

# The temporal relationship between phospholipase activation, diradylglycerol formation and superoxide production in the human neutrophil

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Fluctuations in the amounts of choline, inositol 1,4,5-trisphosphate ( $IP_3$ ) and diradylglycerol have been used to monitor phospholipase activation in the human neutrophil. Stimulation of human neutrophils by formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe) resulted in a rapid activation of both phosphatidylinositol 4,5-bisphosphate breakdown by phospholipase C and phosphatidylcholine breakdown by phospholipase D. Diradylglycerol accumulation occurred more slowly than that of either choline or  $IP_3$  and was inhibited by 30 mM-butanol, suggesting that the bulk was derived from the phospholipase D pathway via phosphatidate phosphohydrolase. Consistent with this is the observation that choline and diradylglycerol are produced in similar amounts. 1,2-Diacylglycerol (DAG) and 1-*O*-alkyl-2-acyl-*sn*-glycerol species accumulated with different time courses, indicating that one or more steps in the phospholipase D pathway was selective for the diacyl species. Superoxide production by fMet-Leu-Phe-stimulated neutrophils paralleled DAG accumulation over the first 5 min, but thereafter this production stopped, despite the fact that DAG remained elevated. We conclude that DAG derived from the phospholipase D pathway is only one of the second messengers important in controlling this functional response.

## INTRODUCTION

Levels of diradylglycerol (DRG; the prefix radyl refers to both ether and acyl linkages) have been observed to increase in many cell types following receptor stimulation (for review see [1]). The major source of this second messenger is considered to be phosphatidylinositol 4,5-bisphosphate ( $PIP_2$ ) hydrolysis catalysed by a specific phospholipase C (PLC) [1]. The other product of this reaction, inositol 1,4,5-trisphosphate ( $IP_3$ ), has a well-established role in mediating intracellular  $Ca^{2+}$  mobilization [1]. However, it is now evident that hydrolysis of other phospholipids can give rise to significant levels of DRG in stimulated cells. The transphosphatidylation property of phospholipase D (PLD) has been exploited by a number of groups of workers to establish the presence of agonist-stimulated PLD activity in different tissues, and phosphatidylcholine (PC) seems to be a major substrate for this enzyme [2–4]. When PLD is stimulated in the presence of a primary alcohol (e.g. butanol), transphosphatidylation results in the formation of a phosphatidyl alcohol at the expense of phosphatidic acid (PA) [5]. The alcohol therefore inhibits the formation of PA and prevents its subsequent conversion to DRG by phosphatidate phosphohydrolase. The relative contributions of the PLC and PLD pathways to DRG formation in the human neutrophil were investigated recently using this property of aliphatic alcohols to block selectively the PLD pathway [6]. The evidence presented demonstrated that (i) 30 mM-butanol inhibited DRG formation from the PLD pathway without affecting  $PIP_2$  hydrolysis by PLC and (ii) the DRG responsible for the fMet-Leu-Phe-stimulated superoxide production in cytochalasin B-treated neutrophils was derived through PLD.

DRG formation is often estimated from prelabelling studies employing radiolabelled fatty acids or glycerol, or by conversion of cellular DRG to [ $^{32}P$ ]PA using diacylglycerol kinase and

