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EVIDENCE FOR DIFFERENT MECHANISMS INVOLVED IN THE FORMATION OF LYSO PLATELET-ACTIVATING FACTOR AND THE CALCIUM-DEPENDENT RELEASE OF ARACHIDONIC ACID FROM HUMAN NEUTROPHILS

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Abstract—Recent studies suggest that the first step in platelet-activating factor (PAF) biosynthesis, 1-alkyl-2-lyso-GPC (lyso PAF) formation, may be initiated by the selective transfer of arachidonate from 1-alkyl-2-arachidonoyl-GPC to an acceptor lyso phospholipid by a CoA-independent transacylase activity (CoA-IT). The present study was designed to determine whether the formation of 1-alkyl-2-lyso-GPC and the release of arachidonic acid can occur by different mechanisms. These experiments examined both the formation of 1-[^{3}H]alkyl-2-lyso-GPC from 1-[^{3}H]alkyl-2-acyl-GPC and the release of arachidonic acid from membrane phospholipids as determined by GC/MS in neutrophil homogenates under various conditions. The addition of unlabelled lyso phospholipids to neutrophil homogenates stimulated the time-dependent formation of 1-[^{3}H]alkyl-2-lyso-GPC from 1-[^{3}H]alkyl-2-acyl-GPC. Without exogenous lyso phospholipids, little 1-[^{3}H]alkyl-2-lyso-GPC was formed in this reaction. The activity which catalyzed the formation of 1-[^{3}H]alkyl-2-lyso-GPC had characteristics identical to CoA-IT as indicated by the fact that both reactions were: independent of Ca^{2+}, Mg^{2+}, CoA and CoA fatty acids, located in microsomal fractions, and stable in 10 mM dithiothreitol. In sharp contrast to the aforementioned reaction, addition of lyso phospholipids did not affect the quantity of arachidonic acid released from membrane phospholipids. Furthermore, there was a Ca^{2+}-independent release of arachidonic acid from membrane phospholipid that was increased 4 to 5-fold after the addition of 5 mM Ca^{2+}. Finally, Ca^{2+}-dependent arachidonic acid release was inhibited by putative phospholipase A_2 inhibitors, aristolochic acid and scalaradial, at concentrations where neither the production of 1-[^{3}H]alkyl-2-lyso-GPC nor Ca^{2+}-independent arachidonic acid release was altered. Together these data imply that there may be different mechanisms involved in the formation of 1-alkyl-2-lyso-GPC and arachidonic acid from membrane phospholipids.

Platelet-activating factor (PAF, 1-alkyl-2-acetyl-GPC)‖ has been shown to possess a wide variety of pro-inflammatory activities [Refs. 1–3 for reviews] and the production of PAF has been studied extensively. One conclusion that can be drawn from these biochemical studies is that PAF biosynthesis is linked in some way to arachidonic acid metabolism. There are several lines of evidence to support such a hypothesis: (1) inflammatory cells which produce large amounts of PAF contain large substrate pools of the potential common precursor, 1-alkyl-2-arachidonoyl-GPC [4–6]; (2) these ether-linked phospholipids containing arachidonate are the major sources of arachidonate lost from membrane phospholipids during cell activation [7–10]; (3) a major portion of the 1-alkyl-2-lyso-GPC (lyso PAF) formed during inflammatory cell activation is derived from 1-alkyl-2-arachidonoyl-GPC [4, 11, 12]; (4) PAF biosynthesis is inhibited in cells depleted of arachidonate and increased in cells where arachidonate pools have been artifically enhanced [13, 14]; amd (5) PAF and eicosanoid biosynthesis appear to have common regulatory points as evidenced by the fact that a number of inhibitors block the production of both classes of mediators [3, 15–17]. Based on these studies, we and others have proposed that the biochemical link between these two lipid classes revolves around common precursor phospholipid pools which give rise to both PAF and eicosanoids. If the aforementioned hypothesis is correct, it is likely that an arachidonate-selective enzyme is required for the hydrolysis of 1-alkyl-2-arachidonoyl-GPC to produce 1-alkyl-2-lyso-GPC and hence PAF.

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‖ Abbreviations: AA, arachidonic acid; 1-alkyl, 1-*O*-alkyl; 1-alkenyl, 1-*O*-alk-1′-enyl; BPB, *p*-bromophenacyl bromide; DTT, dithiothreitol, EGTA, [ethylenebis(oxyethylenenitrilo)]tetra acetic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine; lyso PAF, 1-alkyl-2-lyso-GPC, lyso platelet-activating factor; 2ME, 2-mercaptoethanol; PAF, platelet-activating factor, 1-alkyl-2-acetyl-GPC; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PLA_2, phospholipase A_2; and PS, phosphatidylserine.

