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Induction of cytosolic phospholipase A_2 activity by phosphatidic acid and diglycerides in permeabilized human neutrophils: interrelationship between phospholipases D and A_2

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Relationships between phospholipases are poorly understood, but phosphatidic acid (PA) and diglycerides (DGs), produced by phospholipase D (PLD) and phosphatidate phosphohydrolase actions, might function as second messengers coupling cell stimulation to cellular responses. This study investigates the role of PLD-mediated PA and DG formation in inducing phospholipase A_2 (PLA_2) activity in intact human neutrophils (PMNs) and in PMNs permeabilized with *Staphylococcus aureus* α-toxin. PMNs were labelled with [^{3}H]arachidonic acid (AA) to assess AA release and metabolism and diacylglycerol formation, or with [^{3}H]1-*O*-hexadecyl-2-lyso-glycerophosphatidylcholine for the determination of platelet-activating factor (PAF), PA and alkylacylglycerol production. In intact PMNs primed with tumour necrosis factor α before stimulation with *N*-formyl-Met-Leu-Phe, AA release and metabolism and PAF formation increased in parallel with enhanced PA and DG formation, and inhibition of PA and DG production led to a decrease in both AA release and PAF accumulation. In α-toxin-permeabilized PMNs, AA release and PAF production result from the specific activation of cytosolic PLA_2 ($cPLA_2$). In this system, PA and DG formation were always present when $cPLA_2$ activation occurred; blocking PA and DG production inhibited AA release and PAF accumulation. Adding either PA or DG back to permeabilized cells (with endogenous PA and DG formation blocked) led to a partial restoration of AA release and PAF formation; a combination of PA and DGs reconstituted full $cPLA_2$ activity. These results strongly suggest that products of PLD participate in activating $cPLA_2$ in PMNs.

INTRODUCTION

Neutrophils (PMNs) play a central role in host defence, and production of the lipid mediators leukotriene B_4 (LTB_4) and platelet-activating factor (PAF) is an important aspect of PMN function during normal inflammation. Increased PMN activity has also been associated with both acute and chronic inflammatory states such as sepsis syndrome, adult respiratory distress syndrome, rheumatoid arthritis and asthma, with enhanced production of both PAF and LTB_4 being implicated in the pathogenesis of these disorders (reviewed in [1,2]). The initial and rate-limiting enzyme responsible for both LTB_4 and PAF formation in PMNs is believed to be an *sn*-2-acylhydrolase, phospholipase A_2 (PLA_2) [3,4]. PMNs contain at least two types of Ca^{2+}-dependent PLA_2 [5], a 14 kDa PLA_2 termed 'secretory' PLA_2 ($sPLA_2$) and an 85 kDa enzyme localized in the cytosol of resting cells called 'cytosolic' PLA_2 ($cPLA_2$). Although substantial progress has been made in understanding the role of these enzymes in the generation of LTB_4 and PAF in PMNs, the mechanism that links cell stimulation to actual activation of one or both types of PLA_2 has remained elusive.

Phosphatidic acid (PA), produced by phospholipase D (PLD) activation, and diglycerides (DGs) subsequently formed via cleavage of PA by phosphatidate phosphohydrolase, have been proposed to act as intracellular second messengers to link cell stimulation to cellular responses (reviewed in [6]). PMNs contain PLD(s) that cleave both phosphatidylcholine and phosphatidylinositol in response to physiological stimuli [7–9], and many of the same agonists that induce PLD activity also initiate PLA_2 activation in PMNs. The interrelationships between different pathways involved in phospholipid metabolism are not well understood; in this study the potential role of the products of PLD activation in the induction of PLA_2 activation in PMNs were examined.

Studying mechanisms of phospholipase activation in intact cells has been difficult because cell stimulation leads to the activation of multiple pathways. The delineation of steps involved in a single path is complicated, and the determination of how different paths interact is nearly impossible. Cell-free or isolated enzyme systems have been used for most studies investigating requirements for enzyme activation; however, these systems are not suitable for addressing signal transduction steps involved in enzyme activation or for assessing the interrelationship between enzyme systems. To overcome some of the problems inherent in both approaches, we have used PMNs permeabilized with *Staphylococcus aureus* α-toxin to study the interrelationship between PLD and PLA_2 activation.

Permeabilized cell systems have been used to elucidate signal transduction mechanisms leading to enzyme activation in a number of cell types [10–12]. Many methods are available for cell

Abbreviations used: AA, arachidonic acid; $cPLA_2$, cytosolic PLA_2; cyto B, cytochalasin B; DGs, diglycerides; FMLP, *N*-formyl-Met-Leu-Phe; GTP[S], guanosine 5′[γ-thio]triphosphate; [^{3}H]AA, [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; [^{3}H]lyso-PAF, 1-*O*-[9′,10′-^{3}H]hexadecyl-2-lyso-glycerophosphatidylcholine; HSA, human serum albumin; LTB_4, leukotriene B_4; MAP kinase, mitogen-activated protein kinase; PMNs, polymorphonuclear leucocytes (neutrophils); PA, phosphatidic acid; PAF, platelet-activating factor; Pet, phosphatidylethanol; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; $sPLA_2$, secretory PLA_2; TNFα, tumour necrosis factor α.

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permeabilization [13,14] but the α-toxin of *S. aureus* has several advantages [12,15]. α-Toxin monomers interact on the surface of cells to form 2–3 nm diameter pores in the plasma membrane. The pores prevent leakage of macromolecules from the cell but allow small molecules (less than 1000 Da) added extracellularly to move freely with very rapid penetration and equilibration into the cytosol [10]. α-Toxin monomers (larger than 28 kDa) are too large to pass through the pores, so only membranes facing the extracellular space are affected; intracellular structures are not altered. This model allows cellular enzymes and proteins to be maintained *in situ* but allows potential regulators of enzyme activation to be selectively added. We have used this model to study the requirements for both PLD [11] and PLA_2 [16] activity in PMNs and have shown selective activation of $cPLA_2$ in the absence of detectable $sPLA_2$ activity in the permeabilized cells; further work has demonstrated that both PAF and LTB_4 production occur in this system [17].

In this study the role of PLD-mediated PA and DG formation in the induction of PLA_2 activity was first investigated in intact PMNs primed with tumour necrosis factor α (TNFα) before stimulation with the chemotactic peptide *N*-formyl-Met-Leu-Phe (FMLP). The permeabilized PMN model was then used to demonstrate that: (1) PA and DG formation were always present when $cPLA_2$ activity was evident; (2) blocking PA and DG formation led to a parallel reduction in arachidonic acid (AA) release and metabolism and PAF formation; (3) adding either PA or DGs back to cells partly restored $cPLA_2$ activity; (4) the addition of a combination of PA and DGs at physiologically relevant concentrations completely reconstituted $cPLA_2$ activity. These results strongly suggest that products of PLD activation participate in the induction of PLA_2 activity in human PMNs.

EXPERIMENTAL

Reagents

Stock solutions of FMLP (10 mM; Peninsula Laboratories, Belmont, CA, U.S.A.), and cytochalasin B (cyto B; 5 mg/ml; Aldrich Chemical Co., Milwaukee, WI, U.S.A.) were prepared in DMSO (Sigma, St. Louis, MO, U.S.A.) and stored at −20 °C. NADPH, GTP, guanosine 5′-[γ-thio]triphosphate (GTP[S]) (Boehringer Mannheim, Indianapolis, IN, U.S.A.), acetyl-CoA (Pharmacia, Piscataway, NJ, U.S.A.), $MgATP^{2-}$, ferricytochrome *c* and superoxide dismutase (Sigma) were stored in accordance with manufacturers' directions. Stock solutions of TNFα (100000 i.u./ml; Genzyme, Cambridge, MA, U.S.A.) were prepared in PBS, pH 7.4 (Ca^{2+}- and Mg^{2+}-free; Gibco, Grand Island, NY, U.S.A.), supplemented with 0.1% gelatin (Sigma) and 0.2% (v/v) human serum albumin (HSA; Sigma) and stored at −70 °C. Working solutions of TNFα (10000 i.u./ml) were made in PBS supplemented with 1 mM Ca^{2+} and 0.2% HSA and stored at 4 °C. Pyrogen-free reagents were used wherever possible and all solutions were routinely monitored for endotoxin contamination by the Limulus amoebocyte lysate assay (Cape Cod Associates, Woods Hole, MA, U.S.A.). All organic solvents (Fisher Scientific, Raleigh, NC, U.S.A.) were of HPLC grade or better. Lipid standards, PA (10 mg/ml, derived from egg lecithin) and DGs (10 mg/ml, derived from pig liver) were obtained from Serdary (Ontario, Canada) and Avanti Polar Lipids (Birmingham, AL, U.S.A.) and stored at −20 °C.

