A novel and sensitive assay for phospholipase D in intact cells

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A novel and sensitive assay for phospholipase D (PLD) that measures the incorporation of high specific activity [³H]butan-1-ol into [³H]phosphatidylbutanol has been developed. The assay has been used to measure PLD activation in human neutrophils and platelets. Both the chemotactic peptide fMet-Leu-Phe and opsonised-zymosan stimulated PLD in the human neutrophil. In the platelet, PLD was stimulated by thrombin and collagen but responses were small and only occurred at high agonist concentrations. This assay has a number of advantages over existing techniques and should be valuable for investigating PLD activation in a variety of isolated cells and possibly intact tissues.

Phospholipase D; Transphosphatidylation; Opsonised-zymosan; Neutrophil; Platelet

1. INTRODUCTION

Evidence is rapidly accumulating to indicate that phospholipase D (PLD) has an important role in receptor-effector coupling [1–3]. PLD hydrolyses phospholipids to yield the free polar headgroup and phosphatidic acid. Phosphatidic acid may have a second messenger role [4], but such a role is more clearly recognized for diacylglycerol generated from phosphatidic acid by phosphatidate phosphohydrolase [5,6]. A unique feature of PLD is the ability to catalyse the transphosphatidylation of phospholipids. In this reaction the phosphatidyl moiety is transferred to a primary alcohol to produce the corresponding phosphatidylalcohol [7,8]. The formation of phosphatidylalcohol is therefore a useful measure of PLD activity in stimulated cells.

The methods currently used to measure transphosphatidylation require cellular phospholipids to be prelabelled and cells to be stimulated in the presence of high concentrations (10–200 mM) of aliphatic alcohols; radiolabelled phosphatidylalcohols are then separated by thin-layer chromatography (TLC). This approach has enabled PLD activity to be demonstrated in human neutrophils, NG108-15 cells, NIH-3T3 fibroblasts, hepatocytes and human platelets [9–14]. The major disadvantages of these techniques are that the high concentrations of alcohols required to obtain measurable levels of phosphatidylalcohol may have cytotoxic effects. Further, existing methods only measure PLD-dependent hydrolysis of those phospholipid pools that can be adequately radiolabelled. Thus, we have developed an alternative procedure that measures the PLD-catalysed transfer of high specific activity [³H]butan-1-ol into phosphatidylbutanol (PBut). This technique has two advantages: it uses a much lower concentration of alcohol (16 μM) and does not require cellular phospholipids to be prelabelled. Thus, it should be applicable to many cell types.

Here we report the characterisation of this method using the human neutrophil and its use to investigate receptor-linked activation of PLD in the human platelet.

2. MATERIALS AND METHODS

2.1. *Materials*

Radiochemicals, purchased from Amersham International (Bucks, UK), were [³H]butan-1-ol (12 Ci/mmol) and 1-stearoyl-2-[¹⁴C]-arachidonyl phosphatidylcholine (56 mCi/mmol). Other reagents were from Sigma (Poole, UK) except collagen which was from Hormon-Chemie (Munich, FRG). Organic solvents were of analar grade from BDH (Poole, UK).

2.2. *Cell incubation*

Human peripheral blood neutrophils were purified as described in [15] and suspended in 30 mM Hepes-buffered Hanks balanced salt solution, pH 7.2 (HBH). Cells (2 × 10⁷/ml) were pre-incubated with 100 μCi [³H]butan-1-ol together with 5 μM cytochalasin B, where required, for 5 min at 37°C before the addition of agonist. fMet-Leu-Phe was dissolved in dimethylsulphoxide (DMSO) such that the final DMSO concentration did not exceed 0.2% (v/v), and opsonised-zymosan was suspended in HBH. Final incubation volume was 0.5 ml.

Washed human platelets were prepared according to [16] and suspended in 5 mM Hepes-buffered, calcium-free, Tyrodes solution, pH 7.4 (HBT). Platelets (10⁹/ml) were incubated as described above except that 200 μCi [³H]butan-1-ol was used and CaCl₂ was adjusted

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Abbreviations: PLD, phospholipase D; TLC, thin-layer chromatography; PBut, phosphatidylbutanol; PAF, platelet-activating factor (1-*O*-hexadecyl-2-acetyl-sn-glycerophosphorylcholine)

to 1 mM. Platelet stimulants were each dissolved in HBT containing 2 mg/ml bovine serum albumin.