[ $^{32}P$ ]ATP [7]. These methods do not distinguish between the diacylglycerol (DAG) and the 1-alkyl- or 1-alkenyl-glycerol (AAG) species. It is becoming increasingly important to make this distinction, because it is now known that AAG is formed in significant amounts in human neutrophils stimulated by phorbol 12-myristate 13-acetate,  $Ca^{2+}$  ionophore (A23187) or fMet-Leu-Phe [8–11] and has been detected in other tissues following stimulation [12,13]. Furthermore, PLD hydrolysis of 1-*O*-alkyl-*sn*-glycero-3-phosphorylcholine (1-*O*-alkyl-PC) in human neutrophils and HL60 granulocytes has been clearly demonstrated [2,6,9]. The 1-*O*-alkyl-PA formed is then converted to AAG, presumably by phosphatidate phosphohydrolase. The functional significance of the 1-*O*-alkyl lipid species is unclear, but ether-linked acylglycerols are known to either activate or inhibit protein kinase C, depending upon the composition of the aliphatic chains [14–16]. In this report we have investigated how the activities of the polyphosphoinositide-specific PLC and the PC-hydrolysing PLD change with time after fMet-Leu-Phe stimulation. These changes have been related to the production of DAG and AAG. The contribution of each pathway to the production of DRG in cytochalasin B-treated neutrophils is discussed.

## MATERIALS AND METHODS

Human neutrophils were purified [17] and suspended in 30 mM-Hepes-buffered Hanks balanced salt solution, pH 7.2 [18]. PC pools were labelled with 1-*O*-[ $^3H$ ]alkyl-2-lyso-PC (Amersham International, Amersham, Bucks., U.K.) (80 Ci/mmol) by the method of Pai *et al.* [2]. Cells were preincubated with 5  $\mu$ M-cytochalasin B (Sigma) with or without alcohols for 5 min at 37 °C before the addition of fMet-Leu-Phe (Sigma). Reactions were terminated by addition of chloroform/methanol (1:2, v/v);

Abbreviations used: DRG, diradylglycerol; DAG, diacylglycerol; AAG, 1-*O*-alkyl- or 1-*O*-alkenyl-glycerol;  $IP_3$ , inositol 1,4,5-trisphosphate;  $PIP_2$ , phosphatidylinositol 4,5-bisphosphate; PC, phosphatidylcholine; PA, phosphatidic acid; PLC, phospholipase C; PLD, phospholipase D; 1-*O*-alkyl-PC, 1-*O*-alkyl-*sn*-glycero-3-phosphorylcholine; 1-*O*-alkyl-Pbut, 1-*O*-alkyl phosphatidylbutanol.

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and lipids were extracted as described by Bligh & Dyer [19]. Phosphatidyl alcohols and 1-*O*-alkyl-PA were separated by t.l.c. on silica-gel G plates (Whatman, Maidstone, Kent, U.K.) using the organic phase of 2,2,4-trimethylpentane/ethyl acetate/acetic acid/water (5:11:2:10, by vol.) as the mobile phase. The  $R_f$  values were 0.36 for 1-*O*-alkylphosphatidylbutanol (1-*O*-alkyl-PBut) and 0.13 for 1-*O*-alkyl-PA. Amounts of DRG were measured as described [7], except that mixed micelles containing 5 mol% of phosphatidylserine in Triton X-100 (0.3%, w/v) were used in place of  $\beta$ -octylglucoside/cardiophilin. DAG and AAG were distinguished by further incubation of samples with 500 units of phospholipase  $A_1$  from *Rhizopus arrhizus*, as described in [11]. For the measurement of  $IP_3$ , neutrophils were treated with ice-cold  $HClO_4$  (2%, v/v) and the protein-free supernatant was neutralized with KOH (220 mM). The  $IP_3$  content of the neutralized samples was measured using the adrenocortical binding protein assay as described by Palmer *et al.* [20]. Superoxide production was monitored using cytochrome *c* as an indicator, as described in [21]. Choline was measured in the aqueous phase by a modification of the method of Wang & Haubrich [22]. Briefly, the aqueous phase was evaporated to dryness under  $N_2$  and redissolved in a buffer containing 25 mM-Tris/HCl, 9 mM-MgSO<sub>4</sub>, 2.25 mM-EGTA, 9 mM-NaF and 1 mM-[<sup>32</sup>P]ATP (22.5 mCi/mmol) in a final volume of 200  $\mu$ l. The reaction was run for 2 h at 37 °C and stopped by addition of 0.5 mg of phosphorylcholine/ml. [<sup>32</sup>P]Phosphorylcholine was separated on Dowex 1-X4 anion exchange columns (1.5 ml bed volume) as described [22].