α-Toxin preparation

α-toxin was prepared as previously described [10].

PMN isolation

PMN populations (more than 95% PMNs) were prepared by layering heparinized venous blood from normal adult volunteers over Isolymph (Gallard-Schlesinger Chemical Manufacturing Corporation; 3 ml of blood per ml of Isolymph) for red cell sedimentation. Collected cells were centrifuged through Isolymph to eliminate mononuclear cells; contaminating erythrocytes were removed by hypotonic lysis. PMNs were resuspended in PBS supplemented with 0.1% gelatin (Sigma) at 5×10^7 cells/ml.

Labelling of PMNs with ^3H

Isolated PMNs were prelabelled with [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid ([^{3}H]AA; 100 Ci/mmol; Du Pont–New England Nuclear, Boston, MA, U.S.A.) for assays to measure AA release and metabolism and diacylglycerol formation. [^{3}H]AA in absolute ethanol was added to PMNs at 1 μCi per 3.5×10^7 cells; ethanol levels never exceeded 0.5% of total volume. Cells were incubated for 30 min at 37 °C, washed, and resuspended in PBS at 5×10^7 cells/ml.

For assessment of the formation of PAF, PA, alkylacylglycerol and phosphatidylethanol (Pet), PMNs were labelled with 1-*O*-[9′,10′-^{3}H]hexadecyl-2-lyso-glycerophosphatidylcholine ([^{3}H]lyso-PAF; 56 Ci/mmol; a gift from Dr. R. L. Wykle, Bowman Gray School of Medicine, Winston-Salem, NC, U.S.A.). [^{3}H]Lyso-PAF was suspended by sonication in Ca^{2+}- and Mg^{2+}-free PBS supplemented with 2.5 mg/ml BSA (Sigma) before addition to PMNs (1 μCi of [^{3}H]lyso-PAF in 50 μl of buffer per 3.5×10^7 cells). Cells were labelled for 45 min at 37 °C, washed, then resuspended in PBS at 5×10^7 cells/ml.

Primed stimulation of PLA_2 and PLD in intact PMNs

PMNs (final concentration 5×10^6 cells/ml) were resuspended in PBS containing 1 mM Ca^{2+} for assessment of PLA_2 and PLD activities. Cells were treated with 5 μg/ml cyto B for 5 min at 37 °C and primed with 100 i.u./ml TNFα (or HSA containing buffer) for 30 min. Samples were then stimulated with 1 μM FMLP for an additional 5 min. Cellular lipids were obtained by vortex-mixing cells with chloroform/methanol (1:2, v/v) acidified with 0.15 ml of 9% formic acid (3 ml/ml of cells) and extracting lipids as described below.

Cell permeabilization

Human PMNs were permeabilized with α-toxin in a 'cytoplasmic' buffer consisting of 140 mM KCl, 1 mM MgCl, 1 mM $MgATP^{2-}$, 10 mM glucose, 10 mM Hepes, 0.1% gelatin and 1 mM EGTA, pH 7.4, for 15 min at 37 °C as previously described [10,11].

PLA_2 and PLD activation in permeabilized PMNs

For phospholipase assays, PMNs (final concentration 5×10^6 cells/ml) were permeabilized in cytoplasmic buffer supplemented with 100 μM acetyl-CoA. Cofactors and stimuli (no cyto B was used in permeabilized PMNs) were added 15 min after α-toxin, and reactions were halted by vortex-mixing cells with chloroform/methanol at the indicated times.

Lipid extraction

Lipids were extracted by a modification of the Bligh and Dyer technique [18]. After chloroform/methanol addition, samples

were mixed with 5 μl of phosphatidylcholine (10 mg/ml), 2 ml of chloroform and 1 ml of water (per ml of the original cell solution) and vortex-mixed. After centrifugation, organic layers were retained, solvents were evaporated under nitrogen and samples resuspended in chloroform/methanol (9:1, v/v).

TLC

AA, LTB_4, 5-hydroxyeicosatetraenoic acid (5-HETE) and diacylglycerol from PMNs prelabelled with [^{3}H]AA were separated on Silica Gel G plates (Analtech, Newark, DE, U.S.A.) by development for 30 min in a hexane/ethyl ether/formic acid (90:60:6, by vol.) mixture followed by a 6 min exposure in a second solvent system consisting of hexane/ethyl ether/formic acid (60:90:6, by vol.) [19,20]. PAF, PA, alkylacylglycerol and Pet formation were assessed in PMNs labelled with [^{3}H]lyso-PAF. PAF isolation was performed on Silica Gel G plates in a solvent system of chloroform/methanol/acetic acid/water (50:25:8:4, by vol.) [20,21]. PA, Pet and alkylacylglycerol were isolated by TLC in the organic phase of a solvent system containing ethyl acetate/iso-octane/acetic acid/water (110:50:20:100, by vol.) on Silica Gel 60 TLC plates (Curtin Matheson Scientific, Florence, KY, U.S.A.) [9]. Fractions, based on the location of lipid standards, were scraped from the TLC plates and assayed by liquid-scintillation counting (Model LS 1801, Beckman Instruments).

Lipid preparation for fatty acid mass determination

AA mass was determined as previously described [16] by using octadeuterated AA and trideuterated stearic acid (5 ng/μl of each in methanol; Cayman Chemical Co.) as internal standards and employing separation by reverse-phase chromatography on Bakerbond solid-phase extraction octadecyl columns (Scientific Products, McGraw Park, IL, U.S.A.). Isolated fatty acids were converted into pentafluorobenzyl esters by the addition of 30 μl of di-isopropylethylamine [20% (v/v) in acetonitrile; Pierce, Rockford, IL, U.S.A.] and 30 μl of pentafluorobenzyl bromide [20% (v/v) in acetonitrile; Pierce] and incubation at 40 °C for 40 min. Pentafluorobenzyl esters were extracted and resuspended in hexane.

GLC–MS

Pentafluorobenzyl esters were analysed by negative-ion chemical-ionization GLC–MS [22]. Carboxylate anions at m/z 303 and 311 for AA and octadeuterated AA respectively were monitored with a Hewlett-Packard mass spectrometer (HP 5989A) interfaced to a Hewlett-Packard series II gas chromatograph (HP 5890). Quantities of released AA were determined from a standard curve obtained with octadeuterated AA as the internal standard.

Determination of free Ca^{2+} levels

Free Ca^{2+} levels (1 μM or less) were determined by the fluorescence of fura 2 (free salt, 3 μM; Molecular Probes, Eugene, OR, U.S.A.) in a fluorimeter (SLM 8000, SLM Instruments; excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm).

Statistics and data presentation

Results were tested for statistical significance with Student's t test or analysis of variance followed by Tukey's procedure; values of $P < 0.05$ were considered statistically significant. Results from radiolabelled assays are displayed as the level of ^{3}H label found in specific lipid fractions as a percentage of total label recovered. Total release of [^{3}H]AA was measured by combining ^{3}H found in the fatty acid, LTB_4 and 5-HETE fractions.

RESULTS

TNFα priming of PLA_2 and PLD in human PMNs

Initial studies in this investigation focused on determining the relationship between PLA_2 and PLD activation in intact PMNs. In previous reports the ability of TNFα to prime PMNs for enhanced AA release and metabolism, PAF formation and PA production when cells were subsequently stimulated was characterized [9,20]. Table 1 summarizes the results obtained when cells pretreated with cyto B were primed with 100 i.u./ml TNFα for 30 min and then stimulated with 1 μM FMLP for 5 min. In the absence of priming (PMNs treated with buffer containing HSA, the carrier for TNFα), stimulation with FMLP induced modest increases in AA release (combination of ^{3}H found in the fatty acid, LTB_4 and 5-HETE fractions) and PAF formation. Priming with TNFα did not directly increase AA release and metabolism or PAF formation, but when primed cells were subsequently stimulated with FMLP, total free AA was twice the AA levels found in resting cells (HSA control) and PAF formation was more than tripled. Under the same conditions, PA formation in unprimed cells was significantly increased by FMLP stimulation (3.5-fold increase in PA levels). TNFα did not cause any increase in PA levels in the absence of stimulation but did enhance PA formation in PMNs stimulated with FMLP to more than a 5-fold increase over control values.

