Erythropoietin stimulates G-protein-coupled phospholipase D in haematopoietic target cells

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A murine haematopoietic stem-cell line, B6Sut.EP, responsive to erythropoietin (EPO), has been found to exhibit both early and late changes in diacylglycerol (DAG) and phosphatidic acid (PA) as measured by HPLC and TLC. DAG levels peaked at 5 s with a 28.1 % increase compared with control levels (from 17.3 to 22.2 pmol/10⁶ cells) with a later peak at 30 min (84.2 % increase from 17.3 to 31.9 pmol). These changes were concentration-dependent from 0.025 to 10 units/ml EPO (5 s, EC₅₀ = 0.82 unit/ml; 30 min, EC₅₀ = 0.10 unit/ml). In addition, PA levels increased 752.3 % compared with control levels (from 8.6 to 64.7 µg/10⁶ cells) with an early peak at 20 s, as measured by both HPLC and TLC (5 s, EC₅₀ = 0.07 unit/ml). G-protein regulation was investigated by studying the effects of the non-hydrolysable GTP analogue guanosine 5'-[γ-thio]triphosphate (GTP[S]) on PA synthesis. The addition of GTP[S] (10 µM) in permeabilized cells increased PA content from 6.3 µg to 48.6 µg per 10⁶ cells. In the presence of EPO and GTP[S], PA levels increased to 64.8 µg. An antagonist of G-proteins, guanosine 5'-

[β-thio]diphosphate (GDP[S]), had no effect on control levels of PA (5.9 µg/10⁶ cells) but blocked the effect of EPO on PA (30.6 µg/10⁶ cells). Thus, EPO stimulated both lipid second messengers, DAG and PA. Our results demonstrate DAG kinetics to be biphasic, as observed with a high concentration of EPO, or monophasic, as observed with low concentrations of EPO. The PA accumulation preceding that of DAG in the slower and sustaining phase suggests that PA was not derived from DAG. This was confirmed by the stimulation of PA (without ATP) by GTP[S], effectively excluding phosphorylation of DAG by DAG kinase in the formation of PA. In addition, phospholipase D (PLD) activation was demonstrated with a maximal increase in phosphatidylethanol at 5 min, suggesting that EPO increases PA via a guanine nucleotide-binding protein coupled to PLD. The temporal relationship of the evolution of PA and DAG is further strengthened by experiments with ethanol and propranolol as inhibitors of the DAG/PA phosphohydrolase reaction and R59022 as an inhibitor of the DAG kinase reaction.

INTRODUCTION

Earlier studies from this laboratory [1–4] have implicated phospholipase activation as an important early event in the signal transduction of erythropoietin (EPO). Diacylglycerol (DAG) was shown to increase by 30 min in murine fetal liver cells as were specific lipoxygenase metabolites of arachidonic acid. The importance of DAG in cellular metabolism has been highlighted by the discovery of protein kinase C (PKC), an enzyme that is activated by DAG and serves as its receptor. In addition, DAG can give rise to arachidonic acid liberated from complex lipids via phospholipid-derived DAG by sequential actions of DAG lipase and monoacylglycerol lipase. The details of the generation of DAG from membrane phospholipids have not been identified in target cells for EPO. Assumption of a central importance for phosphatidylinositol (PI)-specific phospholipase C may also be premature. Evidence from Wright et al. [5] and Raben et al. [6] indicates that growth factors activate two or three coupling mechanisms for the stimulation of lipid metabolism. These mechanisms have a common result in the elevation of DAG levels.

Although phosphatidic acid (PA) has been recognized as a central metabolite in phospholipid and triacylglycerol metab-

olism, measurements of PA mass, which is only 2–4 % of the total phospholipids, have been difficult. There are increasing numbers of reports of increased formation of PA via phospholipase D (PLD) [7–11] and linkage of PLD to hormonal action or growth factors. Experiments in hepatocytes indicated that the increase in PA does not require ATP but proceeds via a PLD stimulated by G-protein [7]. The purpose of this study was to identify characteristic temporal patterns of DAG and PA production in response to EPO.

MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) except guanosine 5'-[γ-thio]triphosphate (GTP[S]), guanosine 5'-[β-thio]diphosphate (GDP[S]) and R59022, which were obtained from Boehringer Mannheim, solvents from Mallinckrodt Specialty Chemicals (Paris, KY, U.S.A.), and arachidonic acid and heneicosanoic acid standards from Alltech Associates (Deerfield, IL, U.S.A.). Highly purified recombinant EPO was obtained from McDonnell Douglas (St. Louis, MO, U.S.A.). Neutral lipids were purchased from Supelco

Abbreviations used: DAG, diacylglycerol; EPO, erythropoietin; GDP[S], guanosine 5'-[β-thio]diphosphate; GTP[S], guanosine 5'-[γ-thio]triphosphate; IL-3, interleukin 3; MEM, minimal essential medium; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PET, phosphatidylethanol; PI, phosphatidylinositol; PKC, protein kinase C; PLD, phospholipase D; PPH, PA phosphohydrolase; PS, phosphatidylserine; SB/CD, short-bed/continuous development; VBR, Victoria Blue reagent.