## RESULTS

In confirmation of previous studies, superoxide production in response to fMet-Leu-Phe was found to be rapid in onset and continued at a linear rate for up to 5–6 min (Fig. 1; [23,24]). The termination of the response at this point was not due to depletion of cytochrome *c*, as stimulation by PMA under the same conditions induced a greater reduction of the indicator (results not shown). There is now a substantial amount of evidence to support a role for cellular DAG and protein kinase C in mediating

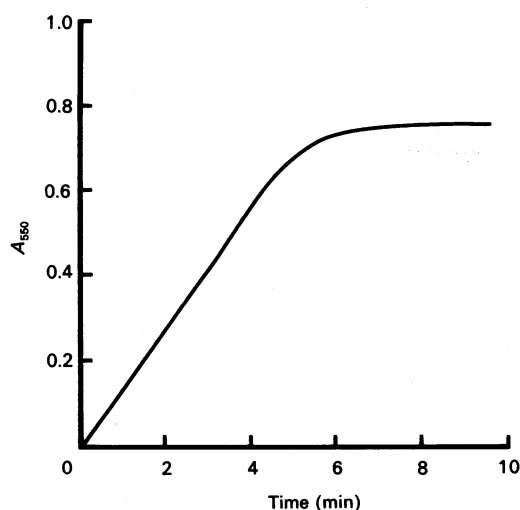


Fig. 1. Superoxide production in fMet-Leu-Phe-stimulated human neutrophils

Neutrophils [(1–2) × 10<sup>6</sup>/ml] were pre-incubated with 5  $\mu$ M-cytochalasin B for 5 min at 37 °C in the presence of cytochrome *c* before addition of 10<sup>-7</sup> M-fMet-Leu-Phe. The change in absorbance at 550 nm was monitored for 10 min. The results shown are from one experiment but are representative of many others.

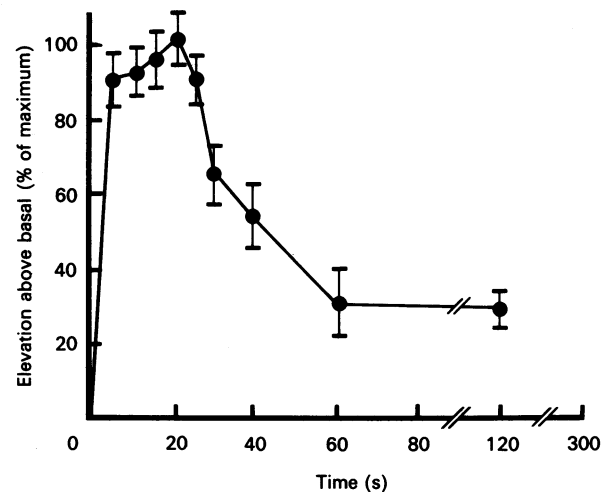


Fig. 2. Time course of fMet-Leu-Phe-stimulated  $IP_3$  formation in human neutrophils

Neutrophils (0.5 ml, 2 × 10<sup>7</sup>/ml) were pre-incubated with 5  $\mu$ M-cytochalasin B for 5 min at 37 °C before addition of 10<sup>-7</sup> M-fMet-Leu-Phe.  $IP_3$  was measured at the times indicated as described in the Materials and Methods section. The results shown are the means ± S.E.M. for three experiments each performed in triplicate.

this response [23,25,26]. We have investigated the source of this DAG by measuring phospholipase activities in fMet-Leu-Phe-stimulated cells. Activation of  $PIP_2$ -specific PLC was assessed by measuring the accumulation of  $IP_3$  (Fig. 2). In the different experiments performed, basal levels of  $IP_3$  varied considerably (6–43 pmol/10<sup>7</sup> cells). In all cases, however, the amount of  $IP_3$  increased transiently in response to fMet-Leu-Phe stimulation, reaching maximum values of three times basal at 5 s (range 17–122 pmol/10<sup>7</sup> cells; Fig. 2).