2.3. Extractions and TLC

Reactions were terminated by adding 1.5 ml chloroform/methanol (1:2, v/v), lipids extracted as described by Bligh and Dyer [17] and chloroform layers evaporated to dryness in streams of nitrogen. Dry lipid extracts were dissolved in 1 ml chloroform and washed 3 times with 2 ml of theoretical upper phase (upper phase of chloroform/methanol/1 M NaCl/H$_2$O (2:2:1:1, v/v)). Radiolabelled products were separated by TLC in a solvent system comprising the organic phase of 2,2,4-trimethylpentane/ethyl acetate/acetic acid/H$_2$O (5:11:2:10 v/v).

2.4. Lipid phosphorus assay

Phosphorus in lipid extracts of cell samples was determined by the method of Bartlett [18].

3. RESULTS

Human neutrophils treated with fMet-Leu-Phe incorporated [^{3}H]butan-1-ol into a product that was chromatographically identical to PBut (Fig. 1A, B).

Early experiments demonstrated the presence of a radiolabelled material that comigrated with PBut in control preparations. Additional studies revealed that this material was a minor contaminant of the high specific activity [^{3}H]butan-1-ol which was readily removed from the lipid extract by washing the lower chloroform layer with theoretical upper phase [17]. Using this washing technique we were able to measure the stimulated incorporation of [^{3}H]butan-1-ol into [^{3}H]PBut by fMet-Leu-Phe-stimulated human neutrophils (Fig. 1A). The identity of a ^{14}C-labelled PBut standard, generated by incubating cabbage phospholipase D with 1-stearoyl-2-[^{14}C]arachidonyl-phosphatidylcholine and butanol, was confirmed by demonstrating that [^{3}H]butan-1-ol was incorporated into a dual-labelled product that was chromatographically identical to PBut (Fig. 1B). High specific activity [^{3}H]butan-1-ol was incorporated into two other products that were more polar than PBut; one remained at the origin of the chromatogram (R_f = 0.03) and the other ran close to the PBut standard (R_f = 0.30). The formation of these products was neutrophil-dependent and production of the more polar material was increased by fMet-Leu-Phe treatment (Fig. 1A). A third broad peak of radioactivity (R_f = 0.6–0.9), present in the absence of cells, was judged to be a [^{3}H]butan-1-ol contaminant that was not removed by the washing procedure (Fig. 1A).

fMet-Leu-Phe stimulated the formation of [^{3}H]PBut which was rapid in onset, peaked by 1 min and sustained for 5 min (Fig. 2). The response was concentration-dependent with a half-maximal (ED$_{50}$) value of 71 ± 22 nM (Fig. 3).

Opsonised-zymosan also stimulated [^{3}H]PBut generation in the human neutrophil, the magnitude of the response was similar to that observed for fMet-Leu-
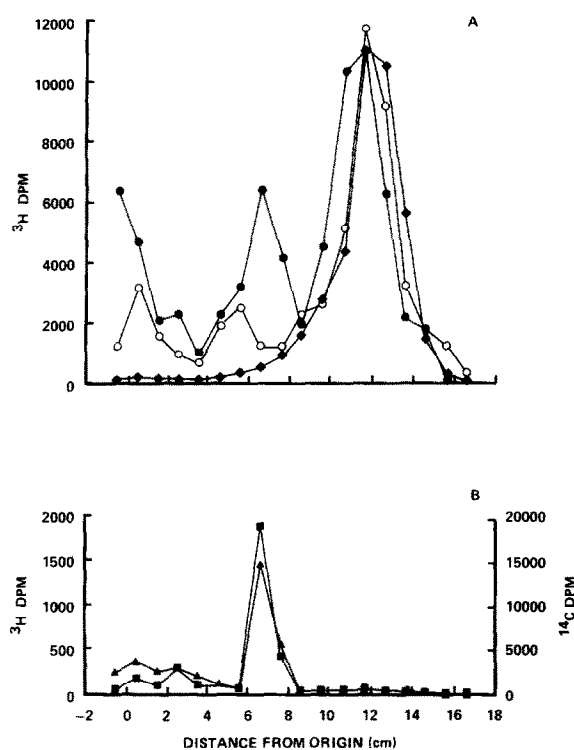