[^{3}H]Lyso-PAF has an alkyl linkage in the sn-1 position, so an assessment of DG formation in [^{3}H]lyso-PAF labelled PMNs monitors only alkylacylglycerol formation. In contrast, when PMNs are radiolabelled with [^{3}H]AA for short periods, most AA is incorporated into the 2 position of the diacyl species of phosphatidylcholine and phosphatidylinositol [3,19,22] and DGs produced will be primarily of the diacylglycerol molecular species. Thus, to characterize DG formation fully during primed stimulation of PMNs, the production of both alkylacylglycerol and

Table 1 Priming of PLA_2 and PLD in human PMNs by TNFα

PMNs (5×10^6 cells/ml) prelabelled with [^{3}H]AA (AA release and metabolism and diacylglycerol formation) or labelled with [^{3}H-]lyso-PAF (PAF, PA and alkylacylglycerol formation) were pretreated with 5 μg/ml cyto B for 5 min at 37 °C. Cells were then primed with TNFα (or HSA) for 30 min before stimulation with FMLP or buffer. Reactions were halted after 5 min and lipids were extracted and analysed as described in the Materials and methods section.

Treatment	AA release (%)*	PAF formation (%)†	PA production (%)‡	DG (alkylacyl) formation (%)§	DG (diacyl) formation (%)‖
HSA control	5.4 ± 0.4	0.8 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	2.1 ± 0.1
HSA to 1 μM FMLP	7.1 ± 0.4	1.5 ± 0.2	2.2 ± 0.2	5.7 ± 0.6	3.1 ± 0.1
100 i.u./ml TNFα	5.6 ± 0.3	0.8 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	2.2 ± 0.1
TNFα to FMLP	10.9 ± 0.5	2.6 ± 0.3	3.1 ± 0.2	7.3 ± 0.3	3.8 ± 0.2

* Arachidonic acid release is shown as the percentage of ^{3}H found as free AA and metabolites; means ± S.E.M. for 10 experiments, duplicate determinations per experiment.

† Means ± S.E.M. for eight experiments, duplicate determinations per experiment.

‡ Means ± S.E.M. for nine experiments, duplicate determinations per experiment.

§ DG (alkylacylglycerol) formation determined in cells labelled with [^{3}H]lyso-PAF; means ± S.E.M. for nine experiments, duplicate determinations per experiment.

‖ DG (diacylglycerol) formation determined in cells labelled with [^{3}H]AA; means ± S.E.M. for 10 experiments, duplicate determinations per experiment.

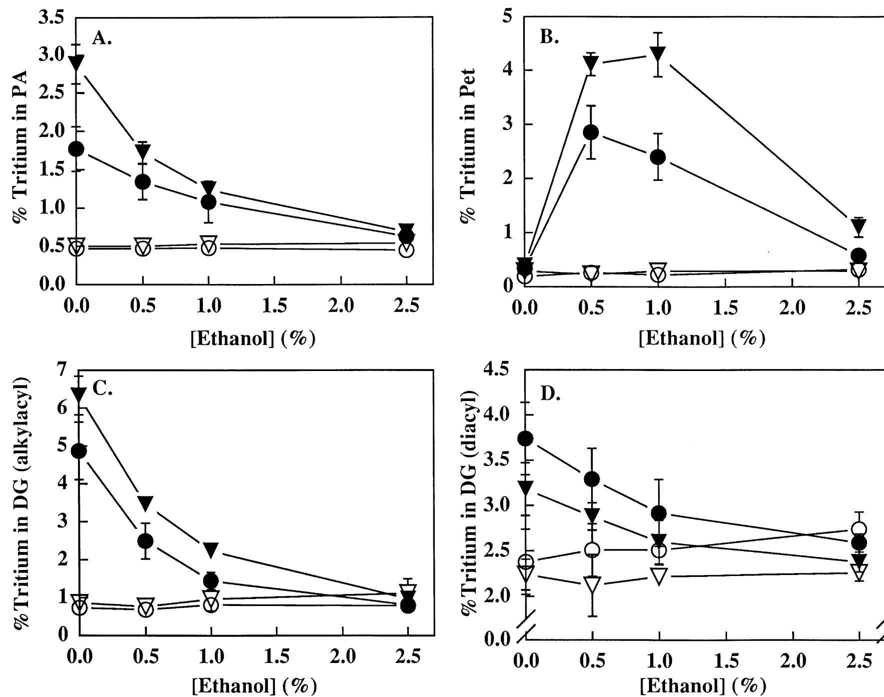

Figure 1 Effects of ethanol on PA and DG production in PMNs primed with TNFα before stimulation with FMLP

PMNs (final concentration 5×10^6 cells/ml) labelled with [^{3}H]lyso-PAF (**A**–**C**) or [^{3}H]AA (**D**) were pretreated with 5 μg/ml cyto B for 5 min before priming with TNFα (or HSA) for 30 min at 37 °C. Immediately before stimulation, 100% ethanol (to yield the indicated final concentrations) was added to cells,. PMNs were stimulated with FMLP (or buffer) and incubated for a further 5 min before extraction and processing of lipids as described in the Materials and methods section. Symbols: ○, HSA control; ●, 1 μM FMLP; ▽, 100 i.u./ml TNFα; ▼. 100 i.u./ml TNFα + 1 μM FMLP. (**A**) PA production; means ± S.E.M. for four experiments, duplicate determinations per experiment. (**B**) Pet formation; means ± S.E.M. for four experiments, duplicate determinations per experiment. (**C**) DG (alkylacyl) formation; means ± S.E.M. for four experiments, duplicate determinations per experiment. (**D**) DG (diacyl) formation; means ± S.E.M. for three experiments, duplicate determinations per experiment.

diacylglycerol was assessed. In unprimed PMNs, stimulation with FMLP caused the formation of very high levels of alkylacylglycerol (nearly a 10-fold increase) but only a modest increase in diacylglycerol formation. Priming with TNFα did not effect basal levels of either form of DG but enhanced the formation of both in cells subsequently stimulated with FMLP. These results demonstrate that both PA and DG formation were present when AA release and PAF formation occurred and that the enhanced PLA_2 activity seen during primed stimulation was paralleled by an increased formation of both PA and DGs.

Role of PLD in PA and DG formation in PMNs primed with TNFα and stimulated with FMLP

PA formation can occur directly through the activation of PLD or indirectly by the sequential actions of phospholipase C (PLC) and DG kinase. Primary alcohols compete with water in a transphosphatidylation reaction during PLD activation to produce Pet rather than PA [23] and can be used to differentiate between PLD- and PLC-mediated PA formation. To characterize which path was involved in the induction of PA formation in cells primed with TNFα before stimulation with FMLP, the effects of increasing concentrations of ethanol on PA (Figure 1A) and Pet (Figure 1B) formation were determined. In the absence of stimulation (PMNs treated with either HSA or TNFα), increasing ethanol concentrations from 0% to 2.5% (v/v) (a final concentration of 2.5% ethanol did not alter cell viability as assessed by Trypan Blue exclusion) had little effect on PA or Pet levels. In cells stimulated with FMLP, with or without TNFα priming, a dose-dependent inhibition of PA formation occurred in response to increasing concentrations of ethanol; a concomitant increase in Pet formation was observed, with maximum levels of Pet attained between 0.5 and 1% ethanol. Above 1% ethanol, Pet levels declined, suggesting enzyme inactivation. Thus PA formed in this system seemed to result primarily from the activation of PLD.

DG production in PMNs might occur through the direct hydrolysis of phospholipids by PLC or by the PLD-mediated formation of PA followed by phosphatidate phosphohydrolase action to form DGs. To assess which path was responsible for the DG production observed during primed stimulation, the effects of ethanol on the formation of both alkylacylglycerol (Figure 1C) and diacylglycerol (Figure 1D) was also assessed. Increasing ethanol concentrations again had little effect on DG levels in cells in the absence of stimulation. However, in cells treated with FMLP, with or without initial priming with TNFα, the addition of ethanol to the system led to a dose-dependent decrease in both alkylacylglycerol and diacylglycerol formation. This result demonstrated that PLD activity followed by phosphatidate phosphohydrolase-mediated cleavage of PA was responsible for the majority of DGs produced in PMNs stimulated with FMLP, with or without TNFα priming.