One candidate enzyme for such a reaction is an arachidonic acid-selective phospholipase A_2 (PLA_2). Such an enzyme activity has been described in human neutrophils [18] as well as in other cells [19–23]. These PLA_2 enzymes demonstrate a preference for arachidonic acid over other fatty acids and can hydrolyze 1-alkyl-2-arachidonoyl-GPC into 1-alkyl-2-lyso-GPC and free arachidonic acid.

Another potential enzyme that would remove arachidonate from phospholipids is a CoA-independent transacylase (CoA-IT). This enzyme activity has been characterized in the human neutrophil [24], platelet [25, 26], U937 cells [27] and other inflammatory cells [20, 28]. An interesting aspect of this enzyme is its unique selectivity for transferring 20 carbon fatty acids, and arachidonate in particular, between phospholipids [24, 27, 28]. Recently, Sugiura and colleagues [29] reported that there is a transacylase activity in human neutrophils that could remove a molecule of arachidonate from 1-alkyl-2-arachidonoyl-GPC and transfer it to 1-alkenyl-2-lyso-GPE, producing 1-alkenyl-2-arachidonoyl-GPE and 1-alkyl-2-lyso-GPC. Moreover, studies in broken cell preparations have demonstrated that the production of 1-alkyl-2-lyso-GPC does not require Ca^{2+} and that 1-alkyl-2-lyso-GPC production and transacylase activity are affected in parallel by inhibitors and heat inactivation [30, 31]. Additionally, the production of 1-alkyl-2-lyso-GPC requires the presence of a suitable, stereoselective lyso phospholipid, which can act as an acyl acceptor for a transacylase reaction [30, 31].

Based on the previous studies there appear to be two candidate enzymes, PLA_2 and CoA-IT, which may be important in the breakdown of 1-alkyl-2-arachidonoyl-GPC. In the present study, we have established a system which will monitor the production of 1-alkyl-2-lyso-GPC and the concomitant release of free arachidonic acid from membrane phospholipids. If 1-alkyl-2-lyso-GPC and arachidonic acid are being produced by one enzyme (such as a PLA_2), then the concentrations of both molecules should move in concert. However, if 1-alkyl-2-lyso-GPC is produced by a transacylase and free arachidonic acid by a PLA_2, then it should be possible to regulate these molecules independently. The data presented here demonstrate that 1-alkyl-2-lyso-GPC and arachidonic acid levels may be regulated independently, supporting the hypothesis that different enzymatic steps maybe involved in 1-alkyl-2-lyso-GPC production and arachidonic acid release.

MATERIALS AND METHODS

Materials

1-[1′,2′-^{3}H]Alkyl-2-lyso-GPC (52 Ci/mmol) was purchased from New England Nuclear (Boston, MA). 1-Alkyl-2-lyso-[^{14}C-N-methyl] GPC (55 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). 1-*O*-Hexadecyl-2-lyso-GPC (lyso PAF) was obtained from Biomol (Plymouth Meeting, PA). Histopaque-1077, aristolochic acid, *p*-bromophenacyl bromide and the remaining chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). Silica gel G plates were from Analtech Inc. (Newark, DE). Essentially fatty acid free bovine serum albumin was obtained from Calbiochem (San Diego, CA). Silica gel columns were from Baker (Phillipsburg, NJ). Arachidonic acid D_8 (5,6,8,9,11,12,14,15) standard was from Cambridge Isotope Laboratories (Woburn, MA). Scalaradial was obtained from Dr. Robert Jacobs (University of California, Santa Barbara).

Preparation of human neutrophils

Human leukocyte packs were purchased from the Biological Specialty Corp. (Landsdale, PA) and neutrophils were isolated by the procedure of Boyum [32], using the histopaque-1077 technique. Briefly, leukocyte packs were centrifuged at 300 *g* for 10 min. The cell pellets were resuspended in phosphate-buffered saline (PBS) composed of 137 mM NaCl, 8.8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl (Dulbecco's, Gibco Laboratories, Long Island, NY) and layered over histopaque-1077. The pellets were collected after centrifugation (300 *g* for 30 min) and washed once in PBS. The cell pellets were exposed briefly to deionized water to lyse any erthrocytes. The remaining cells were collected by centrifugation, suspended in PBS, counted, and identified after cytospinning and staining. The final leukocyte preparation was of greater than 95% purity and viability, as determined by trypan blue exclusion and histological examination.