Hydrolysis of PC by PLD was assessed in neutrophils pre-labelled with 1-*O*-[<sup>3</sup>H]alkyl-lyso-PC by measuring fMet-Leu-Phe-stimulated formation of 1-*O*-[<sup>3</sup>H]alkyl-PA or 1-*O*-[<sup>3</sup>H]alkyl-PBut. The latter product is formed by a transphosphatidylation reaction when cells are stimulated in the presence of 10 mM-butanol. PLD activity was also assessed by measuring the formation of free choline. All three products increased rapidly during the first 1 min after addition of fMet-Leu-Phe (Fig. 3). The increase in 1-*O*-[<sup>3</sup>H]alkyl-PA was transient, peaking at 50 s, whereas the levels of 1-*O*-[<sup>3</sup>H]alkyl-PBut and choline remained elevated for up to 5 min. Thus all three methods indicated that there was a burst of PLD activity following addition of the chemotactic peptide.

The amount of DRG in human neutrophils increased rapidly in response to fMet-Leu-Phe (Fig. 4a), as described previously [23]. An increase was detected as early as 10 s, and by 2 min this had reached a maximum level of about 400 pmol/10<sup>7</sup> cells, which was maintained for at least 10 min. In some experiments (four out of 15), it was possible to detect an initial transient increase in DRG which peaked at about 20–30 s (Fig. 4b).

The phospholipase  $A_1$  activity of the lipase from *Rhizopus arrhizus* was used to determine the proportions of both DAG and AAG species making up the total DRG formed in fMet-Leu-Phe-stimulated neutrophils [11]. In unstimulated neutrophils DAG and AAG were present in approximately equal amounts (38 ± 5 and 40 ± 5 pmol/10<sup>7</sup> cells respectively) (Fig. 5). fMet-Leu-Phe induced a rapid increase in DAG, which reached 210 pmol/10<sup>7</sup> cells by 2 min and returned almost to basal by 10 min. AAG formation was similar in extent to DAG formation but occurred more slowly (Fig. 5). In the presence of 30 mM-