Effects of ethanol on PLA_2 activity in PMNs primed with TNFα and stimulated with FMLP

If PA and DGs participate in the signal transduction system that initiates PLA_2 activity in PMNs, then blocking their formation

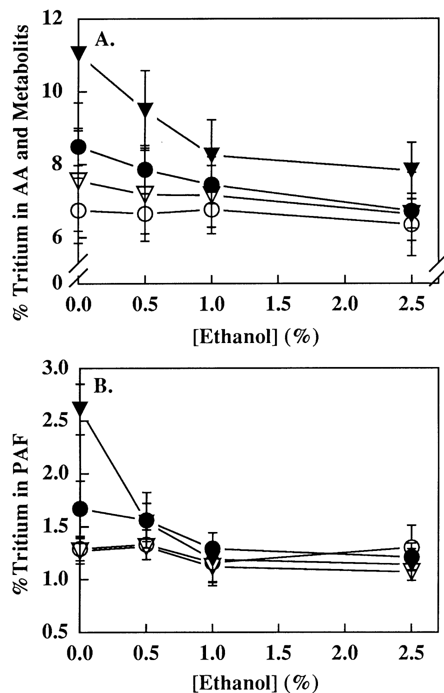

Figure 2 Effects of ethanol on AA release and metabolism and PAF production in PMNs primed with TNFα before stimulation with FMLP

PMNs (final concentration 5×10^6 cells/ml) labelled with [^{3}H]AA (**A**) or [^{3}H]lyso-PAF (**B**) were pretreated with 5 μg/ml cyto B for 5 min before priming with TNFα (or HSA) for 30 min at 37 °C. Immediately before stimulation, 100% ethanol (to yield the indicated final concentrations) was added to cells. PMNs were stimulated with FMLP or buffer and incubated for an additional 5 min before extraction and processing of lipids as described in the Materials and methods section. Symbols: ○, HSA control; ●, 1 μM FMLP; ▽, 100 i.u./ml TNFα; ▼. 100 i.u./ml TNFα + 1 μM FMLP. (**A**) AA release and metabolism; means ± S.E.M. for three experiments, duplicate determinations per experiment. (**B**) PAF formation; means ± S.E.M. for four or five experiments, duplicate determinations per experiment.

should affect AA release and metabolism and PAF accumulation. In Figure 2 the effects of ethanol on PLA_2 activation in PMNs primed with TNFα before stimulation with FMLP were determined. Increasing the ethanol concentration from 0% to 2.5% had little effect on AA (Figure 2A) or PAF (Figure 2B) levels in cells treated with either HSA or TNFα in the absence of stimulation. In both unprimed and primed PMNs stimulated with FMLP, a dose-dependent inhibition in AA release and metabolism and PAF formation was observed: AA release was diminished to near-basal levels by 1% ethanol, and PAF production completely abolished at 0.5% ethanol. These results further support the hypothesis that PA and DGs formed via PLD activity function as second messengers for the induction of PLA_2 activity but could also reflect a non-specific effect of ethanol on PMN metabolism.

AA release and PAF formation in permeabilized human PMNs

To continue the investigation of the interrelationship between PLD activity and induction of PLA_2 activation, PMNs permeabilized with *S. aureus* α-toxin were used. Previous work in this system demonstrated that AA was specifically and maximally released when a combination of 500 nM Ca^{2+}, a guanine nucleotide and FMLP were added to permeabilized PMNs, that radiolabelled AA release paralleled AA mass accumulation, that ATP was required for optimum enzyme activation, that inhibition of phosphorylation with staurosporine decreased the accumulation of free AA, and that the reducing agent dithiothreitol did not abolish AA release [16]. These results were most consistent with $cPLA_2$ being responsible for AA release in permeabilized

Table 2 AA release and metabolism and PAF production in permeabilized human PMNs

PMNs (5×10^6 cells/ml) prelabelled with [^{3}H]AA for measurement of AA release and metabolism, unlabelled for AA mass determination, or labelled with [^{3}H]lyso-PAF for assessment of PAF production were permeabilized with α-toxin in cytoplasmic buffer supplemented with 100 μM acetyl-CoA for 15 min at 37 °C. Guanine nucleotides, Ca^{2+} and FMLP (as indicated) were then added to the permeabilized cells. Reactions were halted after 5 min and lipids were extracted and analysed as described in the Materials and methods section.

Treatment	[^{3}H]AA release (%)*	AA mass (ng/2×10^7 PMNs)†	PAF production (%)‡
Control	4.8 ± 0.4	15.8 ± 1.8	1.0 ± 0.1
1 μM FMLP	4.8 ± 0.4	18.0 ± 3.4	1.0 ± 0.2
500 nM Ca^{2+}	5.2 ± 0.3	13.1 ± 1.9	1.2 ± 0.1
10 μM GTP[S]	6.1 ± 0.4	19.9 ± 5.1	1.5 ± 0.1
10 μM GTP[S] + 500 nM Ca^{2+}	8.9 ± 0.4	53.0 ± 10.6	2.3 ± 0.2
10 μM GTP[S] + 500 nM Ca^{2+} + 1 μM FMLP	10.9 ± 0.7	83.6 ± 17.4	3.3 ± 0.3
100 μM GTP	4.5 ± 0.4	22.1 ± 1.4	1.0 ± 0.2
100 μM GTP + 500 nM Ca^{2+}	5.7 ± 0.6	34.0 ± 6.6	1.4 ± 0.2
100 μM GTP + 500 nM Ca^{2+} + 1 μM FMLP	8.7 ± 0.6	58.5 ± 3.7	2.8 ± 0.2

* Arachidonic acid release from PMNs prelabelled with [^{3}H]AA is shown as the percentage of ^{3}H found as free AA and metabolites; means ± S.E.M. for six to nine experiments, duplicate determinations per experiment.

† Arachidonic acid mass release was determined in unlabelled PMNs; means ± S.E.M. for four experiments, duplicate determinations per experiment.

‡ Means ± S.E.M. for seven to eleven experiments, duplicate determinations per experiment.

Table 3 PA and DG formation in permeabilized human PMNs

PMNs (5×10^6 cells/ml) prelabelled with [^{3}H]lyso-PAF for measurement of PA and alkylacylglycerol formation or labelled with [^{3}H]AA for assessment of diacylglycerol production were permeabilized with α-toxin in cytoplasmic buffer supplemented with 100 μM acetyl-CoA for 15 min at 37 °C. Guanine nucleotides, Ca^{2+} and FMLP (as indicated) were then added to the permeabilized cells. Reactions were halted after 5 min and lipids were extracted and analysed as described in the Materials and methods section.

Treatment	PA formation (%)*	DG (alkylacyl) production (%)†	DG (diacyl) production (%)‡
Control	0.9 ± 0.1	1.1 ± 0.2	1.6 ± 0.1
1 μM FMLP	0.9 ± 0.1	1.1 ± 0.2	1.5 ± 0.1
500 nM Ca^{2+}	1.1 ± 0.2	1.0 ± 0.1	1.7 ± 0.1
10 μM GTP[S]	1.6 ± 0.2	1.3 ± 0.3	1.8 ± 0.1
10 μM GTP[S] + 500 nM Ca^{2+}	2.2 ± 0.1	1.9 ± 0.4	2.4 ± 0.2
10 μM GTP[S] + 500 nM Ca^{2+} + 1 μM FMLP	2.1 ± 0.1	1.8 ± 0.3	2.5 ± 0.2
100 μM GTP	0.9 ± 0.1	1.1 ± 0.2	1.6 ± 0.1
100 μM GTP + 500 nM Ca^{2+}	1.6 ± 0.2	1.2 ± 0.2	1.6 ± 0.1
100 μM GTP + 500 nM Ca^{2+} + 1 μM FMLP	2.3 ± 0.2	1.5 ± 0.2	2.1 ± 0.1

* Phosphatidic acid formation in PMNs prelabelled with [^{3}H]lyso-PAF; means ± S.E.M. for four to eight experiments, duplicate determinations per experiment.

† Alkylacylglycerol formation in PMNs prelabelled with [^{3}H]lyso-PAF; means ± S.E.M. for four to eight experiments, duplicate determinations per experiment.

‡ Diacylglycerol formation in PMNs prelabelled with [^{3}H]AA; means ± S.E.M. for six to nine experiments, duplicate determinations per experiment.