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(Bellefonte, PA, U.S.A.), and 1-palmitoyl-2-oleoylphosphatidic acid was obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.) and was converted into 1-palmitoyl-2-oleoylphosphatidylethanol by reaction in pyridine, trichloroacetonitrile and ethanol as described by Pai et al. [12]. [9,10(N)-³H]Myristic acid (20–40 Ci/mmol) was from Dupont NEN (Boston, MA, U.S.A.). Triarylmethanic dye Victoria Blue reagent (VBR) was purchased from Aldrich Chemical (Milwaukee, WI, U.S.A.) and recrystallized from ethanol.

Preparation of cells

B6Sut.EP cells, isolated following growth in soft agar in the presence of EPO, are a subclone [13] of a previously established murine bone-marrow stem-cell line, B6Sut.A [14]. They proliferate in the presence of EPO or interleukin 3 (IL-3) but do not differentiate. These cells are maintained in RPMI-1640 supplemented with 15% (v/v) fetal bovine serum, 10^{-5} M 2-mercaptoethanol, 2% (w/v) minimal essential medium (MEM) vitamins, 2% (w/v) non-essential amino acids, 1% penicillin/streptomycin, 1% fungizone, 1% L-glutamine and 5% (v/v) conditioned medium from WEHI-3 cells.

Tritiated thymidine incorporation assay

B6Sut.EP cells were resuspended at a concentration of 2×10^6 cells/ml in enriched medium [α MEM containing 10% (v/v) fetal bovine serum, 0.1 μ M 2-mercaptoethanol]. Cells were added to 96-well sterile round-bottomed culture plates such that 200 000 cells were present in each well. EPO was prepared in enriched medium at a $20 \times$ concentration, and 5 μ l was added to each well; total incubation volume in each well was brought up to 200 μ l with enriched medium. After the addition of EPO, plates were incubated for 20 h at 37 °C in a humidified atmosphere of 95% air/5% CO₂, pulsed with [³H]thymidine (Amersham; 1 μ Ci/well), and incubated for an additional 4 h. The cells were then harvested onto a glass-fibre filter mat with a Skatron semi-automatic cell harvester. Filters were air dried, and individual filter discs were removed and counted in a Packard liquid scintillation counter for 1 min. All experimental conditions were performed in quadruplicate in each assay.

Determination of DAG levels

Cells were incubated with EPO (0–20.0 units/ml) for 0–100 min, depending on the experiment. Cellular lipids were extracted by adding sequentially with vortexing 1 ml of chloroform/methanol (1:2, v/v), 0.66 ml of chloroform, and 0.6 ml of deionized water. Boric acid was added to the methanol to minimize acyl migration. After a minimum of 5 min, the samples were centrifuged at 2000 g for 5 min and the aqueous upper layer was discarded. To eliminate acyl migration of DAG, all lipid samples were dried in polypropylene vials (Poly Q scintillation vials; Beckman Instruments, Fullerton, CA, U.S.A.). These vials do not release plasticizers when exposed to chloroform/methanol [15]. Chloroform (1 ml) was added to the bottom layer, and the solution was then filtered through glass wool in a Pasteur pipette. The collected eluate was evaporated under N₂ at room temperature, redissolved in 0.5 ml of chloroform and applied to a 0.5 ml silicic acid column. Neutral lipids were eluted with 4 ml of chloroform, evaporated to dryness and stored at –70 °C under N₂ [16]. Chromatography was performed using a Waters (Milford, MA, U.S.A.) M6000 delivery pump, a U6K injector, a microporasil column (30 cm $\times$ 4.0 mm) and an R-401 differential refractometer at half attenuation (50 mV). The mobile phase was hexane/propan-2-ol/acetic acid (100:1:0.01, by vol.). Flow rate was

2 ml/min. A volume of 0.15 ml containing 10–25 μ g of DAG was typically injected. DAG eluted as a bimodal peak at 9.6–12.5 min after injection, following the non-esterified cholesterol peak. The standard used was 1,2-DAG (either dipalmitoylglycerol, dioleoylglycerol, or 1-stearoyl-2-arachidonoyl-*sn*-glycerol gave the same standard curve, confirming that this quantification does not discriminate between lipids with regard to the number and/or location of double bonds). The detector output was channelled into a convertor (analog/digital) and stored in a computer. Integration of peaks was performed with a CPlot system. The peak integral was calibrated against 1,2-DAG standard and calculated in pmol of DAG per 10^6 cells. The method was reproducible with a typical S.D. of 2 mV at a total of 18–35 mV; recovery was 96–103%. All experiments with DAG were performed in triplicate for each assay (coefficient of variation for three replicate samples was less than 6%).

