonized zymosan [9,10].

While the signal transduction pathways involved in activation of the oxidase are at present far from clear, a variety of second messengers have been proposed to

mediate the respiratory burst. Diradylglycerol mass (both DAG [11] and EAG [12,13]) correlates with superoxide generation under a variety of activation conditions (e.g., fMLP, A23187, concanavalin A), and may produce cellular effects by both protein kinase C-dependent [10,14] and -independent [15] mechanisms. In addition to DAG, other molecules such as arachidonic acid [16–19] and PA [20,21] have been proposed as messengers, although correlations between mass and activation have not been established.

The production of some putative mediators involves the participation of at least two phosphodiesterases: phospholipases C and D. It is well established that in fMLP-activated neutrophils, phosphatidylinositols (PI, PIP, and PIP<sub>2</sub>) are hydrolyzed via phospholipase C to generate DAG plus inositol 1,4,5-trisphosphate, an intracellular calcium mobilizing agent [22,23]. Recently, however, several studies [24,25] have indicated that in fMLP-activated cells, the bulk of diradylglycerol arises not from the action of phospholipases C, but from that of phospholipase D, which acts on choline-containing glycerolipids, and perhaps on phosphatidylinositol [26] to generate PA. The latter is hydrolyzed to form diradylglycerol via the action of PA-phosphohydrolase [25]. The phospholipase D pathway has been demonstrated both by using radiolabeled lipid precursors (in particular 1-*O*-alkyl lysophosphatidylcholine, which is converted by the cell into the corresponding 2-acyl species), and by the observation of

*Correspondence address:* J.D. Lambeth, Department of Biochemistry, Emory University Medical School, Atlanta, GA 30322, USA

*Abbreviations:* DAG, 1,2-diacylglycerol; EAG, 1-*O*-alkyl, 2-acylglycerol; PA, phosphatidic acid; fMLP, formyl-methionyl-leucyl-phenylalanine; PI, phosphatidylinositol; ATP, adenosine 5'-triphosphate; CB, cytochalasin B; PC, phosphatidylcholine; TLC, thin-layer chromatography; PBS, phosphate-buffered saline; PETH, phosphatidylethanol; EtOH, ethanol; GM-CSF, granulocyte/monocyte colony-stimulating factor; PMA, phorbol 12-myristate 13-acetate

transphosphatidylation with added alcohols such as ethanol [27], e.g. to generate phosphatidylethanol, a reaction unique to phospholipase D. Transphosphatidylation competes with hydrolysis, causing the alcohol to inhibit phospholipase D-dependent PA (and secondarily, diradylglycerol) production.

One or more G proteins participate in neutrophil activation. *Bordetella pertussis* toxin, which is known to inactivate some G proteins, inhibits fMLP-activated superoxide production [28–30], arachidonate generation, phosphoinositide hydrolysis, and calcium fluxes [29,31–33]. The phospholipase D-dependent hydrolysis of PC in fMLP-stimulated neutrophils is also pertussis toxin-sensitive [13]. Studies using fluoride, a potent activator of  $G_s$ ,  $G_i$  and transducin [34–36], have also supported a role for G-proteins in neutrophil activation. In contrast to the rapid activation of superoxide by fMLP, fluoride activates after a prolonged lag (about 5 to 8 min). Previously, English and colleagues [37] demonstrated that fluoride activates the phospholipase C-dependent hydrolysis of PIP<sub>2</sub>. In addition, fluoride causes calcium mobilization [4], arachidonic acid release [31], and increased cyclic AMP levels in neutrophils [38]. The present studies investigate fluoride as a stimulus for the generation of diradylglycerols and for the activation of phospholipase D. We find that fluoride is a profound stimulus for diradylglycerol generation, and that it acts by both phospholipase D-dependent and -independent mechanisms.