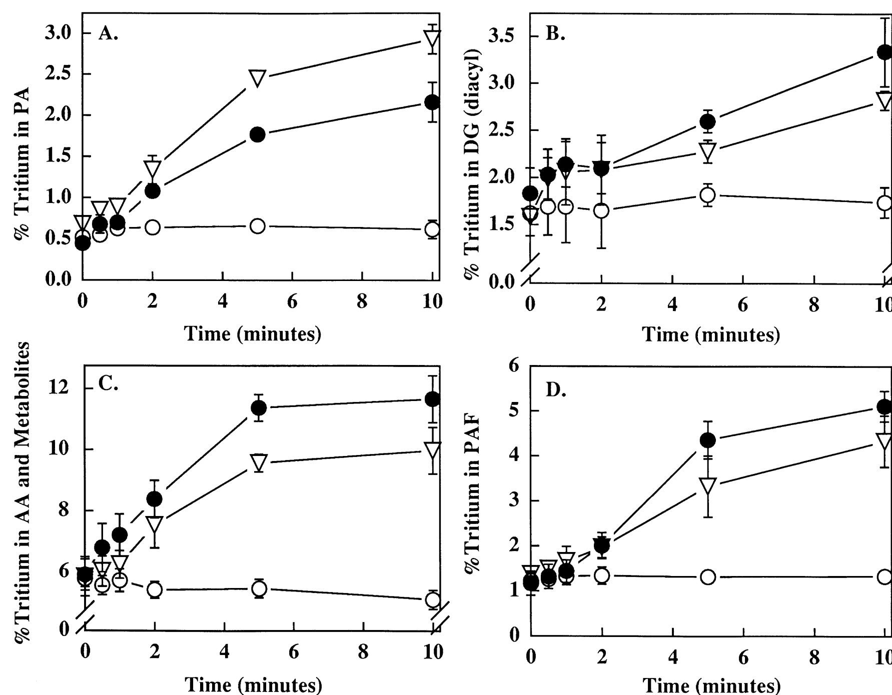

Figure 3 Time course of PLD and PLA_2 activation in permeabilized PMNs

PMNs (final concentration 5×10^6 cells/ml) labelled with [^{3}H]lyso-PAF (**A**, **D**) or [^{3}H]AA (**B**, **C**) were permeabilized with α-toxin in cytoplasmic buffer for 15 min at 37 °C. PMNs were then stimulated and incubated for the indicated times before extraction and processing of lipids as described in the Materials and methods section. Symbols: ○, control; ●, 10 μM GTP[S] + 500 nM Ca^{2+} + 1 μM FMLP; ▽, 100 μM GTP + 500 nM Ca^{2+} + 1 μM FMLP. (**A**) PA production; (**B**) DG (diacylglycerol) formation; (**C**) AA release and metabolism; (**D**) PAF formation. In all panels the results are means ± S.E.M. for three experiments, duplicate determinations per experiment.

PMNs, and further work demonstrated that $sPLA_2$ was inactive in the cytosolic buffer used for studies of permeabilized cells [16]. Studies also investigated whether LTB_4 and PAF formation could occur in the permeabilized PMN system and demonstrated that the production of both lipid mediators occurred in parallel with the activation of $cPLA_2$ [17]. Results from those investigations are summarized in Table 2.

PA and DG production in permeabilized human PMNs

Studies were next focused on determining the effects of Ca^{2+}, guanine nucleotides and FMLP on PA and DG formation in the permeabilized PMN model (Table 3). Previous work demonstrated that mass release of PA in permeabilized PMNs was closely correlated with the formation of radiolabelled PA [11], so the formation of tritiated PA and DGs was monitored in this study. As with AA release and PAF formation, stimulation of permeabilized PMNs with FMLP or the addition of 500 nM Ca^{2+} (to mimic intracellular Ca^{2+} concentrations attained after PMN stimulation) did not induce any changes in PA or DGs (either alkylacylglycerol or diacylglycerol) from control values. The addition of GTP[S] to induce the activation of guanine nucleotide-dependent protein (G-protein) did initiate PA formation but did not significantly increase DG production. A combination of GTP[S] and Ca^{2+} induced the further formation of PA and initiated DG production; stimulation with FMLP had no further effects.

GTP[S] is resistant to hydrolysis and holds G-proteins in the 'on' conformation; it is frequently used in signal transduction studies to enhance enzymic activity. However, under physiological conditions, G-protein activation is transient and GTP is hydrolysed to return G-proteins to the inactive, GDP-bound form. To ensure that PLD activation observed in permeabilized PMNs was not an artifact caused by the use of GTP[S], the natural guanine nucleotide was also used. GTP did not directly initiate PA or DG accumulation in permeabilized PMNs but the addition of Ca^{2+} with GTP did increase PA levels, and stimulation with FMLP further enhanced PA accumulation and initiated the formation of both alkylacylglycerol and diacylglycerol. Thus, although the correlation of PA and DG formation (Table 3) with AA release and PAF production (Table 2) was not perfect, PA formation was present in all cases where $cPLA_2$ activity occurred, and conditions that induced maximum AA release and PAF production also initiated DG formation.

Time course of AA release, PAF formation and PA and DG accumulation in permeabilized human PMNs

For PA and DGs to function as second messengers in the induction of $cPLA_2$ activity in PMNs requires that their production precedes or coincides with the release of AA and the formation of PAF. In control cells, no changes were evident in PA (Figure 3A), DG (Figure 3B), AA (Figure 3C) or PAF (Figure 3D) levels at any time after stimulation. When cells were treated with 500 nM Ca^{2+}, 1 μM FMLP and either 10 μM GTP[S] or 100 μM GTP, PA production and DG formation (diacylglycerol and alkylacylglycerol formation followed a similar time course; results not shown) were slightly increased by 0.5–1 min after cell stimulation. Under the same conditions, AA release and metabolism were also slightly increased by 1 min, whereas PAF formation was delayed but significantly different from control by 2 min. At 5 min after stimulation, PA and PAF formation, and

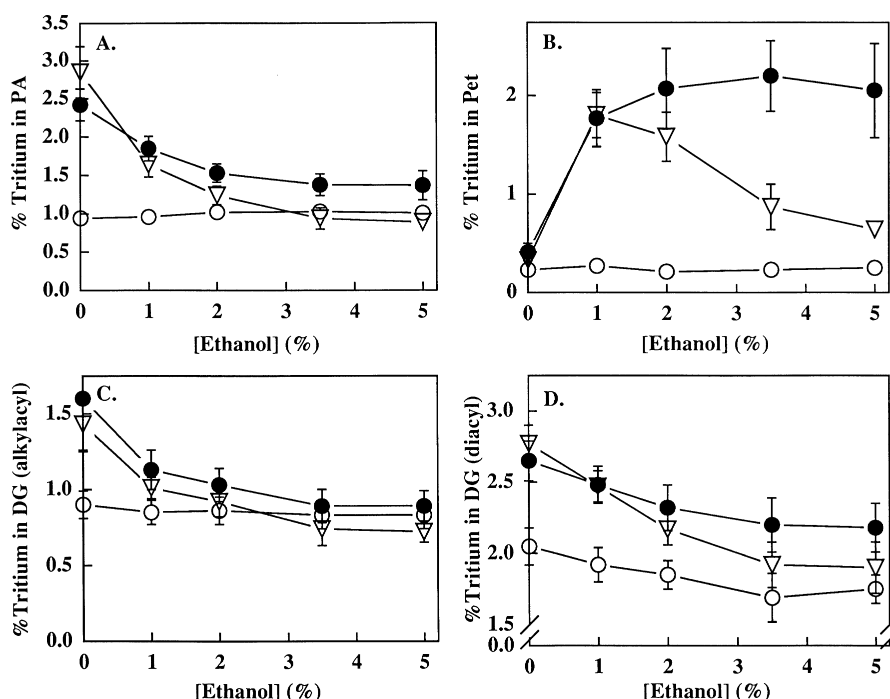

Figure 4 Effects of ethanol on PA and DG production in permeabilized PMNs

PMNs (final concentration 5×10^6 cells/ml) labelled with [^{3}H]lyso-PAF (**A–C**) or [^{3}H]AA (**D**) were permeabilized with α-toxin in cytoplasmic buffer for 15 min at 37 °C. Immediately before stimulation, 100% ethanol (to yield the indicated final concentrations) was added to cells. PMNs were stimulated as indicated and incubated for an additional 5 min before extraction and processing of lipids as described in the Materials and methods section. Symbols: ○, control; ●, 10 μM GTP[S] + 500 nM Ca^{2+} + 1 μM FMLP; ▽, 100 μM GTP + 500 nM Ca^{2+} + 1 μM FMLP. (**A**) PA production; means ± S.E.M. for four experiments, duplicate determinations per experiment. (**B**) Pet formation; means ± S.E.M. for four experiments, duplicate determinations per experiment. (**C**) DG (alkylacyl) formation; means ± S.E.M. for four experiments, duplicate determinations per experiment. (**D**) DG (diacyl) formation; means ± S.E.M. for three to five experiments, duplicate determinations per experiment.