TLC of PA

Two methods were used. In the first, PA was separated from other lipids by multidimensional chromatography on 20 cm $\times$ 8 cm silica gel 60 F-254 plates (Merck). Samples (lipid from 10^5 cells) and dipalmitoyl PA standard were applied in 20 μ l of chloroform. The plates were developed with butanol/acetic acid/water (6:1:1, by vol.) to the top of the plate (8 cm). The plates were then thoroughly dried for 40 min in a lyophilizer, and the top 1 cm of the plate containing the neutral lipids was removed. The plates were turned through 180° and then developed with chloroform/methanol/NH₄OH (10:3:0.6, by vol.) to the top of the plate. The plates were air-dried for 30 min, stained with Coomassie Brilliant Blue R250 (0.03% in 30% methanol/100 mM NaCl) for 30 min, and destained for 5 min in 30% methanol/100 mM NaCl. The plates were air-dried and scanned with an Ultrosan laser densitometer. The output was channelled to a Hewlett-Packard analog/digital converter, and the data were stored in an LIS Sunquest computer. Integration of TLC bands was performed using the CPlot system. Standard curves of dipalmitoyl PA (0.25, 0.5 and 1 μ g) were constructed for each plate. The variation of the standard curve due to saturated (C_{16:0} PA) compared with unsaturated (C_{18:2} PA) and egg PA is very small. Recovery of added PA was 80% [17].

The second method for separation of PA is a two-dimensional system, which permits a good separation of PA from other phospholipids and at the same time separation of phospholipid classes. The method requires lipid extracts to be applied in 20 μ l of chloroform to 20 cm $\times$ 10 cm silica gel 60 plates using two different solvent systems. In the first, the plates were developed with ethyl acetate/iso-octane/acetic acid (50:15:8, by vol.). This system allows a good separation of PA, with the other phospholipids staying at the origin. In the second system, which permits an analysis of total phospholipids including PA, the plates were developed with chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, by vol.). Lipids were stained with Coomassie Blue as described above. Using this system, it was found necessary to include standard egg PA (5 μ g sample) to localize this lipid on the plate [18]. Recovery of added PA was 74%. All experiments for PA used both TLC methods, each in triplicate. The results were compared and a mean of the resulting PA was computed.

HPLC of phospholipids and PA

The washed lipid extract was dried under N₂ and redissolved in hexane/propan-2-ol/water (40:54:6, by vol.). Chromatography was performed with a Waters Associates (Milford, MA, U.S.A.) model 6000A or model M-45 solvent delivery system equipped

with a model U6K injector and a model 450 variable-wavelength detector, which was operated at 205 nm. The lipid extract was separated into phospholipid classes by chromatography on a 250 mm × 4.6 mm Hibar II column packed with 10 μ m LiChrospher Si-100. The eluting solvent was prepared by the addition of 490 ml of HPLC-grade propan-2-ol to 62 ml of 25 mM potassium phosphate (pH 7.0). After mixing, 367 ml of HPLC-grade hexane and 100 ml of ethanol were added. The solvent was filtered through a 0.5 μ m-pore-size type FH Millipore filter (Millipore Corp., Bedford, MA, U.S.A.), and 0.6 ml of acetic acid was added. The final solvent composition was hexane/propan-2-ol/25 mM phosphate buffer/ethanol/acetic acid (367:490:62:100:0.6, by vol.). The flow rate was 0.3 ml/min at the start; PA was eluted at 18 min between phosphatidylethanolamine (PE) (14 min) and PI (22 min). After the elution of phosphatidylserine (PS) at 33 min, the flow rate was increased to 1.5 ml/min and the phosphatidylcholine (PC) was eluted at 85–88 min. Detection was by absorption at 205 nm (full scale 2.0 absorbance units) [19].

Quantification of PA mass after HPLC was performed by a rapid, highly sensitive (about 24.00 A units/ μ mol of PA) method using complexation with the dye VBR [20]. Samples of standard PA dissolved in chloroform and aliquots of PA extracts from HPLC prepared in 20 μ l of methanol, were added to 0.5 ml of VBR solution in an ethylene glycol/glycerol (1:1, v/v) mixture (13.5 mg/100 μ l), and chloroform added to make a final volume of 1.0 ml. Tubes were shaken, 1.0 ml of ethyleneglycol added, and the biphasic system was mixed vigorously. After centrifugation for 3 min (2 min at 1000 g, 1 min at 500 g), the upper phase was removed by aspiration, and the lower phase was transferred to a cuvette for absorbance measurement at 590 nm. Recovery of added PA was 96%, better than that with TLC methods.