## 2. MATERIALS AND METHODS

Hespan (6.2% hetastarch in 0.9% NaCl) was obtained from American Hospital Supply Corp. Lymphocyte separation medium (6.2% Ficoll, 9.4% sodium diatrizoate) was purchased from Organon Teknika-Cappel (Durham, NC). [ $\gamma$ -<sup>32</sup>P]ATP (4000 Ci/mmol) was from ICN Radiochemicals. 1-*O*-[9,10-<sup>3</sup>H]Octadecyl-2-lyso-*sn*-glycero-3-phosphocholine ([<sup>3</sup>H]alkyl-lyso PC, 110 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Cytochalasin B, fMLP, phospholipase C (*Bacillus cereus* and *Clostridium perfringens*), octyl- $\beta$ -D-glucopyranoside, ATP, fetal bovine serum, sodium fluoride and AlCl<sub>3</sub> were from Sigma. Cardiolipin (bovine heart), L- $\alpha$ -1,2-dioleoylglycerol and L- $\alpha$ -1,2-dioctanoylglycerol were shipped on dry ice from Avanti Polar Lipids (Birmingham, AL). *Escherichia coli* diglyceride kinase was obtained from Lipidex (Middletown, WI). *Rhizopus* lipase was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Silica gel 60 TLC plates were from EM Science. EAG was prepared and purified from 1-*O*-hexadecyl-2-oleoyl PC as described [39,40]. All other reagents and solvents were of the highest quality available commercially.

Human neutrophils were isolated from the peripheral blood of healthy adult donors as described previously [41]. In all cases, informed consent was obtained. Isolated cells were resuspended in PBS/glucose (2.6 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgCl<sub>2</sub>, 136 mM NaCl, 8 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.6 mM CaCl<sub>2</sub>, 5.5 mM glucose, pH 7.4) for superoxide generation and estimation of diradylglycerol, and in Hepes/saline buffer (25 mM Hepes, 125 mM NaCl, 0.7 mM MgCl<sub>2</sub>, 0.5 mM EGTA, 10 mM glucose and 1 mg/ml of fatty acid-free bovine serum albumin, pH 7.4) for labeling of cells with [<sup>3</sup>H]alkyl-lyso PC.

Superoxide was measured as described previously [9] by monitoring superoxide dismutase-inhibitable cytochrome *c* reduction, using an SLM/Aminco DW 2000 spectrophotometer in the dual wavelength mode (*A*<sub>549</sub>–*A*<sub>540</sub>). A difference extinction coefficient of 21 mM<sup>-1</sup>·cm<sup>-1</sup> was used to calculate the quantity of cytochrome *c* reduced [42]. The 'maximal rate' refers to highest rate achieved after the initial lag (10–20 s for fMLP and 5–8 min for fluoride) which follows addition of agonist. Lipids were extracted according to Bligh and Dyer [43]. Diradylglycerol was quantified by conversion to [<sup>32</sup>P]phosphatidic acid using *E. coli* diglyceride kinase and [ $\gamma$ -<sup>32</sup>P]ATP by the method of Preiss et al. [44] with modifications [45]. 1-*O*-Alkyl versus 1-acyl containing species were differentiated as described [46] using selective destruction by *Rhizopus* lipase of 1-acyl containing species.

The choline phospholipid pool of neutrophils ( $2 \times 10^7$  cells/ml) was labeled with [<sup>3</sup>H]alkyl-lyso PC according to Billah et al. [25]. Incubations and lipid extractions were carried out as above, except that 2% acetic acid rather than 1 M NaCl was used in the extraction. [<sup>3</sup>H]PA, [<sup>3</sup>H]PETH, and [<sup>3</sup>H]EAG were analyzed on silica gel 60 TLC plates separated in the following solvent systems: chloroform/methanol/acetic acid (90:10:10, by volume) for PA (*R*<sub>f</sub>, 0.47) and PETH (*R*<sub>f</sub>, 0.70), and chloroform/methanol/acetic acid (98:2:1, by volume) for EAG (*R*<sub>f</sub>, 0.67). After visualization of standards by iodine, followed by autoradiography, appropriate regions were scraped and quantified by scintillation counting as described [45].