AA release and metabolism, had reached near-maximal levels, whereas DG production continued to increase throughout the 10 min time course. Thus PA and DG formation either coincided with or preceded significant $cPLA_2$ activation.

Effects of ethanol on PA and DG formation in permeabilized human PMNs

To determine whether PA and DG formation in permeabilized PMNs was the result of PLD activation, the effects of ethanol on PA and DG formation were assessed. In the absence of stimulation, increasing ethanol levels from 0% to 5% had no effect on basal levels of PA (Figure 4A), Pet (Figure 4B) or alkylacylglycerol (Figure 4C), but did slightly decrease diacylglycerol levels (Figure 4D). Increasing ethanol concentrations from 0% to 5% induced a dose-dependent decrease in both PA and DG formation in cells stimulated with 10 μM GTP[S], 500 nM Ca^{2+} and 1 μM FMLP (60% decrease in PA, 80% inhibition of alkylacylglycerol formation and 55% lowering of diacylglycerol concentration at 2% ethanol). The decrease in PA and DG formation in cells stimulated with GTP, Ca^{2+} and FMLP was even more pronounced (85%, 95% and 85% decreases in PA, alkylacylglycerol and diacylglycerol respectively with 2% ethanol). In concert with decreased PA and DG formation, a concomitant increase in Pet production occurred between 0% and 2% ethanol. These results demonstrate that the majority of PA and DGs produced in the permeabilized PMNs under conditions optimal for $cPLA_2$ activation were the result of PLD activation.

Effects of ethanol on AA release and PAF formation in permeabilized human PMNs

To determine whether blocking PLD-mediated PA and DG formation would effect AA release and metabolism and PAF formation, permeabilized PMNs were treated with increasing concentrations of ethanol immediately before the addition of Ca^{2+}, guanine nucleotides and FMLP. Ethanol had no effect on baseline levels of free AA and metabolites (Figure 5A) or PAF (Figure 5B) in unstimulated PMNs. With increasing concentrations of ethanol, a dose-dependent decrease in AA release and metabolism was observed in PMNs stimulated with a guanine nucleotide, Ca^{2+} and FMLP, with 2% ethanol inducing 45% inhibition in GTP[S]-treated PMNs and a 72% decrease in GTP-treated cells. Similar effects on PAF formation were observed: 2% ethanol inhibited PAF formation by 65% in GTP[S]-treated cells and by 68% after GTP stimulation. These results continued to support the hypothesis that $cPLA_2$ activation in PMNs could be influenced by products of the PLD pathway.

PA and DG induction of $cPLA_2$ activity in permeabilized human PMNs

To explore more fully the PLD mediation of $cPLA_2$ activation, PA and DGs were added to permeabilized PMNs where endogenous PA and DG formation were decreased by ethanol pretreatment, and the effects on AA release and metabolism and PAF production were monitored. Levels of PA and DGs (0 to

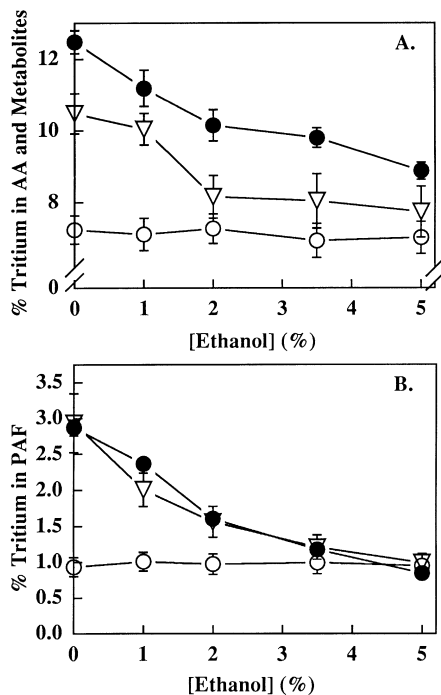

Figure 5 Effects of ethanol on AA release and metabolism and PAF production in permeabilized PMNs

PMNs (final concentration 5×10^6 cells/ml) labelled with [^{3}H]AA (**A**) or [^{3}H]lyso-PAF (**B**) were permeabilized with α-toxin in cytoplasmic buffer for 15 min at 37 °C. Immediately before stimulation, 100% ethanol (to yield the indicated final concentrations) was added to cells. PMNs were stimulated as indicated and incubated for an additional 5 min before extraction and processing of lipids as described in the Materials and methods section. Symbols: ○, control; ●, 10 μM GTP[S] + 500 nM Ca^{2+} + 1 μM FMLP; ▽, 100 μM GTP + 500 nM Ca^{2+} + 1 μM FMLP. (**A**) AA release and metabolism; means ± S.E.M. for four experiments, duplicate determinations per experiment. (**B**) PAF formation; means ± S.E.M. for four experiments, duplicate determinations per experiment.

10 μg per 5×10^6 PMNs) used for these experiments were within the physiologically relevant range on the basis of stimulated PA and DG mass production in intact PMNs [9] and PA mass measurements in permeabilized PMNs, where basal values of PA in unstimulated cells were 0.53 ± 0.1 μg per 10^7 cells and increased to 1.45 ± 0.2 μg per 10^7 PMNs after stimulation (results not shown).

Adding increasing concentrations of PA (Figure 6, filled symbols) to permeabilized PMNs had no significant effect on levels of free AA and metabolites (Figure 6A) or PAF (Figure 6B) in control cells in which endogenous PA and DG formation was blocked with 2 % ethanol when compared with cells without ethanol (open symbols). In PMNs pretreated with 2 % ethanol and then stimulated, there was a dose-dependent increase in AA release and metabolism in response to PA addition, with maximum levels occurring at 1–2 μg added PA. PA at 2 μg restored 60 % of the AA release and metabolism induced with GTP[S] [calculated by expressing the difference between the percentage of ^{3}H in cells with 2 μg of lipid added and the percentage of ^{3}H in ethanol-treated cells (zero lipid) as a percentage of the difference between cells stimulated in the absence of ethanol and cells treated with the same agonists in the presence of ethanol] or 67 % of AA accumulation initiated with GTP in concert with Ca^{2+} and FMLP when compared with levels generated in the absence of ethanol (open symbols). PAF production was similarly affected, with PA addition (2 μg/ml) inducing 55 % of maximum production in GTP[S]-stimulated PMNs and 60 % restoration of PAF formation in cells treated with GTP.

In resting permeabilized PMNs, DGs had no significant effect on AA release and metabolism until a concentration of 10 μg of DGs was added (Figure 6C), and had no effect on PAF formation (Figure 6D). In stimulated cells, the lowest concentration of DGs (0.5 μg/ml) increased AA release, with no further enhancement occurring with higher DG concentrations. DG addition also induced an increase in PAF formation in stimulated cells: maximum PAF generation was induced with 2 μg of DGs. DGs were not as effective as PA in reconstituting $cPLA_2$ activity, with 36 % restoration of AA release and 17 % of PAF production in GTP[S]-treated PMNs (at 2 μg of DGs) and 47 % of AA and 49 % of PAF in GTP-stimulated cells. Thus both PA and DGs were able to partly reconstitute AA release and PAF formation when endogenous PA and DG formation was blocked in permeabilized PMNs, lending further support to the hypothesis that products of PLD activation participate in the induction of $cPLA_2$ activity.

Because neither PA nor DGs were able to restore fully the release and metabolism of AA or the production of PAF individually, the effects of adding both lipids simultaneously were monitored. The results indicate that whereas adding the combination of 2 μg/ml PA and 2 μg/ml DGs had no effects on basal AA (Figure 7A) or PAF (Figure 7B) levels in control cells, they had additive effects on $cPLA_2$ activity, with the combination fully reconstituting AA release and PAF production in cells stimulated with either GTP[S] or GTP in combination with Ca^{2+} and FMLP. These results suggest that the PLD-mediated production of both PA and DGs is necessary for full activation of $cPLA_2$ to occur.