Transphosphatidylation experiments for demonstration of PLD activation

We chose the transphosphatidylation reaction in which an exogenous alcohol acts as acceptor for PA to demonstrate activation of PLD. Thus, in the presence of exogenous ethanol, activation of PLD will result in the elevation of both PA and the product of the transphosphatidylation, phosphatidylethanol (PEt). Cells were prelabelled with [3 H]myristic acid (1 μ Ci/ml) for 8 h, chased in isotope-free medium for 1 h, and incubated with EPO in the presence of ethanol (2%, v/v) for the specified amount of time. The lipids from washed cells were extracted by a short-bed/continuous development (SB/CD) TLC using an SB/CD chamber (Regis Chemical Co., Morton Grove, IL, U.S.A.) on unmodified silica gel G plates. Cellular PA and PEt were resolved using position 5 on the SB/CD chamber for 50 min and a solvent system of benzene/chloroform/pyridine/formic acid (45:38:4:2.2, by vol.) [15]. The standards, including PA and PEt, were visualized with $\text{CuSO}_4/\text{H}_3\text{PO}_4$ and charred. R_f values of 0.52–0.54 were found for PEt and 0.23–0.24 for PA, whereas phospholipids (PC, PE, PI and PS) remained at the origin (R_f 0.02) and neutral lipids moved near the solvent front (R_f 0.92). Since PA and PEt standards co-migrated with the radiolabelled peaks, the chromatogram was scraped in 2-cm zones, and the ^3H -labelled PA and PEt were quantified by scintillation counting. Parallel lipid samples were used to determine inorganic phosphorus mass. ^3H -labelled DAG was also determined by TLC using the SB/CD chamber, developing the plates first in only diethyl ether at position 1 for 2 min and, after drying, developing in the same direction at position 3 for 50 min in a benzene/hexane/diethyl ether/formic acid (65:50:2:0.2, by

vol.) solvent system [20]. 1,2-DAG spots were scraped and [^3H]DAG quantified by scintillation counting.

Permeabilization of B6Sut.EP cells for experiments with non-hydrolysed GTP analogues

Concentrated B6Sut.EP cells were diluted to 2×10^6 /ml as in the tritiated thymidine incorporation assays and preincubated for 1 min with saponin (15 μ g/ml). They were then treated for 30 s with GTP[S], GDP[S] (both at concentrations of 10 μ M), and/or EPO at 30 s and 30 min. The reaction was terminated after addition of 1 ml of chloroform/methanol (1:2, v/v). Preliminary studies showed that PA formation in the saponin-treated cells was maximal within 60 s of the addition of EPO. Thus, EPO was always added after the addition of GTP[S] to the permeabilized cells.

Membrane phosphorus phospholipid determination

Membrane preparations from cells were incubated as above, and the extract equivalent to 1 μ g of protein was applied to silica gel 60F plates. Phospholipids were separated using the two-dimensional system of Abdel-Latif et al. [17]. Plates were stained with Coomassie Blue, and the area containing the lipids removed. PC and PE were extracted with 2 ml of chloroform/methanol/water (5:5:1, by vol.) plus 5 μ l of NH_4OH . PI and PA were extracted with the same solvent plus 20 μ l of 1 M HCl. Samples were filtered and evaporated to dryness under N_2 . Perchloric acid (50 μ l; 11.6 M) and sulphuric acid (50 μ l; 2.5 M) were added, and the samples were heated at 200 $^\circ\text{C}$ for 60 min. Water (1 ml) was added while the tubes were hot, and the samples assayed for PO_4 [21].

RESULTS

In Table 1 tritiated thymidine incorporation, a measurement of DNA synthesis and an indicator of proliferation, was assessed in B6Sut.EP cells in response to 0–20 units/ml of EPO. Increasing thymidine incorporation occurred in a dose-dependent manner with a maximal effect observed between 2 and 20 units/ml. Because EPO is known to bind to receptors at the cell surface and initiate a series of events that include the activation of PKC [4], we first analysed the time-course of activation of DAG, which is

Table 1 [^3H]Thymidine incorporation of B6Sut.EP cells after EPO treatment

Cells were plated at 200 000 cells/well in the presence of 0–20 units/ml EPO for 20 h at 37 $^\circ\text{C}$. [^3H]Thymidine was added and incubated for a further 4 h. Cells were then harvested, and filters were counted for radioactivity. Three experiments were performed in quadruplicate and the means $\pm$ S.E.M. determined.

EPO activity (units/ml)	[^3H]Thymidine incorporation (c.p.m./min)
0	12019 $\pm$ 1260
0.002	12400 $\pm$ 1257
0.02	12842 $\pm$ 762
0.2	19258 $\pm$ 1036
2	23466 $\pm$ 653
20	33562 $\pm$ 3395

Figure 1 Time-course of DAG accumulation in EPO-treated B6Sut.EP cells

Cells were incubated with EPO (1 unit/ml) for up to 120 min. (A) Time-course over 0–120 s; (B) time-course over 0–90 min. Lipids were extracted as described in the Materials and methods section. Collected eluates were evaporated, redissolved and applied to a silicic acid column. Chromatography was then applied and integration of peaks performed. Three experiments were performed in triplicate and the means $\pm$ S.D. determined.