## 3. RESULTS

### 3.1. Effect of ethanol on superoxide generation by neutrophils stimulated with fMLP and fluoride

As shown in Fig. 1 (panel A), fMLP stimulates superoxide generation following a brief lag (about 15 s), and superoxide generation ceases after about 2 min. When cells are pretreated with cytochalasin B, the kinetics of activation are similar, but superoxide generation is enhanced at later time points since the respiratory burst continues beyond 2 min. In both cases, 0.8% ethanol (data not shown) or 1.6% ethanol nearly completely inhibited superoxide generation, as reported previously [27].

In contrast to fMLP, fluoride activates after a prolonged lag (panel C), and superoxide generation continued beyond 20 min (note the difference in scales in upper and lower panels). Unlike fMLP, superoxide generation in response to fluoride was inhibited only slightly by ethanol at either 0.8 (not shown) or 1.6% (Fig. 1, panel C). However, the lag was prolonged (2–5 min for three experiments). Fluoride produced only about a third of the maximal rate of superoxide generation as fMLP. This was apparently due to a nonspecific effect of fluoride on NADPH-oxidase activity, since in the presence of fluoride, the rate of fMLP-stimulated superoxide generation was the same as that seen with fluoride alone (data not shown). Viability of neutrophils as judged by Trypan blue exclusion was greater than 95% at both 0.8 and 1.6% ethanol, and did not change with either agonist. Data, expressed as maximal rates of superoxide generation, are summarized in Table I.

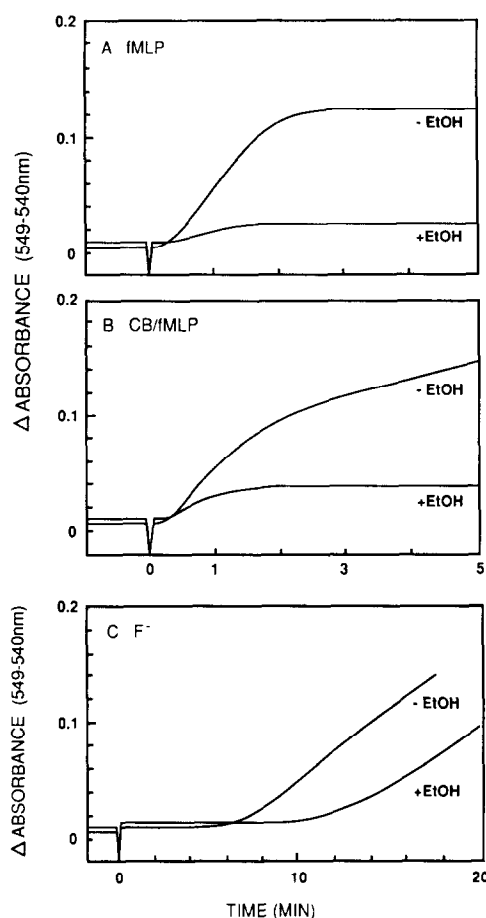


Fig. 1. Effect of ethanol on superoxide production in neutrophils stimulated with fMLP or fluoride. Preincubation and activation conditions were as in Table I. Cells were preincubated without (panels A and C) or with (panel B) cytochalasin B (CB). Cells were then activated with fMLP or fluoride, as indicated at time zero in the presence or absence of ethanol (EtOH) and superoxide generation was monitored continuously as superoxide dismutase inhibitable cytochrome *c* reduction. The results are representative of three experiments carried out on separate preparations.

### 3.2. Fluoride and fMLP activation of diradylglycerol generation, and effects of ethanol

Cytochalasin B enhances and prolongs fMLP-stimulated diradylglycerol generation (both DAG and EAG), which peaks at about 3 min [9,12,46–48]. In the present studies using cytochalasin-pretreated cells\*, fMLP caused a marked increase in total diradylglycerol, which consisted of both DAG and EAG (Table II). The occurrence of the latter implies an origin from phospholipid sources other than phosphoinositides which are comprised almost exclusively of diacyl linkages [49]. Ethanol reduced the production of both species to basal levels.

