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Phospholipase D activation is functionally linked to superoxide generation in the human neutrophil

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Neutrophils stimulated with formylmethionyl-leucylphenylalanine (fMet-Leu-Phe) in the presence of butanol and ethanol formed phosphatidylalcohols through a phopholipase D mechanism. The alcohols inhibited phosphatidic acid and diradylglycerol (DRG) formation, but did not block inositol 1,4,5-trisphosphate release. fMet-Leu-Phe-stimulated superoxide production was inhibited by alcohol concentrations which blocked DRG formation, whereas opsonized-zymosan-stimulated superoxide production was only partially decreased. These results suggest that phospholipase D activation is functionally linked to superoxide production in the human neutrophil.

INTRODUCTION

Receptor-coupled activation of phospholipase C (EC 3.1.4.3) yields two second messengers, namely inositol 1,4,5-trisphosphate and diacylglycerol (DAG). Inositol 1,4,5-trisphosphate mobilizes intracellular Ca^{2+}, and DAG regulates protein kinase C (PKC) activity, which has an important role in stimulating superoxide production by the neutrophil [1–5]. Recent evidence has indicated that inositol lipid hydrolysis is not the only source of diradylglycerols (DRG) in activated cells [6–8]. A number of agonists elicit functional responses in the absence of inositol 1,4,5-trisphosphate formation or an increase in intracellular Ca^{2+} [9–16]. Furthermore, many of these responses are accompanied by raised DRG levels or increased phosphatidylcholine (PtdCho) turnover [9–12,15,16].

In HL60 granulocytes, formylmethionyl-leucylphenylalanine (fMet-Leu-Phe) activates a phospholipase D (EC 3.1.4.4), which releases phosphatidic acid (PtdA) from PtdCho [17,18]. PtdA can be further hydrolysed by phosphatidate phosphatase (EC 3.1.3.4) to DRG [19]. In the presence of ethanol the activation of phospholipase D results in the formation of phosphatidylethanol through a transphosphatidylation reaction together with a decrease in PtdA generation [17,18,20–24]. In the present study aliphatic alcohols have been used to investigate (i) phospholipase D activation by fMet-Leu-Phe in the human neutrophil, (ii) the importance of this pathway for DRG generation and (iii) the significance of this signal-transduction pathway in stimulating superoxide production.

MATERIALS AND METHODS

Human neutrophils were purified [25] and suspended in 30 mM-Hepes-buffered Hanks balanced salt solution, pH 7.2 [26]. PtdCho pools were labelled with [^{3}H]1-*O*-alkyl-2-lyso-PtdCho (Amersham International, Amersham, Bucks., U.K.) by the method of Pai *et al.* [17]. Cells were preincubated with 5 μM-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 adding chloroform/methanol (1:2, v/v); lipids were extracted as described by Bligh & Dyer [27]. Phosphatidylalcohols and 1-*O*-alkyl-PtdA were separated by t.l.c. on silica-gel G plates (Whatman, Maidstone, Kent, U.K.) with the use of two solvent systems: (A) chloroform/methanol/propan-1-ol/ethyl acetate/KCl (0.25%, w/v) (25:13:25:25:9, by vol.); (B) the organic phase of 2,2,4-trimethylpentane/ethyl acetate/acetic acid/water (5:11:2:10, by vol.). Amounts of DRG were measured as described [28], except that mixed micelles containing 5 mol% of phosphatidylserine in Triton X-100 (0.3%, w/v) were used in place of β-octyl glucoside/cardiolipin. Amounts of inositol 1,4,5-trisphosphate were measured as reported in [29] and superoxide production was monitored as described in [30]. [^{14}C]Butan-1-ol was purchased from New England Nuclear, Stevenage, Herts., U.K.

RESULTS

When [^{3}H]1-*O*-alkyl-2-lyso-PtdCho was added to human neutrophils 41 ± 6% was taken up and 82 ± 4% of this was acylated to form [^{3}H]1-*O*-alkyl-PtdCho. The addition of fMet-Leu-Phe stimulated the production of 1-*O*-alkyl-PtdA from 1-*O*-alkyl-PtdCho (Fig. 1). The fMet-Leu-Phe-stimulated production of [^{3}H]1-*O*-alkyl-PtdA was substantially decreased in the presence of butanol, and a novel product was formed (Fig. 1). This product was identified as phosphatidylbutanol by comparison of its mobility in two different solvent systems with a [^{3}H]1-*O*-alkyl-PtdCho (extracted from prelabelled neutrophils). The incorporation of [^{14}C]butan-1-ol into this product by fMet-Leu-Phe-stimulated neutrophils provided additional evidence that the material was phosphatidylbutanol (results not shown).

fMet-Leu-Phe-induced [^{3}H]phosphatidylbutanol and [^{3}H]1-*O*-alkyl-PtdA formation was rapid, and levels of both lipids peaked within 1 min (Fig. 2). The amount of [^{3}H]1-*O*-alkyl-PtdA declined rapidly thereafter; by contrast, [^{3}H]phosphatidylbutanol levels were main-

Abbreviations used: DAG, diacylglycerol; PKC, protein kinase C; DRG, diradylglycerol; 'radyl' refers to acyl, alkyl and alkenyl substituents; PtdCho, phosphatidylcholine; PtdA, phosphatidic acid; IC_{50}, concentration causing half-maximal inhibition.