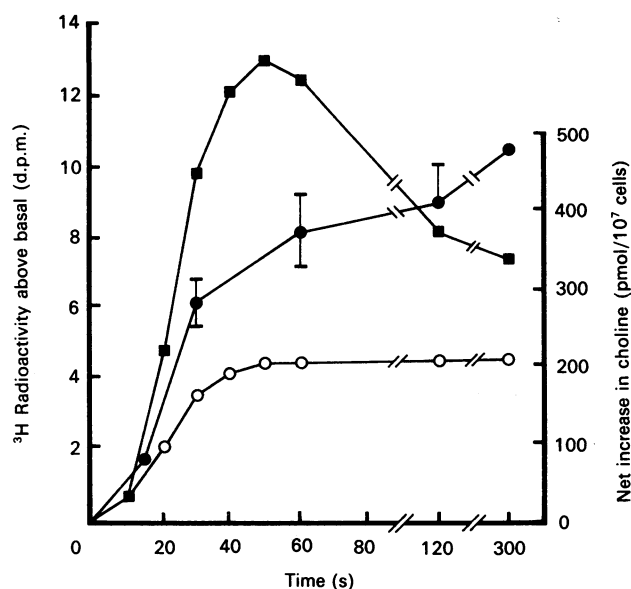


Fig. 3. Time course of fMet-Leu-Phe-stimulated PLD activation

Neutrophils were incubated with 1-*O*-[<sup>3</sup>H]alkyl-lyso-PC to label the 1-*O*-alkyl-PC pools. The cells were pretreated with cytochalasin B as described in the legend to Fig. 1 before addition of 10<sup>-7</sup> M-fMet-Leu-Phe. At the times indicated, lipids were extracted and the labelled products were analysed for 1-*O*-[<sup>3</sup>H]alkyl-PA (■) and 1-*O*-[<sup>3</sup>H]alkyl-PBut (○) by t.l.c. Results are the means of duplicates from one experiment which is representative of three others. The variation between duplicates was less than or equal to 5%. Choline formation (●) was measured as described in the Materials and methods section using non-labelled cells which were treated with cytochalasin B and fMet-Leu-Phe as described before. These results are means ± S.E.M. from three experiments, each performed in duplicate. The mean basal level of choline in neutrophils was 180 ± 27 pmol/10<sup>7</sup> cells (*n* = 5).

butanol [<sup>3</sup>H]PA formation was inhibited by 91 ± 8% (results not shown) and AAG production was abolished (Fig. 5). Under these conditions DAG formation continued, but at a very much lower rate (Fig. 5).

## DISCUSSION

The description by Priess *et al.* [7] of a sensitive method for determining the amount of cellular DRG has greatly improved our interpretation of the role of this second messenger in signal transduction. The assay avoids complications arising from changes in the specific activity of metabolites in isotopically labelled cells. The use of this assay, together with similar assays to determine the amounts of IP<sub>3</sub> [20] and choline, has allowed us to measure accurately fluctuations in the levels of these products in stimulated neutrophils.

fMet-Leu-Phe induced a rapid activation of both PIP<sub>2</sub>-specific PLC and PC breakdown by PLD. Analysis of the amounts of IP<sub>3</sub> has confirmed the conclusions drawn from experiments using isotopically labelled cells [27]. Our results demonstrate that the onset of PLD activation is only slightly slower than that of PLC activation. It is not possible to say whether the products from PIP<sub>2</sub> hydrolysis play a role in PLD activation as suggested by Billah *et al.* [28], or whether there is a specific receptor-coupled PLD.

The transient increase observed in [<sup>3</sup>H]PA (Fig. 3) suggests that PLD activation may be short-lived. It should be noted, however, that [<sup>3</sup>H]PA formation is the least reliable measure of PLD activity, because this species can be formed by PLC breakdown of 1-*O*-[<sup>3</sup>H]alkyl-PC to [<sup>3</sup>H]AAG and subsequent phosphorylation by diacylglycerol kinase. A transient activation of PLD is consistent with the sustained increase in [<sup>3</sup>H]PBut levels (Fig. 3), as these novel phosphatidyl alcohols appear to be metabolically stable. If PLD activation was transient, then choline levels would have been expected to fall as a result of further metabolism. This latter anomaly may be explained if the bulk of the choline was released from the cell and was, as a result, unavailable for further metabolism. The measurement of total choline performed in this study would not make this distinction. Thus it is not clear at present if PLD activity remains elevated or whether it returns to basal levels after the initial burst.

Large amounts of DAG and AAG are formed in fMet-Leu-Phe-stimulated neutrophils (Fig. 5; [10,11]). There is some disagreement in the literature over the exact time course, but in all cases the production of AAG was found to lag behind that of DAG. The use of primary alcohols selectively to block the PLD