DISCUSSION

Significant progress has been made in understanding the steps involved in the activation of $cPLA_2$ in PMNs and other cell types. Translocation of $cPLA_2$ from the cytosol to cell membranes [24–26] and phosphorylation of $cPLA_2$ leading to enhanced enzymic activity [27–30] have been proposed as major mechanisms controlling enzyme activity, but many questions remain unanswered. For example, whereas only nanomolar concentrations of Ca^{2+} are necessary for the translocation of $cPLA_2$ from cytosol to membrane [24–26] and these concentrations can be achieved by many agonists that interact with membrane receptors on PMNs [31], direct stimulation of PMNs by agonists such as FMLP or complement component C5a lead to only limited AA release, or LTB_4 or PAF production [19–21,32,33]. Stimulation of PMNs with FMLP readily induces the phosphorylation of $cPLA_2$ (D. Bass, personal communication) but causes little AA release in intact PMNs. Activation of protein kinase C (PKC) has been implicated in the phosphorylation of $cPLA_2$ [28,34,35] but direct activation of this kinase by phorbol 12-myristate 13-acetate (PMA) or cell-permeant DGs initiates little AA release [4,19,21,36]. Further, although the activation of the mitogen-activated protein kinase (MAP kinase) cascade, of Raf, and initiation of Ras activity have all been linked to $cPLA_2$ activity, the mechanism(s) coupling cell stimulation to their activation have not been elucidated [37,38].

In this paper we advance the hypothesis that products of PLD activation participate in the signal transduction pathway linking cell stimulation to PLA_2 activation. Several previous reports have suggested that a link exists between PA production and PLA_2 activation. PLD-mediated formation of PA can be substantially reduced via a transphosphatidylation reaction in the

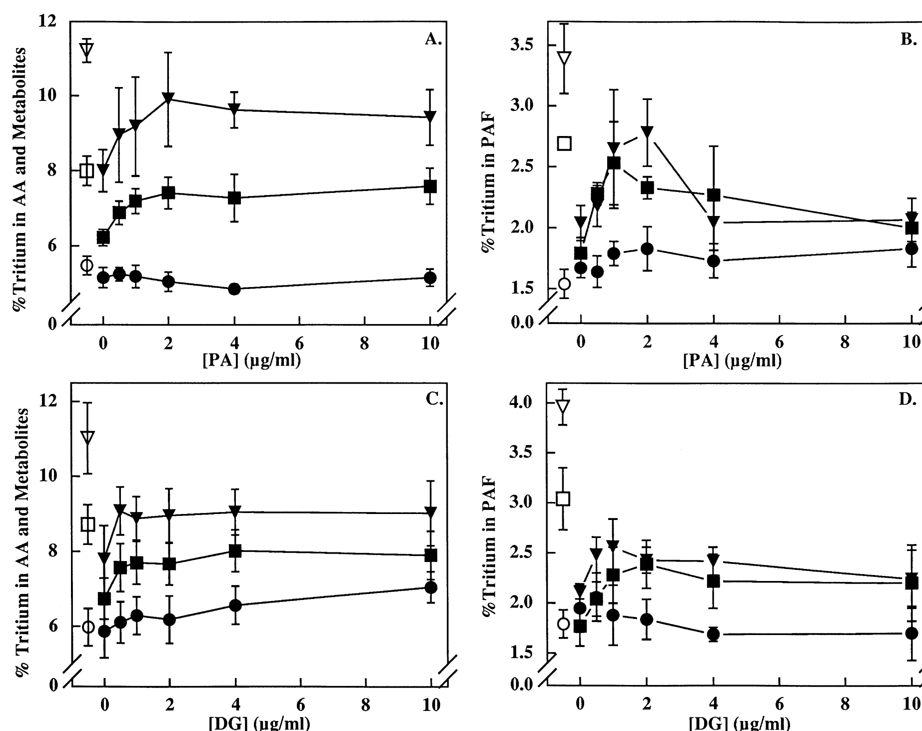

Figure 6 Effects of PA and DGs on AA release and metabolism and PAF production in permeabilized PMNs

Commercially prepared PA (derived from egg lecithin) or DGs (derived from pig liver) were resuspended in 100% ethanol to yield the indicated concentrations (final concentration 2% ethanol). PMNs (final concentration 5×10^6 cells/ml) labelled with [^{3}H]AA (**A**, **B**) or [^{3}H]lyso-PAF (**C**, **D**) were permeabilized with α-toxin in cytoplasmic buffer for 15 min at 37 °C. Immediately before stimulation, ethanol containing PA or DGs was added to cells. PMNs were stimulated as indicated and incubated for an additional 5 min before extraction and processing of lipids as described in the Materials and methods section. Open symbols, controls without ethanol (with endogenous PA and DG formation occurring); filled symbols, cells treated with 2% ethanol (endogenous PA and DG production blocked); ○,●, control; ▽,▼, 10 μM GTP[S] + 500 nM Ca^{2+} + 1 μM FMLP; □,■, 100 μM GTP + 500 nM Ca^{2+} + 1 μM FMLP. (**A**) AA release and metabolism in response to PA addition; means ± S.E.M. for three to five experiments, duplicate determinations per experiment. (**B**) AA release and metabolism in response to DG addition; means ± S.E.M. for four experiments, duplicate determinations per experiment. (**C**) PAF formation in response to PA addition; means ± S.E.M. for three to five experiments, duplicate determinations per experiment. (**D**) PAF formation in response to DG addition; means ± S.E.M. for four experiments, duplicate determinations per experiment.

presence of a primary alcohol [23], and ethanol has been shown to decrease platelet AA release and metabolism in response to stimulation (reviewed in [39]). Further work demonstrated that ethanol treatment of platelets decreased the release of $sPLA_2$ and other granule constituents from cells after stimulation with thrombin, but had no direct effect on $sPLA_2$ enzymic activity [40]. In contrast, ethanol treatment significantly blocked $cPLA_2$ activity when the platelet enzyme was isolated and activity was measured against phospholipid vesicles. In peritoneal macrophage, ethanol treatment decreased AA release and metabolism in response to zymosan stimulation but not Ca^{2+} ionophore-induced AA release [41]. These results suggested that ethanol was affecting a step in the signal transduction path leading to PLA_2 activation and were supported by findings that ethanol abolished AA release in response to the direct activation of G-proteins by fluoroaluminate. Although activation of PLD was not addressed in those studies, the results could be consistent with effects induced by ethanol blocking the PLD-mediated formation of PA. PA facilitates the degranulation of several cell types including platelets [42]; blocking PA accumulation via a transphosphatidylation reaction could reduce platelet degranulation [42] and thus inhibit $sPLA_2$ release. Physiological PLD activation is dependent on G-protein activity (reviewed in [6]); the effects of ethanol on G-protein-induced AA release in macrophages could result from an ethanol-mediated decrease in PA formation.

Further support for a role for endogenous PA production in the induction of PLA_2 activity was obtained in a study where membranes isolated from PMA-pretreated platelets were stimulated with GTP[S] in the presence of 50 μM Ca^{2+} [43]. PA production occurred in parallel with AA release in response to varying concentrations of PMA and guanine nucleotides; PA formation preceded AA release; and ethanol inhibited PA generation and AA release in parallel. In another report, addition of PA to phospholipid vesicles enhanced the activity of an AA-specific PLA_2 isolated from macrophages [44].

Suggestions that DGs function in inducing PLA_2 activity have also come from several sources. Cell-permeant DGs do not directly activate PLA_2 but enhance enzyme activity if cells are subsequently stimulated [19,21,36]. Diacylglycerols are the physiological activators of PKC (reviewed in [45]), and although direct activation of PKC with PMA initiates little PLA_2 activity in PMNs [4,46] it does prime cells for enhanced enzymic activity on further stimulation [35,36,46]. Alternatively, DGs might influence PLA_2 in a PKC-independent manner. Both the diacylglycerol, 1-oleoyl-2-acetylglycerol, and its alkylacylglycerol analogue, 1-*O*-octadecenyl-2-acetylglycerol, primed PLA_2 activity in PMNs for subsequent activation by FMLP or Ca^{2+} ionophores [19,21]; however, whereas OAG directly activates PKC (reviewed in [47]), most reports indicate that alkylacylglycerols do not activate PKC activation and may inhibit it [48,49].