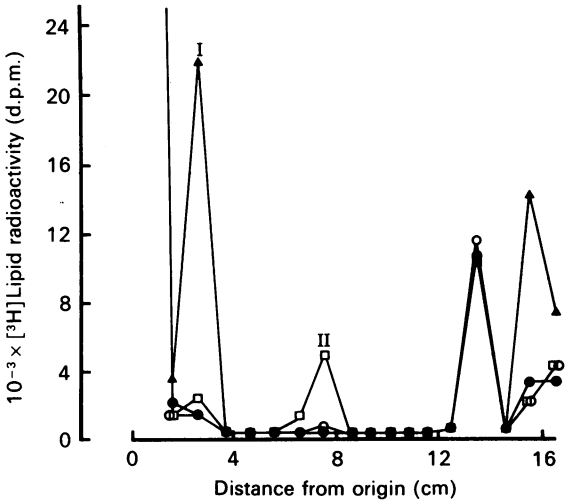

Fig. 1. Formation of [^{3}H]PtdA and [^{3}H]phosphatidylbutanol by fMet-Leu-Phe-stimulated human neutrophils

Neutrophils were prelabelled with [^{3}H]1-*O*-alkyl-2-lyso-PtdCho as described in the Materials and methods section. Cell suspensions (0.5 ml; 2×10^7 cells/ml) were pre-incubated for 5 min in the absence (filled symbols) or presence (open symbols) of butanol (30 mM) before addition of vehicle (●, ○) or 1×10^{-7} M-fMet-Leu-Phe (▲, □), then incubated for 5 min at 37 °C. Lipids were separated by t.l.c. using solvent system B. Bands (1 cm wide) were scraped from the chromatogram and the ^{3}H radioactivity measured by scintillation spectrometry. I, PtdA; II, phosphatidylbutanol.

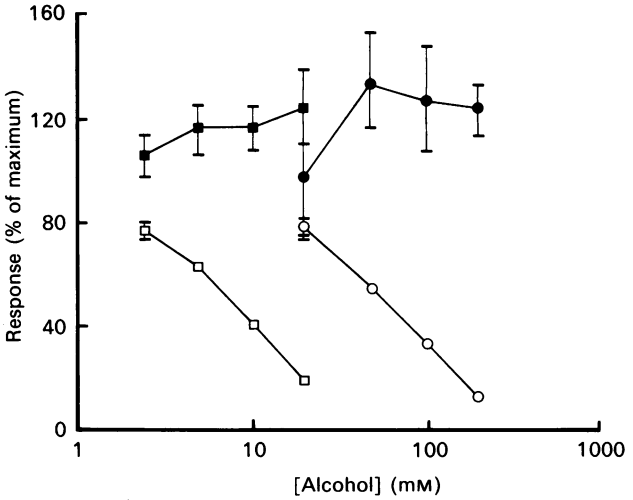

Fig. 3. Effect of butanol and ethanol on DRG and inositol 1,4,5-trisphosphate formation in fMet-Leu-Phe-stimulated human neutrophils

Human neutrophil suspensions (0.5 ml; 2×10^7 cells/ml) were preincubated with butanol (■, □) or ethanol (●, ○) for 5 min before addition of fMet-Leu-Phe (1×10^{-7} M). inositol 1,4,5-trisphosphate levels (filled symbols) were measured after 20 s and DRG levels (open symbols) determined after 5 min. Results are the mean ± S.E.M. for three or four separate experiments using neutrophils from different donors. Resting DRG levels were 74 ± 15 pmol/10^7 cells ($n = 4$) and increased to 411 ± 63 pmol/10^7 cells ($n = 4$) in the absence of the alcohols. Inositol 1,4,5-trisphosphate levels increased by 30 ± 8 pmol/10^7 cells ($n = 7$) in control experiments. Where error bars are not shown, the S.E.M. values fall within the dimensions of the symbol.