Treatment of human neutrophils

Neutrophils were labeled with 1-[^{3}H]alkyl-2-lyso-GPC as follows. Cells ($2–3 \times 10^7$ cells/mL) were incubated in a buffer of the following composition (mM): NaCl, 118; KCl, 4.4; $NaHCO_3$, 24.9; KH_2PO_4, 1; D-glucose, 11.1; $CaCl_2$, 1; $MgCl_2$, 1.1; and 0.1% bovine serum albumin (BSA). 1-[^{3}H]Alkyl-2-lyso-GPC (0.5 to 1 μCi/10^7 cells) was added and the cells were incubated for 1 hr at 37°. Labeled neutrophils were washed twice with PBS containing 1 mM [ethylenebis(oxyethylenenitrilo)]tetra acetic acid (EGTA), 1% BSA and then resuspended at a density of 40×10^6 cells/mL in PBS containing 1 mM EGTA. The cells were ruptured by N_2 cavitation (750 psi, 10 min), and then centrifuged at 1000 *g* for 5 min to remove remaining intact cells; the resulting homogenate was used immediately for subsequent experiments.

Homogenates of human neutrophils were treated with various combinations of unlabeled 1-alkyl-2-lyso-GPC, EGTA, $CaCl_2$ and/or compounds and incubated at 37° for 0–16 min. Each sample represented the homogenate from approximately 1×10^8 cells in a volume of 2.5 mL. Following the treatment and incubation period, the reactions were terminated by the addition of 2.5 mL chloroform:methanol (1:2, v/v) to the samples. [$^2H_8$]Arachidonic acid (100 or 200 ng) was added as an internal standard, and the lipids were extracted by the method of Bligh and Dyer [33].

Assay for 1-[^{3}H]*alkyl-2-lyso-GPC production*

An aliquot of the chloroform extracted material was separated by TLC on Silica G plates developed in chloroform:methanol:glacial acetic acid:water (50:25:8:4, by vol.), and visualized by radioscanning

Table 1. Comparison of the transacylase activities involved in the acylation of 1-[^{3}H]alkyl-2-lyso-GPC and the deacylation of 1-[^{3}H]alkyl-2-acyl-GPC

Sample	Acylation of 1-alkyl-2-lyso-GPC	Deacylation of 1-alkyl-2-acyl-GPC
Control*	124,300 ± 19,000	11,500 ± 80
10 mM DTT	124,100 ± 3,510 (99.8%)	12,300 ± 490 (107%)
10 mM 2ME	111,800 ± 19,700 (90%)	11,300 ± 450 (98%)
Homogenate†	270,000 ± 5,260	2,330 ± 130
Supernatant	5,900 ± 610 (0.1%)	Not detectable (0%)
Microsomes	7,210,000 ± 665,000 (99.9%)	25,900 ± 880 (100%)

* Homogenates of human neutrophils were treated with vehicle or 10 mM DTT or 10 mM 2ME for 10 min at 37°. Then the homogenates were assayed for the acylation of 1-[^{3}H]alkyl-2-lyso-GPC and the deacylation of 1-[^{3}H]alkyl-2-acyl-GPC as described in Materials and Methods. Data (dpm/mg protein) are the means ± SD of triplicate determinations; values in parentheses are percentages of the control.

† Homogenates of human neutrophils obained after a 500 *g* centrifugation were further separated into supernatant and microsomal fractions (the supernatant of a 20,000 *g*, 20-min spin was then centrifuged for 100,000 *g*, 60 min). The activities (expressed as means ± SD) in the resulting fractions (dpm/mg protein) were determined in triplicate. The relative percentage in the supernatant and microsomal fractions are given in parentheses.

(Bioscan); the area corresponding to 1-[^{3}H]alkyl-2-lyso-GPC was scraped and quantified by liquid scintillation counting. An assay examining the acylation of 1-[^{3}H]alkyl-2-lyso-GPC was performed as described previously [27].

Assay for free arachidonic acid

The balance of the chloroform extract for each sample was evaporated to dryness and the material resuspended in hexane. The hexane was passed through a Silica solid phase column (500 mg), washed twice with hexane and a fatty acid enriched fraction eluted with hexane:ethyl ether (1:1, v/v). Solvents were removed from the samples under a stream of nitrogen and then the samples were converted to pentafluorobenzyl esters as previously described [34, 35] using pentafluorobenzyl bromide and diisopropylethylamine in acentonitrile. Solvents were removed and samples were suspended in hexane. GC/MS analysis was performed with a Finnigan MAT TSQ 700 GC/MS/MS/DS (San Jose, CA) operated as a single stage quadrupole system, as previously described [34, 35]. Typically 3–5% of the total arachidonate in all phospholipids was released after the addition of 5 mM Ca^{2+} for 16 min to neutrophil homogenates.

Chromatography of lipids

Lipids were extracted [33] from the neutrophil homogenates (2.5 mL, 100 × 10^6 cells) and separated into choline-, ethanolamine- and inositol-containing phospholipids using a normal-phase HPLC system [36]. The arachidonate content of each phospholipid class was measured by GC/MS analysis following base hydrolysis of fatty acids from each phosphlipid class [7, 9]. Examination of the major phospholipid classes in the homogenates which contain arachidonate indicated that phosphatidyl ethanolamine (PE) contained the bulk of the arachidonate (82%) with smaller quantities in phosphatidylcholine (PC) and phosphatidylinositol/phosphatidylserine (PI/PS) (data not shown).