the endogenous activator of PKC. By means of HPLC, we measured the kinetics of DAG accumulation in response to 1 unit/ml EPO. In three experiments (Figure 1A), DAG levels peaked at 5 s with a 28.1 % increase compared with control levels (from 17.3 to 22.2 pmol/10⁶ cells). By 20 s DAG levels were no longer changing. Thus, the early phase of DAG increase is rapid and transient. In addition, there were later changes seen that peaked at 30 min (Figure 1B), and the increase compared with control levels was even greater (84.2 % increase from 17.3 to 31.9 pmol). This second phase of DAG increase decayed slowly. By 90 min DAG levels had nearly returned to baseline levels. These changes were concentration-dependent in response to EPO from 0.025 to 10 units/ml (Figure 2A). The shape of the dose–response curve was markedly different at 5 s compared with that at 30 min. For the 30-min time point, a plateau effect was reached at much lower concentrations of EPO than was seen

Figure 2 DAG and PA responses to various concentrations of EPO

(A) DAG at early (5 s) (○) and late (30 min) (□) times; (B) PA dependence on EPO concentration. Concentrations of EPO from 0 to 10 units/ml were tested for their effects on DAG production (as determined by HPLC), or PA production (as determined by TLC and HPLC). Each point represents the mean of two different experiments in triplicate.

at 5 s. The EC₅₀ value at 5 s was calculated to be 0.82 unit/ml and at 30 min, 0.10 unit/ml.

In this study, PA was measured by both TLC (Figure 3A) and HPLC (Figure 3B). In both cases PA levels increased maximally at 20 s. Levels increased from 8.6 to 64.7 μg/10⁶ cells (7.5-fold increase). The rapid accumulation of DAG preceded that of PA, thus PA could be derived from a rapidly phosphorylated pool of DAG only at very fast kinetic times. PA accumulation precedes that of DAG for the slow and sustained phase, suggesting with a higher probability that PA was not derived from DAG. The changes in PA were also concentration-dependent in response to EPO from 0.025 to 10 units/ml (Figure 2B) with an EC₅₀ of 0.073 unit/ml at 5 s.

To assess the mechanism of PA accumulation, the involvement of G-proteins was examined by studying the effects of the non-hydrolysable GTP analogue GTP[S] on PA synthesis. These

Figure 3 Effect of EPO on PA accumulation as determined by TLC (A) and HPLC (B)

Cells were pretreated with EPO (0.2 unit/ml), and PA was measured from 0 to 250 s as described in the Materials and methods section. Two experiments are shown.

studies were performed with cells in which the permeability of the plasma membrane was increased by exposure to low concentrations of saponin. In the permeabilized cells, the addition of GTP[S] (10 mM) increased PA from $6.3 \pm 0.9 \mu\text{g}$ to $48.6 \pm 1.6 \mu\text{g}$ per 10^6 permeabilized cells. EPO + GTP[S] increased levels to $64.8 \pm 1.8 \mu\text{g}$. GDP[S], a G-protein antagonist, had no effect on control levels of PA ($5.9 \pm 0.8 \mu\text{g}/10^6$ permeabilized cells) but blocked the effect of EPO on PA ($30.6 \pm 1.8 \mu\text{g}/10^6$ permeabilized cells). This was performed without ATP (Table 2). At high concentrations of GTP[S], ATP had a slight effect on the accumulation of PA. Potential phospholipid sources of PA were investigated by incubating B6Sut.EP permeabilized cells with GTP[S] in the presence and absence of EPO (Table 3). The major phospholipids were separated as described, washed, and assayed for PO_4 content. Whereas PA levels rose from 3.2 ± 1.0 to $21.4 \pm 3.09 \mu\text{g}/10^6$ permeabilized cells, PI levels remained the same. PE decreased significantly ($P < 0.05$) at 20 min but not at

Table 2 Effect of GTP analogues on PA

B6Sut.EP cells were first preincubated for 1 min with saponin (15 $\mu\text{g}/\text{ml}$) then treated for 30 s with EPO (0.2 unit/ml), GTP[S] (10 μM), GDP[S] (10 μM), or ATP (10^{-4} M). PA levels were measured as described in the Materials and methods section. Values are the means $\pm$ S.E.M. of three separate experiments conducted in triplicate.

Treatment	PA level ($\mu\text{g}/10^6$ permeabilized cells)
Control	6.3 ± 0.9
GTP[S]	48.6 ± 1.6
GTP[S] + EPO	64.8 ± 1.8
GDP[S]	5.9 ± 0.8
GDP[S] + EPO	30.6 ± 1.8
GTP[S] + ATP	83.2 ± 2.4

20 s. PC declined from $92.2 \pm 13.6 \mu\text{g}/10^6$ cells to half that (42.6 ± 6.8) at 20 s and $55.3 \pm 7.9 \mu\text{g}/10^6$ cells ($P < 0.0001$) at 20 min, implicating PC and possibly PE as the source of PA in the cells. These results show both rapid and slow basal turnover of PC and slow turnover of PE associated with an increase in both DAG and PA mass. As the total phospholipid mass remained relatively constant, this implies that PE and PC hydrolysis products are direct precursors of PA and DAG synthesis.