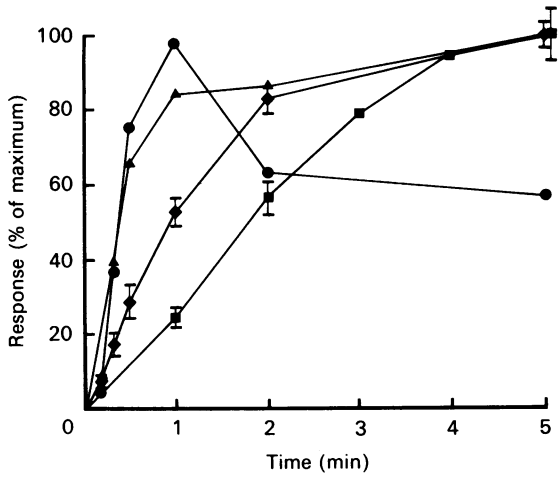

Fig. 2. Time courses for production of [^{3}H]phosphatidylbutanol, [^{3}H]1-*O*-alkyl-PtdA, DRG and superoxide production in fMet-Leu-Phe-stimulated human neutrophils

Results are expressed as percentage of maximum response, which for [^{3}H]phosphatidylbutanol (▲) and [^{3}H]1-*O*-alkyl-PtdA (●) were 5267 and 12918 d.p.m. respectively. Results shown are from a single representative experiment. Maximum responses for DRG (◆) and superoxide (■) were 443 ± 36 pmol/10^7 cells and 195 ± 6 nmol/10^7 cells respectively and are means ± S.E.M. for the three to five experiments using neutrophils from different donors. fMet-Leu-Phe was used at 1×10^{-7} M throughout. Error bars (not shown) did not exceed the size of the symbol.

tained for up to 5 min. The rate of DRG production was much lower, but was sustained for up to 5 min (Fig. 2). The kinetics of superoxide production are also shown in Fig. 2. Superoxide generation is subsequent to DRG formation, consistent with DRG being involved in either initiating or sustaining the respiratory burst.

Butanol and ethanol inhibited DRG formation in fMet-Leu-Phe-stimulated neutrophils in a concentration-dependent manner (Fig. 3). The IC_{50} (concentration causing half-maximal inhibition) values for butanol and ethanol were 8 mM and 60 mM respectively. Inhibition reached 80% with butanol at 20 mM and 85% with ethanol at 200 mM. In contrast, the alcohols at these concentrations did not inhibit fMet-Leu-Phe-induced inositol 1,4,5-trisphosphate formation (Fig. 3). Incubation of neutrophils with butanol and ethanol also caused a concentration-dependent inhibition of fMet-Leu-Phe-stimulated superoxide production (Fig. 4), with IC_{50} values of 12.5 mM and 125 mM respectively.

In contrast, the opsonized-zymosan-evoked response was much less sensitive to inhibition by the alcohols, being only partially decreased by concentrations that almost completely blocked fMet-Leu-Phe-stimulated superoxide production (Fig. 4). Butanol (27.5 mM) reduced the opsonized-zymosan response by 31.4%, whereas ethanol (200 mM) inhibited opsonized-zymosan-stimulated superoxide generation by 20.3%. It is important to note that butanol and ethanol, at identical

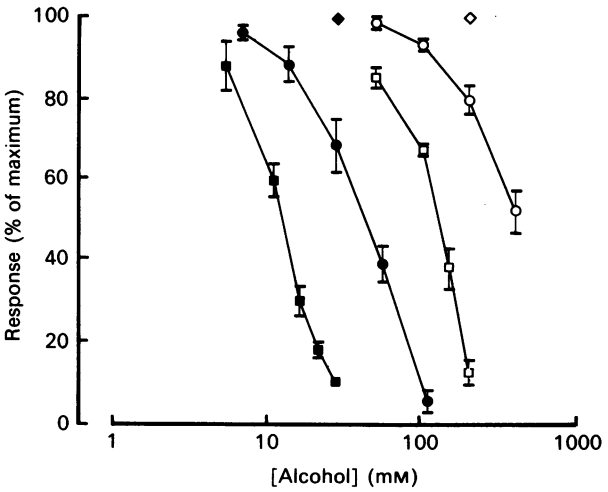

Fig. 4. Effect of butanol and ethanol on superoxide production

Human neutrophil suspensions [1 ml; $(0.5–2.5) \times 10^6$ cells/ml] were preincubated with butanol (filled symbols) or ethanol (open symbols) for 5 min before the addition of 1×10^{-7} M-fMet-Leu-Phe (■, □), opsonized zymosan (4 mg/ml) (●, ○) or 1×10^{-7} M-phorbol 12-myristate 13-acetate (◆, ◇). Results are means ± S.E.M. for three to five experiments using neutrophils from different donors.

concentrations, had no effect on phorbol 12-myristate 13-acetate-stimulated superoxide generation (Fig. 4).