\* Diradylglycerol generation by fMLP in non-cytochalasin B-pretreated cells is too small and transient to be reliably measured; we have therefore not carried out the experiment in Table II on non-treated neutrophils

As shown in Fig. 2, fluoride is a potent stimulus for the generation of diradylglycerol (filled squares); both DAG (open squares) and EAG (open circles) are formed. As with superoxide generation, there was a prolonged lag prior to the onset of diglyceride formation. In more extended time courses (not shown), diradylglycerol levels (both species) peaked at about 40 min and levels declined slightly by about 1 h. As shown in Table II the maximum quantity of diradylglycerol achieved by fluoride (1708 pmol/10<sup>7</sup> cells) was significantly higher than that produced in response to fMLP (483 pmol/10<sup>7</sup> cells). With fluoride stimulation (Table II), ethanol caused only a partial inhibition of diradylglycerol generation (overall 33% inhibition compared with 100% with fMLP). EAG generation was more sensitive to ethanol than was DAG (50% versus 16% inhibition, respectively). Even in the presence of ethanol, quantities of both diradylglycerols remained more than 2-fold higher than levels seen in fMLP-stimulated cells without ethanol.

### 3.3. Fluoride and fMLP activation of choline glycerolipid hydrolysis, and effects of ethanol

1-O-[<sup>3</sup>H]Alkyl-lyso PC, when added to neutrophils, becomes rapidly acylated to form the corresponding diradyl choline glycerolipid [50]. This methodology has been used [24,25] to label the PC pool, and to demonstrate that fMLP causes a rapid generation first of [<sup>3</sup>H]PA, followed by formation of [<sup>3</sup>H]EAG. Ethanol inhibited essentially all of the [<sup>3</sup>H]EAG, and more than 85% of the [<sup>3</sup>H]PA production [24], with concomitant formation of [<sup>3</sup>H]phosphatidylethanol, but was without effect on inositol phosphate release

Table I

Effect of ethanol on superoxide generation in neutrophils activated by fMLP and fluoride

Preincubation	Superoxide	
	nmol/min per 10 <sup>6</sup> cells	% of max
Buffer/fMLP	8.8	100
Ethanol/fMLP	1.0	11
CB/buffer/fMLP	7.9	100
CB/ethanol/fMLP	1.9	24
Buffer/fluoride	2.7	100
Ethanol/fluoride	2.1	78

Cells (1 × 10<sup>6</sup>/2.5 ml) were incubated at 37°C for 5 min with or without cytochalasin B (CB; 5 μM). Ethanol (1.6%) or incubation buffer was then added, and the incubation was continued for an additional 5 min, prior to the addition of fMLP (1 μM). For fluoride activation, cells were preincubated for 5 min at 37°C, ethanol or buffer was added and the incubation was continued for an additional 5 min prior to addition of fluoride (20 mM) containing 160 μM AlCl<sub>3</sub>. Superoxide generation (cytochrome *c* reduction) was monitored continuously as in Fig. 1, and the maximal rate was calculated as in section 2. Although absolute rates of superoxide generation differ among preparations, qualitatively similar data were obtained using neutrophil preparations from three donors

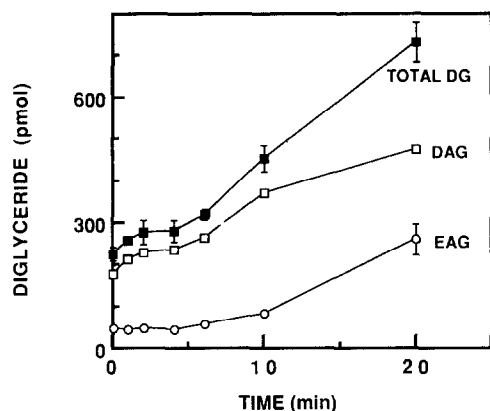


Fig. 2. Diradylglycerol generation in fluoride-stimulated neutrophils. Separate incubations of cells (each  $1 \times 10^7$  cells in 1.0 ml incubation buffer) were incubated for 10 min at  $37^\circ\text{C}$  prior to the addition of 20 mM fluoride. At the indicated times, the entire incubation was transferred to chloroform/methanol, and diglyceride species were quantified as in section 2. Shown are total diglycerides (filled squares), 1,2-diacylglycerol (DAG, open squares) and 1-O-alkyl, 2-acyl glycerol (EAG, circles). Points shown are the average and standard error of 3 incubations using a single preparation. Data are representative of four experiments on separate neutrophil preparations.