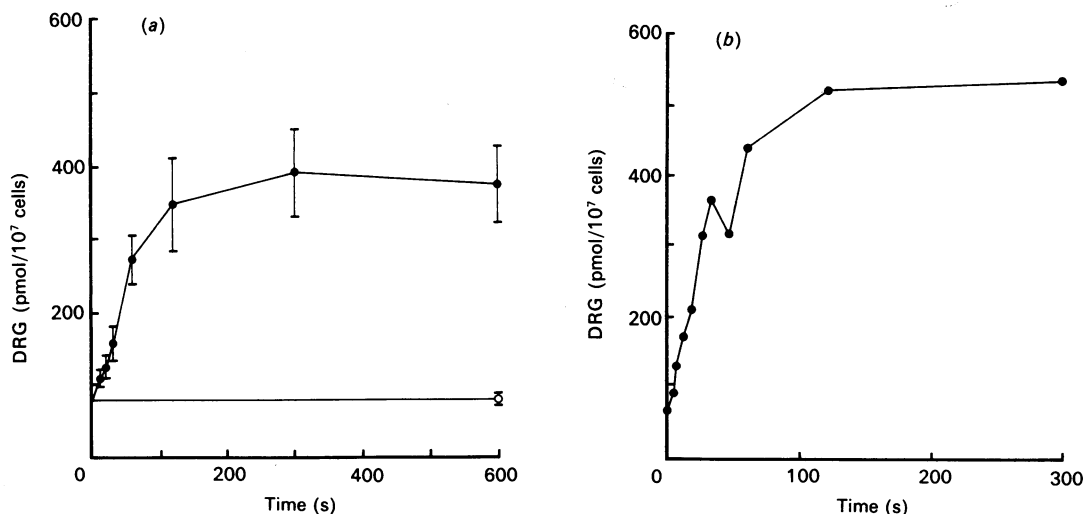


Fig. 4. Time course of fMet-Leu-Phe-stimulated DRG formation

Neutrophils (0.5 ml, 2 × 10<sup>7</sup>/ml) were pre-incubated with 5 μM-cytochalasin B for 5 min at 37 °C before addition of 10<sup>-7</sup> M-fMet-Leu-Phe (●) or vehicle (○). At the times indicated, lipids were extracted and DRG levels were determined using *Escherichia coli* DAG kinase as described in the Materials and methods section. (a) Means ± S.E.M. of results from three experiments; (b) means of duplicate determinations from one experiment representative of a number of others.

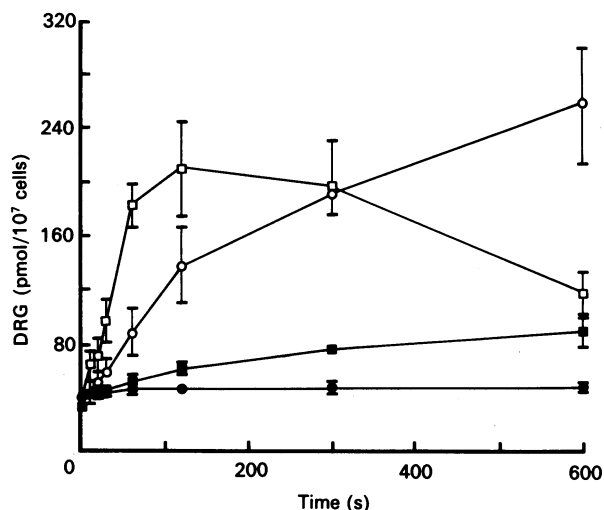


Fig. 5. Time course of fMet-Leu-Phe-stimulated DAG and AAG formation in the presence and absence of butanol

Neutrophils (0.5 ml,  $2 \times 10^7$ /ml) were pre-incubated with 5  $\mu$ M-cytochalasin B in the presence (■, ●) or absence (□, ○) of 30 mM-butanol for 5 min at 37 °C before addition of  $10^{-7}$  M-fMet-Leu-Phe. DAG (■, □) and AAG (●, ○) were determined using *E. coli* DAG kinase and *Rhizopus* lipase as described in the Materials and methods section. The results shown are the means  $\pm$  S.E.M. from three experiments. The increase in DAG was significantly greater than the increase in AAG at the 60 s time point (level of significance > 95 %).

pathway suggests that this is the main source of both DAG and AAG in the neutrophil [6]. Here we have demonstrated that, even at early times, butanol inhibited more than 90 % of DRG production. Further support for the contention that  $PIP_2$  is a minor source of DAG under these conditions comes from the observation that the amount of  $IP_3$  formed in fMet-Leu-Phe-stimulated neutrophils was much less, and occurred significantly earlier, than the major peak in DAG levels. It is possible that the small transient increase in DRG sometimes observed at 30 s corresponds to the small amount of DRG expected to result from  $PIP_2$  hydrolysis. The large variation seen in this response is probably due to the fact that an increase in DRG of the magnitude more often observed for  $IP_3$  production (20–30 pmol/ $10^7$  cells) cannot easily be detected against basal DRG levels of approx. 100 pmol/ $10^7$  cells.