In order to determine whether PLD was responsible for the conversion of PC into PA, cells were prelabelled with [^3H]myristic acid, stimulated with EPO in the presence of ethanol, and the production of PEt was measured. The accumulation of PEt has been useful in various cell culture model systems as an indicator of hormone/growth factor-activated PLD [7–11]. [^3H]Myristic acid preferentially labelled PC (80%) and PE (6%), but not PI or PS. Neutral lipids were also labelled (12%). We found that EPO stimulated a transphosphatidylation reaction in B6Sut.EP cells (Table 4). In cells that had been prelabelled with [^3H]myristic acid, EPO had no effect on the cellular PEt, whereas EPO increased cellular PA levels by 9-fold. In the presence of ethanol, the EPO-stimulated increase in PA was reduced and cellular PEt levels increased 8 times over control (ethanol only) values. These results suggest that PEt is generated at the expense of PA. It is known that PLD catalyses the transphosphatidylation reaction [15]. In the presence of ethanol, EPO-stimulated transphosphatidylation reactions were observed as a rapid increase in PEt (Figure 4) with maximal levels at 5 min, but PEt still increased at 12–14 min. This raised the probability that phospholipids hydrolysed by PLD and generating PA are subsequently hydrolysed to DAG by PA phosphohydrolase (PPH). To examine this hypothesis, stimulation by EPO was performed in the presence of ethanol and then [^3H]DAG was measured (Table 4). If the PPH pathway is active, formation of PEt in the presence of ethanol should prevent DAG formation. (PEt is an unsuitable substrate for PPH.) Although the decrease in [^3H]DAG at 2 min incubation with ethanol + EPO was only 12% and statistically not significant, after 5 min incubation with ethanol + EPO the inhibition was 30% when compared with the stimulated level with EPO only (Table 4). Thus, the temporal increases in PA and PEt in alcohol- and EPO-stimulated cells (Table 4 and Figure 4) are consistent with a pathway involving a precursor/product relationship.

To determine the contribution made by the PLD/PPH pathway to EPO-stimulated DAG formation or the possibility that some early changes in PA might be derived from DAG kinase-catalysed phosphorylation of DAG, mass determinations of DAG and PA in the presence of the inhibitors ethanol, propranolol (also a PA inhibitor, which has been shown to cause

Table 3 Effects of GTP[S] on phospholipid levels in the presence and absence of EPO

Cells were first preincubated for 1 min with saponin (15 µg/ml) with or without 10 µM GTP[S] in the absence or presence of EPO (0.2 unit/ml) for 20 s and 20 min. Values are expressed as nmol/10⁶ permeabilized cells. The phospholipids were separated and analysed for phosphorus as described in the Materials and methods section. Values are the means ± S.E.M. of three separate experiments, each in triplicate. Statistical significance: ^a*P* < 0.0001, ^b*P* < 0.05, ^cN.S.

GTP[S]	EPO	Phospholipid content (nmol/10 ⁶ permeabilized cells)				
		PC	PI	PE	PA	DAG
—	—	92.2 ± 13.6	36.4 ± 3.8	44.2 ± 3.9	3.2 ± 1.0	4.8 ± 1.1
+	—	79.8 ± 7.3	35.6 ± 4.1	45.0 ± 4.1	26.0 ± 2.2	10.9 ± 1.8
+	+ 20 s	42.6 ± 5.8 ^a	30.2 ± 3.6 ^c	43.0 ± 4.0 ^c	34.2 ± 5.5 ^a	16.3 ± 3.0 ^a
+	+ 20 min	55.3 ± 7.9 ^a	36.3 ± 4.2 ^c	34.6 ± 3.8 ^b	21.4 ± 3.9 ^a	28.6 ± 3.4 ^a

Table 4 Analysis of EPO-stimulated PLD activity in B6Sut.EP cells

Cells were prelabelled with [³H]myristic acid for 8 h, and after change of the medium, incubated with 2% (v/v) ethanol (EtOH) for 2 min, EPO (2 units/ml) for 2 min, or EPO + ethanol for 2 min. Analysis of labelled PA, PEt and DAG was performed as described in the Materials and methods section. Each value represents the mean ± S.D. of three separate experiments, each in triplicate. In parentheses are values of [³H]DAG after incubations with ethanol and EPO for 5 min.

Treatment	PA		PEt		PA/PEt ratio	DAG	
	³ H (c.p.m./µmol of P _i)	% of control	³ H (c.p.m./µmol of P _i)	% of control		³ H (c.p.m./µmol of P _i)	% of control
Control (EtOH only)	29 ± 2	100	10 ± 1	100	2.9	16 ± 1	100
EPO	272 ± 8	938	14 ± 4	140	67	36 ± 4	225
EPO + EtOH	56 ± 6	193	83 ± 9	830	0.67 (22 ± 3)	28 ± 4 (137)	175