[27], confirming that the phospholipase D but not the phospholipase C pathway was ethanol-sensitive. To test whether PC was degraded in response to fluoride, the choline glycerolipid pool was radiolabeled as above. Fluoride caused a large increase in the generation of both  $[^3\text{H}]\text{PA}$  and  $[^3\text{H}]\text{EAG}$ , as shown, respectively, in the middle and lower panels of Fig. 3 (open squares). Generation of both species was characterized by a lag which was similar to that seen for both superoxide generation (Fig. 1) and diglyceride mass (Fig. 2).

In contrast to the complete inhibition by ethanol of fMLP-stimulated  $[^3\text{H}]\text{EAG}$  generation [24],  $[^3\text{H}]\text{EAG}$  production in response to fluoride was partially in-

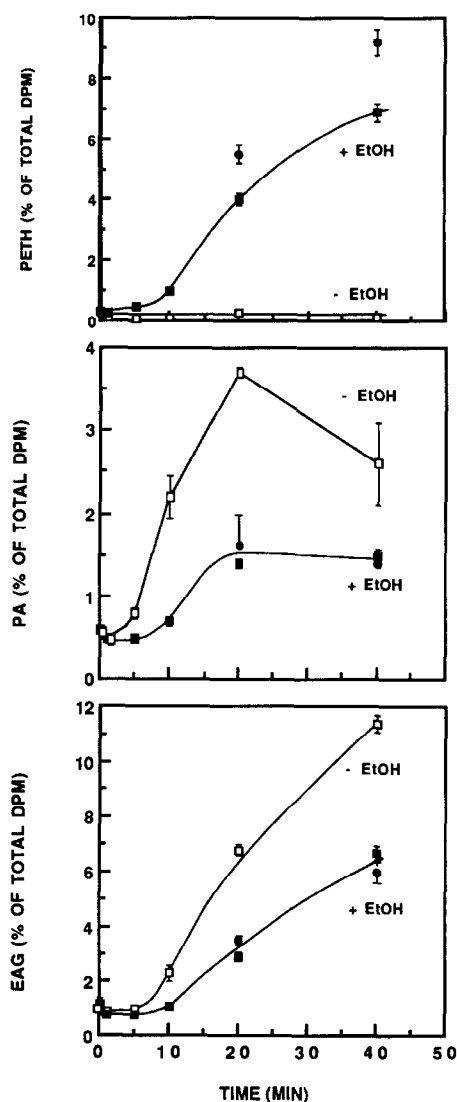


Fig. 3. Effect of ethanol on  $[^3\text{H}]\text{PETH}$ ,  $[^3\text{H}]\text{PA}$  and  $[^3\text{H}]\text{EAG}$  in fluoride-stimulated neutrophils. Cells ( $5 \times 10^6$  cells/0.5 ml), in which the PC pool was labeled, were stimulated with fluoride at time zero, and lipid extracts were analyzed for  $[^3\text{H}]\text{PETH}$  (upper panel),  $[^3\text{H}]\text{PA}$  (middle panel) and  $[^3\text{H}]\text{EAG}$  (lower panel) as described in section 2. Incubations were carried out in the presence of either 0.8% (filled squares) or 1.6% (filled circles) ethanol, or an equal volume of incubation buffer (open squares). Points and error bars represent the mean and standard error of three incubations from the same neutrophil preparation. The experiment shown is representative of three, using separate preparations.