PC is a major substrate for PLD in HL60 cells and rat hepatocytes [28,29]. This also seems to be true in the human neutrophil, since the amount of choline produced following stimulation by fMet-Leu-Phe (Fig. 3) is similar to the amount of DRG derived from the PLD pathway (Fig. 5). The PC pool in neutrophils consists of approximately equal amounts of diacyl and 1-*O*-alkyl species [30,31]. Therefore a non-selective PLD activity would produce diacyl-PA and 1-*O*-alkyl-PA in equal amounts. The different rates of production of the DRG species may indicate that two distinct PLD activities exist, one being specific for diacyl-PC and the other for 1-*O*-alkyl-PC. Alternatively, phosphatidate phosphohydrolase may hydrolyse diacyl-PA preferentially.

There is a substantial amount of evidence supporting a role for protein kinase C in the production of superoxide by the neutrophil. For example, phorbol esters and cell-permeant DAGs are potent stimulators of this response [25,26], and Rider & Niedel [23] have reported that superoxide production in the neutrophil occurs in parallel with DRG production. We can confirm that in the first few minutes after stimulation by fMet-Leu-Phe a similar relationship applies to DAG and superoxide production. How-

ever, after 5 min the production of superoxide has virtually stopped (Fig. 1), despite DAG remaining at a maximum level (Fig. 5). There are several possible explanations for the dissociation between response and second messenger at later times. Firstly, an increase in DAG alone may not be a sufficient stimulus for sustained superoxide production. Alternatively, the loss of the functional response may reflect the production of an endogenous inhibitor. In this regard it is worth noting that 1-*O*-alkyl-2-acyl-*sn*-glycerols with one or two carbon aliphatic moieties at the *sn*-2 position have been reported to inhibit protein kinase C, whereas those with an unsaturated chain at the *sn*-1 position activate this enzyme [14–16]. An unsaturated ether-linked chain was found to be the most abundant species in the AAG fraction from PMA-stimulated neutrophils [10], but the composition in fMet-Leu-Phe-stimulated cells has not been reported. Clearly further work is required before the control of superoxide production is properly understood.

By measuring PLC and PLD activities in the fMet-Leu-Phe-stimulated human neutrophil, we have shown that sustained production of DAG, and a response dependent upon this second messenger, result from a PLD-dependent hydrolysis of phospholipids. The contribution of a  $PIP_2$ -specific PLC to DAG production in the cytochalasin B-treated neutrophil is much smaller, but may have greater significance at early times (< 10 s), i.e. before the onset of PLD activation.