Fig. 1. Incorporation of [^{3}H]butan-1-ol into [^{3}H]PBut by fMet-Leu-Phe-stimulated human neutrophils. Neutrophils pretreated with cytochalasin B were incubated with 1 μM fMet-Leu-Phe (●) or vehicle (○) for 5 min at 37°C in the presence of [^{3}H]butan-1-ol as described in section 2. 1 cm bands were scraped from the chromatogram and radioactivity quantified by liquid scintillation spectrometry. A TLC profile generated in the absence of cells is also shown (♦). (B) Shows the TLC profile of a PBut standard prepared by incubating 1-stearoyl-2-[^{14}C]arachidonyl phosphatidylcholine with cabbage PLD in the presence of [^{3}H]butan-1-ol (■, ^{14}C dpm; ▲, ^{3}H dpm).
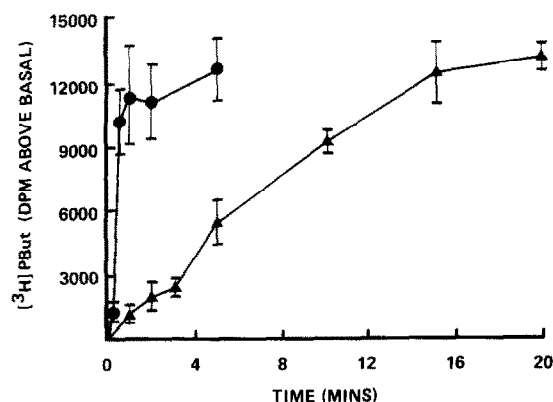

Fig. 2. Time course of fMet-Leu-Phe and opsonised-zymosan-stimulated [^{3}H]PBut formation in human neutrophils. Neutrophils were stimulated with 0.1 μM fMet-Leu-Phe (●) or 4 mg/ml opsonised-zymosan (▲) in the presence of [^{3}H]butan-1-ol for the indicated times as described in section 2. Data are the mean ± SE for 3 separate experiments using cells from different donors. Basal values were 5140 ± 1014 dpm and did not change during the 30 min incubation period.
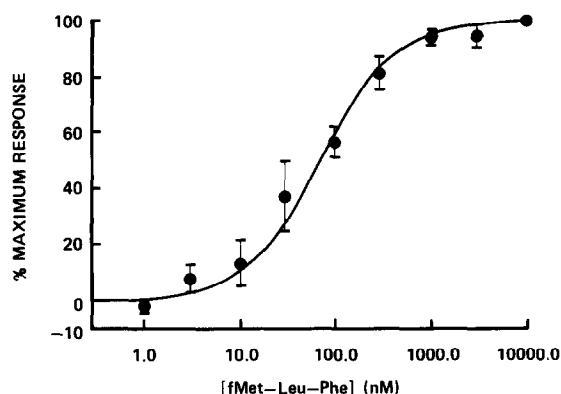
Fig. 3. Dose-response curve for fMet-Leu-Phe-stimulated [³H]PBut formation in human neutrophils. Cytochalasin B-treated neutrophils were incubated with fMet-Leu-Phe in the presence of 100 μCi [³H]butan-1-ol for 5 min at 37°C. Lipids were extracted and separated by TLC as described in section 2. Data are the mean ± SE from 3 different experiments using cells from different donors. The maximum response for [³H]PBut formation measured using 10 μM fMet-Leu-Phe was 24403 ± 15597 dpm (mean ± SE for 3 experiments).

Phe (0.1 μM) but the rate of formation was much slower (Fig. 2).

Incubation of human platelets with high specific activity [³H]butan-1-ol produced labelled products having a TLC profile similar to that obtained from neutrophils. Addition of thrombin stimulated formation of a tritiated product that was chromatographically identical to the [¹⁴C]PBut standard (results not shown). In order to obtain significant amounts of this product, the platelet density needed to be 10^9/ml and the amount of [³H]butan-1-ol had to be increased to 200 μCi/assay. Under these conditions thrombin concentrations of at least 0.5 U/ml were required to obtain significant increases in [³H]PBut (Fig. 4). The effects of other stimulatory agonists on [³H]PBut formation in human platelets are also shown in Fig. 4. Collagen
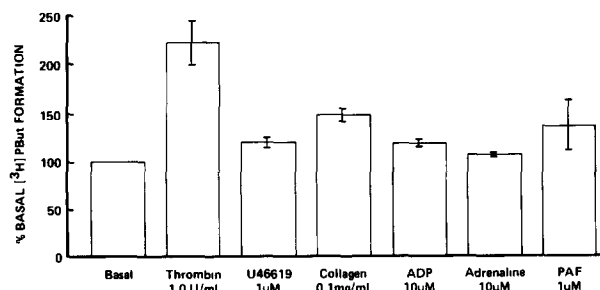

Fig. 4. Formation of [³H]PBut in human platelets stimulated by thrombin, U46619, collagen, ADP, adrenaline and PAF. Platelets were treated with the appropriate stimulus for 5 min at 37°C in the presence of [³H]butan-1-ol and the [³H]PBut formed was separated by TLC as described in section 2. Data are the means ± SE from 3–4 separate experiments using platelets from different donors. Mean basal values were 3732 ± 861 dpm.