Table II

Effect of ethanol on diradylglycerol generation in neutrophils stimulated with fMLP and fluoride

Treatment group	pmol/ $10^7$ cells		
	Diradylglycerol (total)	EAG	DAG
CB (no fMLP)	185 $\pm$ 15	64 $\pm$ 6	121
CB/buffer/fMLP	483 $\pm$ 28	209 $\pm$ 40	274
CB/ethanol/fMLP	179 $\pm$ 25	69 $\pm$ 6	110
No additions	209 $\pm$ 10	82 $\pm$ 7	127
Buffer/fluoride	1708 $\pm$ 136	802 $\pm$ 69	906
Ethanol/fluoride	1209 $\pm$ 140	430 $\pm$ 14	779

Preincubation and activation conditions were as in Table I. Reactions were quenched in chloroform/methanol at 3 min for fMLP and 40 min for fluoride, and diglycerides were analyzed as described in section 2. Values shown are the means  $\pm$  SE of three incubations. Since DAG levels were determined subtractively (i.e. total diglyceride minus EAG), standard error is not shown for the DAG column

hibited (about 50%, Fig. 3, lower panel).  $[^3\text{H}]\text{Phosphatidylethanol}$  formation (upper panel, Fig. 3) occurred in parallel with inhibition, demonstrating the phospholipase D-dependent transphosphatidyl reaction. The extent of inhibition was similar to that seen for ethanol inhibition of EAG mass (52%, Table II). More pronounced ethanol inhibition of  $[^3\text{H}]\text{PA}$  generation was seen (about 70%, Fig. 3, middle panel), but this was still somewhat less than the 85% inhibition by ethanol when fMLP was the agonist [24] suggesting that some of the  $[^3\text{H}]\text{PA}$  was

derived from [ $^3\text{H}$ ]EAG via the action of diglyceride kinase.

#### 4. DISCUSSION

The present studies support the importance of the phospholipase D pathway in receptor-mediated phosphatidate and diradylglycerol generation and in cell activation. Our studies also imply that fluoride stimulation produces the same mediators, but by both phospholipase D (ethanol-sensitive) and phospholipase C (ethanol-resistant) mechanisms. For diradylglycerol mass, this stimulus is far more potent than fMLP (including with cytochalasin B or other forms of priming), consistent with its derivation from multiple mechanisms and phospholipid pools. Regarding phospholipase C activation, English and colleagues [37] previously showed that fluoride is an extremely potent stimulus for phosphoinositide hydrolysis and inositol phosphate release. Based on ethanol inhibition, the present studies suggest that phospholipase C also acts on PC under these conditions. Ethanol inhibition experiments (Table II) also indicate that the phospholipase D mechanism is potently activated by fluoride, in fact to a higher absolute level than that seen with cytochalasin/fMLP. It seems reasonable to assume that fluoride acts on both phospholipases C and D via activation of the relevant G protein(s) to which these phosphodiesterases are coupled, and therefore results in the observed large mass of diradylglycerol.

The present studies provide an additional example of the correlation between diradylglycerol levels and superoxide generation. Such a correlation has previously been noted with concanavalin [47], ionomycin [47], and opsonized zymosan [9] and fMLP (not only when added alone, but also when cells had been primed with GM-CSF [51], low doses of PMA [45], and cytochalasin B [12,52]). For fluoride (present studies), the delayed onset and prolonged duration of diradylglycerol generation correlates reasonably well with the onset and duration of fluoride-activated superoxide generation. In addition, whereas ethanol inhibits both superoxide and diradylglycerol generation in fMLP-activated cells, fluoride-stimulated superoxide and diradylglycerol generation are both relatively resistant to the effects of the alcohol. However, the present studies do not rule out the possibility that phosphatidate could participate in activation (e.g., see [20,21]), since the kinetics of its generation are similar to those for both diglyceride and superoxide generation (Fig. 3). Phosphatidate could be generated in part from phospholipase C-derived diradylglycerol via the action of diglyceride kinase, as may be the case in Fig. 3. Due to limitations in the sensitivity of available assays for phosphatidic acid, little information is currently available on its mass in activated neutrophils and its possible relationship to activation.

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