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## REFERENCES

- Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–193
- Pai, J.-K., Siegel, M. I., Egan, R. W. & Billah, M. M. (1988) *J. Biol. Chem.* **263**, 12472–12477
- Bocckino, S. B., Wilson, P. B. & Exton, J. H. (1987) *FEBS Lett.* **225**, 201–204
- Rubin, R. (1988) *Biochem. Biophys. Res. Commun.* **156**, 1090–1096
- Dawson, R. (1967) *Biochem. J.* **102**, 205–210
- Bonser, R. W., Thompson, N. T., Randall, R. W. & Garland, L. G. (1989) *Biochem. J.* **264**, 617–620
- Preiss, J. E., Loomis, C. R., Bishop, R. W., Stein, R., Neidel, J. E., & Bell, R. M. (1986) *J. Biol. Chem.* **261**, 8597–8600
- Agwu, D. E., McPhail, L. C., Chabot, M. C., Daniel, L. W., Wykle, R. L. & McCall, C. E. (1989) *J. Biol. Chem.* **264**, 1405–1413
- Billah, M. M., Eckel, S., Mullmann, T. J., Egan, R. W. & Siegel, M. I. (1989) *J. Biol. Chem.* **264**, 17069–17077
- Dougherty, R. W., Dubay, G. R. & Niedel, J. E. (1989) *J. Biol. Chem.* **264**, 11263–11269
- Tyagi, S.-R., Burnham, D. N. & Lambeth, J. D. (1989) *J. Biol. Chem.* **264**, 12977–12982
- Daniel, L. W., Waite, M. & Wykle, R. L. (1986) *J. Biol. Chem.* **261**, 9128–9132
- Hii, C. S. T., Kokke, Y. S., Pruimboom, W. & Murray, A. W. (1989) *FEBS Lett.* **257**, 35–37
- Daniel, L. W., Small, G. W. & Schmitt, J. D. (1988) *Biochem. Biophys. Res. Commun.* **151**, 291–297
- Kramer, I. M., van der Bend, R. L., Tool, A. T. J., van Blitterswijk, W. J., Roos, D. & Verhoeven, A. J. (1989) *J. Biol. Chem.* **264**, 5876–5884
- Ford, D. A., Miyake, R., Glaser, P. E. & Gross, R. W. (1989) *J. Biol. Chem.* **264**, 13818–13824
- Tateson, J. E., Randall, R. W., Reynolds, C. H., Jackson, W. P., Bhattacharjee, P., Salmon, J. A. & Garland, L. G. (1988) *Br. J. Pharmacol.* **94**, 528–539
- Hanks, J. H. & Wallace, R. E. (1949) *Proc. Soc. Exp. Biol. Med.* **71**, 196–200
- Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917

20. Palmer, S., Hughes, K. T., Lee, D. Y. & Wakelam, M. J. O. (1988) *Cell. Signalling* **1**, 147–156
21. Choen, H. J. & Chovaniec, M. E. (1978) *J. Clin. Invest.* **61**, 1081–1087
22. Wang, F. L. & Haubrich, D. R. (1975) *Anal. Biochem.* **63**, 195–201
23. Rider, L. G. & Niedel, J. E. (1987) *J. Biol. Chem.* **262**, 5603–5608
24. Korchak, H. M., Vosshall, L. B., Haines, K. A., Wilkenfeld, C., Lundquist, K. F. & Weissmann, G. (1988) *J. Biol. Chem.* **263**, 11098–11105
25. DiVirgilio, F., Lew, D. P. & Pozzan, T. (1984) *Nature (London)* **310**, 691–693
26. Penfield, A. & Dale, M. M. (1984) *Biochem. Biophys. Res. Commun.* **125**, 332–336
27. Burnham, D. N., Tyagi, S.-R., Uhlinger, D. J. & Lambeth, J. D. (1989) *Arch. Biochem. Biophys.* **269**, 345–353
28. Billah, M. M., Anthes, J. C., Egan, R. W., Mullmann, T. J. & Siegel, M. I. (1990) in *Biology of Cellular Transducing Signals* (Vanderhock, J. Y., ed.), Plenum Publishing, New York, in the press
29. Bocckino, S. B., Blackmore, P. F., Wilson, P. B. & Exton, J. H. (1987) *J. Biol. Chem.* **262**, 15309–15315
30. Mueller, H. W., O'Flaherty, J. T., Greene, D. G., Samuel, M. P. & Wykle, R. L. (1984) *J. Lipid Res.* **25**, 383–388
31. Tencé, M., Jouvin-Marche, E., Bessou, G., Record, M. & Benveniste, J. (1985) *Thromb. Res.* **38**, 207–214

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