inhibition of DAG and accumulation of PA in mammalian cells [10]) and R59022 (a DAG kinase inhibitor) were performed at the times shown in Figures 1 and 3 to define the accumulation of PA and DAG with EPO stimulation (Table 5). Cells were preincubated with the inhibitors prior to the stimulation with EPO and the optimal concentrations of ethanol, propranolol and R59022 were determined by experiments with increasing concentrations of inhibitors (results not shown). First, preincubation with R59022 showed only a minimal increase (not statistically significant) at 20 s incubation but did not influence the time-course of accumulation of PA stimulated with EPO nor the DAG accumulation at any level. Basal levels of DAG and PA in unstimulated cells were unchanged by preincubation of cells with R59022. However, R59022 treatment did not influence the accumulation of DAG and PA even at 30 min after stimulation. A significant decrease in the levels of DAG stimulated by EPO in cultures pretreated with ethanol occurred after 5 and 30 min with concomitant increases of approx. 60, 200 and 400% at 5 s, 20 s and 1 min, respectively, in the levels of PA. As shown in Table 5, propranolol pretreatment enhanced the accumulation of PA significantly at 20 s and 5 min but mostly inhibited the formation of DAG at 5 s, 5 min, 30 min, and even 60 min.

DISCUSSION

Growth factors stimulate a variety of cellular responses, including the generation of DAG. Some of these responses are rapid and transient, such as stimulation with epidermal growth factor [5], while others are slower and sustained, such as IL-3-stimulated cell proliferation [22]. The relationship between these responses and DAG requires a knowledge of the kinetics of DAG production [23]. The results presented here demonstrate DAG accumulation kinetics to be biphasic with high concentrations of

EPO, or monophasic with low concentrations of EPO. Furthermore, although some investigators have demonstrated a relationship between DAG production and hydrolysis of PI, our results point to additional sources for DAG production.

The results of these studies in a murine stem-cell line, B6Sut.EP, in terms of the DAG response to EPO, are similar to our previous observations in fetal liver cells [4]. However, in B6Sut.EP cells, we have identified a rapid accumulation of PA in response to EPO which was not observed in the more heterogeneous population of murine fetal liver cells studied. Although in EPO-stimulated cells the increase in PA preceded the sustained phase of DAG formation, it remains possible that PA could be derived from a rapid phosphorylation of the DAG pool. If so, PA accumulation should have been inhibited by the DAG kinase inhibitor R59022. We found that R59022 was without significant effect on the time-course of formation and accumulation of PA. These observations indicated that most of the PA pool is generated through the activation of PLD.

Our findings that EPO induced transphosphatidylation reactions strengthen the evidence that EPO hydrolyses PC and/or PE via the activation of PLD. The temporal relationship of the evolution of PA and DAG indicates the activation of EPO-stimulated PLD and suggests that PA serves, in part, as a precursor of DAG. This conclusion is strengthened by the results with the inhibitors presented in Table 5. PLD hydrolyses PC producing free choline and PA, which in turn can be hydrolysed by PA phosphatase to form DAG. When cells were incubated with GTP[S], PA increased and PC decreased to a similar extent. The effects of ethanol and propranolol provide evidence for the presence of an active PPH and indicate that part of DAG accumulation in EPO-treated cells may derive from PA via PPH. However, our results do not exclude DAG formation by a phospholipase C-mediated hydrolysis of PC or PE. A number of

Figure 4 Time-course for PA and PET formation in response to EPO, ethanol, or a combination of both

Cells were prelabelled with [^3H]myristic acid as described in the Materials and methods section. The experiments shown are representative of three separate experiments, each in triplicate; Δ , ethanol alone; $\blacktriangle$, EPO alone; $\circ$, EPO + ethanol. Duplicate determinations were within 2% of the mean.

recent studies suggest that PLD is a critical component in stimulus–response coupling, although details of its role are still lacking [7–11]. The pluripotent colony-stimulating factor, IL-3, influences the survival and proliferation of a wide spectrum of haematopoietic progenitor cells. Like EPO, the signal transduction mechanism linking its receptor and lipid second messenger generation remains ill defined, but there is evidence for

both tyrosine kinase and PKC activation [24–28]. Depending on the cell type studied, PLD may also play a role. Robinson et al. [22] found that IL-3 treatment of CFTL-12 cells, a murine mast-cell line that requires IL-3 for growth, caused an acute increase in the mass of both alkylacylglycerol and DAG consistent with PC as a significant source. These cells were analysed by metabolically radiolabelling with [^3H]myristate and by analysis of the benzoyl derivatives of DAG by HPLC. The net mass of DAG produced by CFTL-12 cells during a 30-min incubation with IL-3 was 1.7 nmol/ 10^7 cells.

A recent study [27] in which a megakaryoblastic leukaemic cell line, MO7E, was prelabelled with [^3H]glycerol or [^3H]choline demonstrated rapid release of [^3H]phosphorylcholine and a decrease in [^3H]glycerol-labelled PC in response to IL-3 stimulation. In addition, IL-3 did not induce PA accumulation, suggesting that PLD is not activated in MO7E cells. DAG generation is independent of PKC activation, whereas in CFTL-12 cells DAG generation appeared to be dependent on PKC [22]. Although there are discrepancies in terms of PLD involvement in the mechanism of action of IL-3 depending on the cell type, there is a relative consensus that IL-3, like EPO, induces the turnover of PC in responsive cells but does not appear to stimulate inositol phospholipid turnover [24–28].