Table I

PLD levels in neutrophils and platelets

Tissue	Lipid phosphorus (μg)	PBut formed (pg)	pg PBut formed per μg lipid phosphorus
Neutrophils (1 × 10⁷ cells)	1.3 ± 0.3	734 ± 469	549
Platelets (5 × 10⁸ cells)	12.5 ± 3.1	135 ± 27	11

Human neutrophils were stimulated with 1×10^{-5} M fMet-Leu-Phe in the presence of [³H]butan-1-ol (12 Ci/mmol) and platelets incubated with 1 U/ml thrombin plus [³H]butan-1-ol as described in section 2. Results are the mean ± SE from 3 separate experiments

caused a small but significant increase in [³H]PBut, but ADP, the thromboxane mimetic U46619, adrenaline and platelet-activating factor (PAF) did not significantly increase [³H]PBut above resting levels, even at high concentrations.

In order to compare levels of PLD in neutrophils and platelets, the amounts of PBut formed in the assay were expressed in terms of the total lipid phosphorus present in each tissue (Table I). Measurement of PLD in platelets required larger amounts of tissue than in neutrophils but platelets produced much less PBut, even under conditions where PLD activation was maximal (Figs. 3, 4). When PBut formation is expressed as a function of the total lipid phosphorus it is clear that the platelet exhibits only 2% of the PLD activity of the neutrophil.

4. DISCUSSION

Measuring the formation of phosphatidylalcohols affords a simple and convenient method for monitoring PLD activation in cells. In this study the incorporation of high specific activity [³H]butan-1-ol into [³H]PBut has been used to quantify PLD activity in the human neutrophil and platelet. Neutrophils and platelets convert [³H]butan-1-ol into at least 3 different products which can be successfully separated by TLC.

The use of [³H]butan-1-ol to monitor PLD activation has a number of advantages over existing methods. Firstly, prelabelling of phospholipid pools with radiolabelled precursors is avoided. This is particularly important when the precursor may be converted to a biologically active product as with, for example, 1-O-octadecyl-2-lysophosphatidylcholine (lyso-PAF) or arachidonic acid. Problems concerning labelling to isotopic equilibrium are eliminated and the formation of PBut from *all* phospholipid substrates can be measured, not just those pools that are radiolabelled with a suitable precursor. Secondly, the high specific activity of the [³H]butan-1-ol ensures that only very

low concentrations need to be used; i.e. levels that are 1000 times less than those used in existing methods. The advantages are 3-fold: (i) only a small percentage of the phospholipid substrate(s) is converted to PBut and hence total phospholipid metabolism through the PLD pathway is not substantially altered; (ii) the possibility of cellular toxicity from aliphatic alcohols is reduced; and (iii) the chemical stability of stimulants (either particulate or soluble) is not compromised.

The production of [³H]PBut by cytochalasin B-treated neutrophils stimulated with fMet-Leu-Phe confirms other reports showing that a PLD activity is linked to chemotactic peptide receptors [9,10,19]. The ability of cytochalasin B to 'prime' neutrophils and dramatically increase both second messenger generation and functional responsiveness is well recognised [20–23]. The rapid but sustained generation of [³H]PBut by the neutrophil supports other observations which indicate that phosphatidylalcohols are metabolically stable [24]. The metabolic stability of PBut has allowed us to measure long-term responses in the neutrophil and in particular PLD activation by particulate stimuli. Opsonised-zymosan stimulates a slow activation of PLD that continues for 20–30 min. Furthermore, activation by this phagocytic stimulus does not require cytochalasin B pretreatment and is independent of a 'priming' event.

Unlike the neutrophil, the human platelet exhibits only modest PLD activity. Indeed, measurable PBut formation only occurs at thrombin concentrations greater than those required to induce aggregation and secretion [25]. The only other agent that was found to activate PLD in the platelet was collagen and, like the response to thrombin, transphosphatidylation was only observed with a concentration of collagen greater than that needed to stimulate functional responses [26].

In conclusion, the technique described in this report has a number of advantages over existing procedures and should be useful for evaluating the physiological significance of PLD activation in a variety of isolated cells and possibly intact tissues.

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