In this study we first demonstrated that in intact PMNs primed

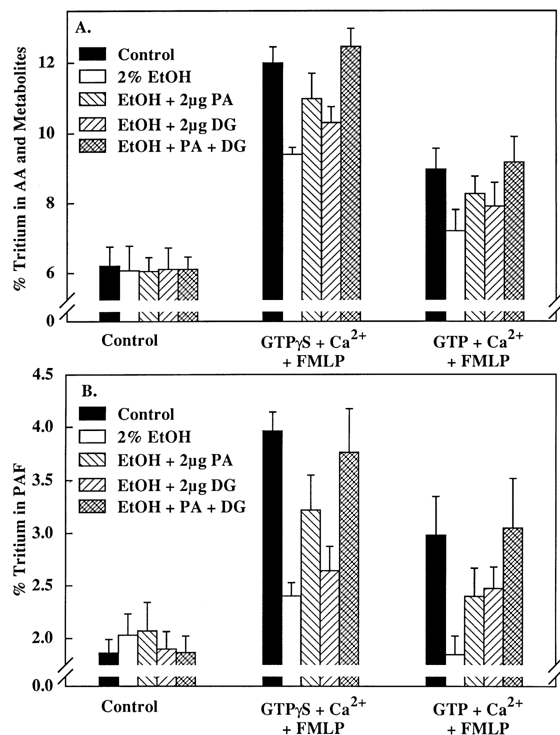

Figure 7 Combined effects of PA and DGs on AA release and metabolism and PAF production in permeabilized PMNs

Commercially prepared PA and DGs were resuspended in 100% ethanol (EtOH) to yield final concentrations of 2 μg/ml (final ethanol concentration 2%). PMNs (final concentration 5×10^6 cells/ml) labelled with [^{3}H]AA (**A**) or [^{3}H]lyso-PAF (**B**) were permeabilized with α-toxin in cytoplasmic buffer for 15 min at 37 °C. Immediately before stimulation, ethanol containing PA or DGs, or both, was added to permeabilized PMNs. Cells were then stimulated with 500 nM Ca^{2+}, 1 μM FMLP and 10 μM GTP[S] (GTPγS) or 100 μM GTP (as indicated) for 5 min before processing of lipids from duplicate samples as described in the Materials and methods section. (**A**). AA release and metabolism; means $\pm$ S.E.M. for three or four experiments, duplicate determinations per experiment. (**B**) PAF production; means $\pm$ S.E.M. for four or five experiments, duplicate determinations per experiment.

with TNFα before stimulation with FMLP, enhanced PA and DG formation was correlated with increased AA release and PAF production. Further work showed that blocking PA and DG formation during primed stimulation led to parallel decreases in the release and metabolism of AA and formation of PAF. Studies in permeabilized PMNs, a system where $cPLA_2$ is specifically activated in the absence of $sPLA_2$ activity [16], demonstrated that PA formation was always present when $cPLA_2$ activity occurred and that both PA and DG production were detected under conditions that maximized AA release and PAF formation. PA and DG formation preceded or coincided with $cPLA_2$ activation; blocking endogenous PA and DG formation in the permeabilized PMN system led to a concomitant decrease in AA release and PAF production. In PMNs where endogenous PA and DG formation were prevented by ethanol pretreatment, neither PA nor DGs caused direct activation of $cPLA_2$ in control (unstimulated) cells, but adding either PA or DGs (at physiologically relevant concentrations) back to permeabilized PMNs stimulated with a guanine nucleotide, Ca^{2+} and FMLP resulted in a partial, dose-dependent, restoration of both AA release and metabolism and PAF formation. In addition, adding a combination of PA and DGs fully reconstituted $cPLA_2$ activity in stimulated cells. These results strongly support the hypothesis that the products of PLD activation function in the signal pathway leading to the activation of $cPLA_2$.

PLA_2 activation could be influenced by PA at several points in the signal transduction path. PKC has been implicated in the phosphorylation of $cPLA_2$ [28,34,35] and in the activation of several components of the MAP kinase cascade [30,50] and of Raf [51]; a number of studies have shown that PA can activate PKC [52–55]. PA also has been shown to stimulate MAP kinase activity directly [56] and to participate in the translocation and activation of Raf [57]. Thus PA might influence $cPLA_2$ activation by inducing the phosphorylation of one or more of the proteins involved in the signal transduction path. Alternatively, PA could affect $cPLA_2$ activity by inducing the activation of the low-molecular-mass GTPase, Ras. In studies on the interaction of GTPase-activating proteins and GTPase-inhibiting proteins with Ras in cell-free systems, it was shown that PA inhibited GTPase-activating protein activity and increased GTPase-inhibiting protein activity, leading to prolongation of Ras activation [58,59]. Another point where PA might affect $cPLA_2$ is at cellular membranes. Adding PA or other anionic phospholipids to substrate vesicles led to increased AA release upon addition of an AA-specific PLA_2 and decreased the Ca^{2+} requirement for PLA_2 activation, suggesting that the physical structure of the membrane affects PLA_2 activity [44]. Increased access of PLA_2 to substrates was also proposed as the mechanism to account for the initiation by PA of AA release in platelets [60,61]. Thus multiple roles for PA in the induction of $cPLA_2$ can be envisaged.

DGs might influence $cPLA_2$ activity by inducing PKC activation; PKC-mediated phosphorylation has been implicated in the activation of several proteins in the path leading to $cPLA_2$ activation [28,30,34,35,50,51]. Alternatively, a potential role for PKC-independent modulation of PLA_2 by DGs might involve the regulation of GTP binding to low-molecular-mass GTPases. In a study of NADPH oxidase activation in a cell-free system, both endogenously produced DGs and DGs added to membrane preparations were shown to enhance the translocation of oxidase components to cellular membranes in a GTP[S]-dependent manner [62] and it was demonstrated that DGs were influencing GTP[S] uptake by a GTP-binding protein. DGs might also influence PLA_2 activity by affecting the structure of cellular membranes, allowing an increased access of PLA_2 to substrates [63]. Whether one of these mechanisms or an alternative path could account for DG induction of $cPLA_2$ activity in permeabilized PMNs is currently under investigation.

Although PA and DG formation were required for full $cPLA_2$ activation in the permeabilized PMN system, they were not sufficient to induce AA release or PAF formation directly in the absence of cell stimulation (Figure 7). $cPLA_2$ activity is dependent on translocation of the enzyme from the cytosol to cellular membranes, and Ca^{2+} is required for enzyme movement [24–26]. The inability of PA and DGs to initiate $cPLA_2$ activity directly might be due to a lack of translocation of the enzyme to its substrate in cell membranes. Conversely, the near-total inhibition of PA and DG production did not completely eliminate PLA_2 activity in either intact or permeabilized stimulated PMNs (Figures 2 and 5). The incomplete inhibition of AA release and PAF production by ethanol in permeabilized PMNs might result from effects of Ca^{2+} on $cPLA_2$ translocation. In stimulated permeabilized PMNs, Ca^{2+} is held constant at 500 nM, a level sufficient to induce the translocation of $cPLA_2$. Phosphorylation of $cPLA_2$ is necessary for full enzymic activity [27–30] but non-phosphorylated $cPLA_2$ can still cleave phospholipids [64]. If PA and DGs affect $cPLA_2$ activity by inducing phosphorylation of the enzyme, then translocation of non-phosphorylated enzyme in ethanol-treated PMNs could result in a decrease in $cPLA_2$

activity but not a total inhibition. PA or DG modulation of $cPLA_2$ activity by regulating phosphorylation also might explain the incomplete blockage of AA release by ethanol after primed stimulation of intact PMNs. Priming PMNs induces phosphorylation and increases the activity of $cPLA_2$ (measured in cell-free assays) before stimulation [27]. This suggests that TNFα priming could induce the phosphorylation of $cPLA_2$ to partly by-pass a PA/DG-dependent step in the signal transduction path and thus protect PMNs from complete inhibition of PLA_2 activity in the presence of ethanol. Alternatively, the less effective inhibition of AA release by ethanol in cells treated with GTP[S] than in those treated with GTP (Figure 5A) suggests that PA and/or DGs may be affecting a G-protein or low-molecular-mass GTPase-dependent portion of the signal transduction path. The poorly hydrolysed guanine nucleotide could maintain GTP-dependent proteins in the 'on' position, rendering them less sensitive to regulation by PA and/or DGs. Finally, redundant mechanisms for activation of $cPLA_2$ might exist in PMNs; the residual $cPLA_2$ activity found after PLD inhibition might result from the activation of a PA/DG-insensitive pathway.

In this study we have demonstrated: that a correlation exists between the formation of PA and DGs and the initiation of AA release and the production of PAF in both intact and permeabilized PMNs; that either exogenously added PA or DGs can partly reconstitute $cPLA_2$ activity when endogenous formation of the two lipids is blocked; and that a combination of the two products of PLD activation can completely restore AA release and metabolism and PAF formation. The results from this study have firmly established a role for PLD-mediated PA and DG formation in the signal transduction pathway leading to the activation of $cPLA_2$ in PMNs and give further insight into the interactions occurring between pathways involved in phospholipid metabolism.

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