There is also relative consensus that tyrosine kinase activation in response to granulocyte/macrophage colony-stimulating factor, EPO and IL-3 is an earlier event in the signalling cascade than is PKC activation [11,12,28]. Based on Exton's studies [23], those of Rao and Mufson [27], Quelle and Wojchowski [13], and our studies, the evidence thus far suggests a model for the mechanisms of action of IL-3 and EPO in which the ligand-specific receptors stimulate PC hydrolysis by phospholipase C and/or PLD through specific G-proteins. The mechanisms for prolonged PC hydrolysis may involve tyrosine phosphorylation of the relevant phospholipases as suggested by Exton [23]. Rao and Mufson [27], furthermore, suggest that IL-3 receptor engagement results in tyrosine-kinase-dependent phosphorylation of proteins that regulate p21^{ras} activity. Evidence from several systems [29] has suggested that PC-specific phospholipase C is a downstream effector of activated p21^{ras} , which has been shown to be activated by EPO [30]. The ras activation of this phospholipase would generate DAG and activate PKC. Alternatively, both EPO and IL-3 could directly activate phospholipase C by tyrosine phosphorylation. Subsequent effects of this cascade could be Raf-1 kinase activation followed by mitogen-activated protein kinase (MAP kinase), eventually resulting in the activ-

Table 5 Temporal changes in total DAG and PA mass following addition of EPO in the presence and absence of inhibitors

Cells were incubated for 5 min with 30 μM ethanol, 250 μM propranolol, 250 μM R59022 or buffer only, and then stimulated with EPO (2 units/ml) for 0, 5, 20 s, and 1, 5, 30, and 60 min. Incubations were stopped by addition of ice-cold methanol, and then lipids were extracted. DAG and PA were determined as described in the Materials and methods section. The data are means $\pm$ S.D. of four experiments and are given relative to EPO-stimulated control values. A value of 28–34 pmol of DAG/ 10^6 cells represents 100% DAG and 35–52 μg of PA/ 10^6 cells represents 100% PA. The statistics performed were a mixed-model ANOVA for each experiment followed by Dunnett's test. $^aP < 0.0001$ versus EPO only control; $^bP < 0.0005$; $^cP < 0.005$; $^dP < 0.05$.

Time	Pretreatment with ...	DAG (% of basal level)				PA (% of basal level)			
		EPO only	Ethanol	Propranolol	R59022	EPO only	Ethanol	Propranolol	RS9022
Control		100 $\pm$ 4	98 $\pm$ 3	98 $\pm$ 2	101 $\pm$ 3	100 $\pm$ 5	100 $\pm$ 4	106 $\pm$ 3	98 $\pm$ 6
5 s		128 $\pm$ 7	131 $\pm$ 7	104 $\pm$ 6 ^c	132 $\pm$ 6	363 $\pm$ 15	420 $\pm$ 9 ^b	380 $\pm$ 16	359 $\pm$ 14
20 s		110 $\pm$ 4	108 $\pm$ 4	110 $\pm$ 2	111 $\pm$ 6	752 $\pm$ 20	955 $\pm$ 22 ^a	789 $\pm$ 34 ^d	748 $\pm$ 18
1 min		101 $\pm$ 5	103 $\pm$ 5	95 $\pm$ 2	102 $\pm$ 4	428 $\pm$ 6	840 $\pm$ 16 ^a	919 $\pm$ 9 ^a	430 $\pm$ 10
5 min		112 $\pm$ 6	82 $\pm$ 6 ^b	98 $\pm$ 2 ^d	110 $\pm$ 8	83 $\pm$ 4	106 $\pm$ 5	130 $\pm$ 8 ^c	85 $\pm$ 6
30 min		183 $\pm$ 15	152 $\pm$ 10 ^a	104 $\pm$ 7 ^a	187 $\pm$ 12	96 $\pm$ 2	100 $\pm$ 3	103 $\pm$ 4	98 $\pm$ 4
60 min		150 $\pm$ 9	137 $\pm$ 3	125 $\pm$ 5 ^c	150 $\pm$ 6	99 $\pm$ 3	95 $\pm$ 6	99 $\pm$ 11	100 $\pm$ 6

ation of MAP kinase. In B6SUt.EP cells, PKC β is associated with enhanced proliferation in response to IL-3 and EPO [31]. It is localized in the nucleus as a result of stimulation and may be related to early-immediate gene expression observed in response to these growth factors [27,32].

The control of PC hydrolysis by G-proteins, PKC and tyrosine kinases appears to be important in the mechanism of action of several haematopoietic growth factors. Although the role of DAG is partially understood, the significance of PA formation is not clear. Further studies are necessary to define the physiological significance of agonist-stimulated PC breakdown.

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