DISCUSSION

The formation of phosphatidylalcohols affords a simple and valid technique for measuring phospholipase D activation in intact cells [17,20]. The use of [^{3}H]1-*O*-alkyl-2-lyso-PtdCho to label choline phospholipid pools was first described by Pai *et al.* [17], who were able to show unequivocally the existence of a receptor-linked phospholipase D in HL60 cells [17,18]. Using very similar techniques we have examined the activation of phospholipase D in intact human neutrophils. Like the HL60 cell, peripheral-blood neutrophils exhibit transphosphatidylation reactions when stimulated by fMet-Leu-Phe in the presence of alcohols. The incorporation of [^{14}C]butan-1-ol into a product that is chromatographically identical with [^{3}H]phosphatidylbutanol provided additional evidence that fMet-Leu-Phe receptors are linked to phospholipase D.

There is now a considerable body of evidence to support the concept that there is more than one pathway for DRG generation in activated cells [6–8]. Hydrolysis of phosphatidylinositol 4,5-bisphosphate is well recognized as one source, and phospholipases C that degrade PtdCho have been described [7]. The latter pathway does not appear to operate in the neutrophil, since fMet-Leu-Phe does not stimulate phosphocholine release in these cells [31]. Another possibility is phospholipase D coupled with phosphatidate phosphatase. The formation of phosphatidylalcohols and [^{3}H]1-*O*-alkyl-PtdA in response to fMet-Leu-Phe clearly indicates that a phospholipase D enzyme is activated in neutrophils. Production of both phosphatidylbutanol and 1-*O*-alkyl-PtdA is an early event in fMet-Leu-Phe-stimulated cells and clearly precedes superoxide production (Fig. 2), suggesting an important functional role for phospholipase D in neutrophil activation. Formation of 1-*O*-alkyl-PtdA also precedes DRG release (Fig. 2), and the generation of both lipid species is inhibited by the alcohols (Figs. 1 and 3), which strongly suggests that the phospholipase D–phosphatidate phosphatase pathway is an important source of DRG in fMet-Leu-Phe-stimulated neutrophils. Hydrolysis of phosphatidylinositol 4,5-bisphosphate was not inhibited by butanol or ethanol at concentrations which blocked DRG formation, indicating that this phospholipase C pathway contributes very little to the DRG that is released in response to fMet-Leu-Phe. Further, this observation indicates that the reported change in membrane fluidity induced by aliphatic alcohols [32] does not result in a non-specific inhibition of receptor-coupled phospholipases.

The evidence in favour of a receptor-linked phospholipase D pathway is compelling. However, the functional significance of phospholipase D activation has been difficult to determine, since a potent and selective inhibitor of this enzyme has not yet been described. In the present study we have illustrated that aliphatic alcohols have some value in this respect. They are known to have dramatic but selective effects on neutrophil responses. Ethanol, propanol and butanol, at non-toxic concentrations, inhibit superoxide generation and lysosomal-enzyme release, but enhance chemotactic responses to fMet-Leu-Phe [32]. We have confirmed that the alcohols inhibit superoxide generation by interfering with signal transduction and find that inhibition of this functional response correlates very closely with inhibition of DRG formation. The IC_{50} values for inhibition of DRG formation and superoxide production by the alcohols were similar, but not identical. Indeed, a decrease in DRG of at least 50% was required before a significant inhibition of superoxide production was observed. A possible explanation for this is that the measurement of DRG does not distinguish between alkyl- and acyl-substituted glycerols. Since it is only the 1,2-diacyl-*sn*-glycerol species that activates protein kinase C, leading to superoxide production, it appears that, in the absence of alcohol, the amount of diacylglycerol produced was supramaximal for protein kinase C activation.

Superoxide generation stimulated by opsonized zymosan was only partially inhibited by ethanol and butanol at concentrations which completely blocked fMet-Leu-Phe-stimulated superoxide production. Furthermore, transphosphatidylation reactions in opsonized-zymosan-activated neutrophils were much smaller than in fMet-Leu-Phe-stimulated cells (results not shown). These observations lend further support to the argument that the alcohols were selective in their action. Opsonized zymosan also stimulates a large and sustained generation of DRG in the neutrophil, and recent studies have indicated that particulate stimuli induce a much greater and protracted degradation of the phosphoinositides than do soluble stimuli such as fMet-Leu-Phe and leukotriene B_4 [33,34].

In summary, the present study indicates that a large part of the DRG formed in fMet-Leu-Phe-stimulated neutrophils results from the activation of a receptor-linked phospholipase D coupled with phosphatidate phosphohydrolase. More importantly, these data provide strong evidence that this second-messenger pathway is essential for fMet-Leu-Phe-stimulated superoxide generation by these cells. In addition, it is possible that the

phospholipase D pathway may be less important for transducing signals from particulate stimuli such as opsonized zymosan.

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