Protein

Protein concentrations were assessed by the method of Bradford [37] using reagents from Bio-Rad (Richmond, CA).

Data analysis

Each experiment was performed two to four times on different human donors with repeated experiments demonstrating similar results. As the magnitude of the responses varied between donors, data from different experiments were not averaged and representative results from one experiment are shown.

RESULTS

Defining the assays

1-Alkyl-2-lyso-GPC production: CoA-independent transacylase. We demonstrated previously that 1-[^{3}H]alkyl-2-lyso-GPC incubated with human neutrophils is metabolized to 1-[^{3}H]alkyl-2-acyl-GPC containing predominantly arachidonate at the *sn*-2 position of the molecule [24]. Using this selective labeling protocol, greater than 95% of the label associated with the cell was 1-[^{3}H]alkyl-2-acyl-GPC and was assumed to be 1-[^{3}H]alkyl-2-arachidonoyl-GPC. Homogenates from cells labeled as described above were prepared and the formation of 1-[^{3}H]-alkyl-2-lyso-GPC was stimulated by exogenous lyso phospholipids. It was hypothesized that the addition of exogenous lyso phospholipids provided the crucial acceptor for the CoA-IT reaction, and the formation of 1-[^{3}H]alkyl-2-lyso-GPC represented the rapid transfer of arachidonate out of 1-[^{3}H]alkyl-2-acyl-GPC into that acceptor lyso phospholipid. To further test the concept that CoA-IT catalyzed this reaction, the characteristics of this activity were compared to

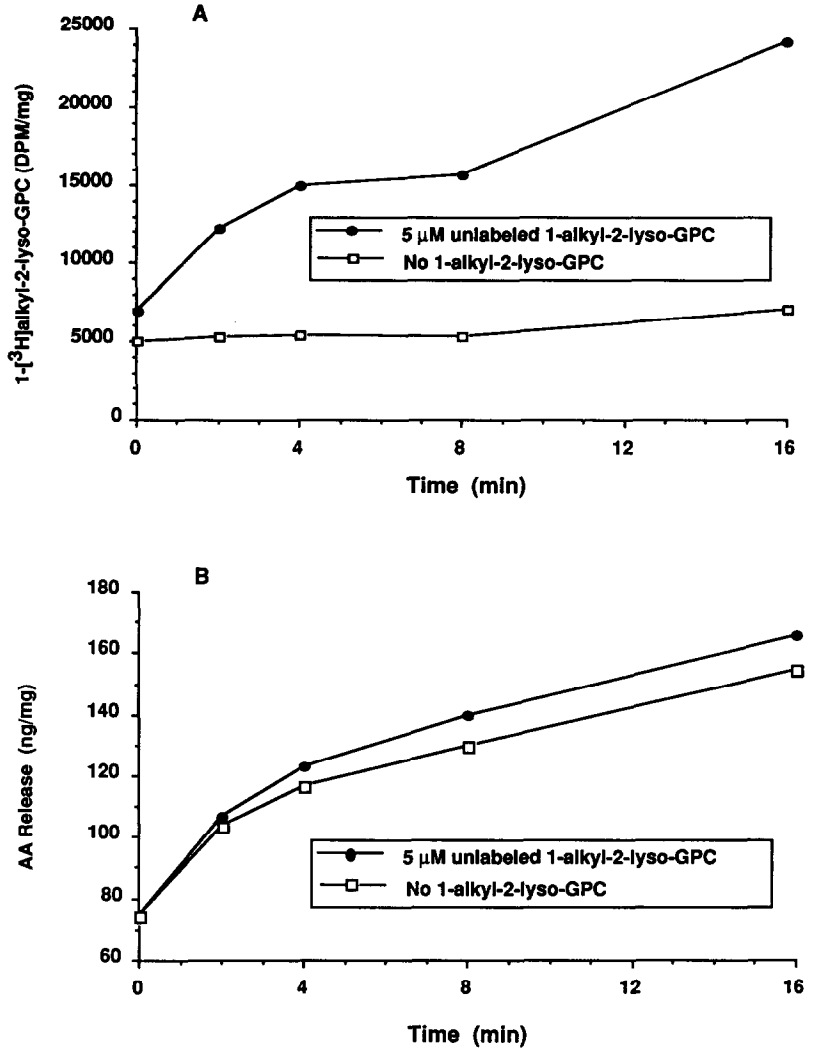

Fig. 1. Effect of unlabeled 1-alkyl-2-lyso-GPC on the production of 1-[^{3}H]alkyl-2-lyso-GPC and the release of arachidonic acid (AA). Human neutrophil homogenates containing 1-[^{3}H]alkyl-2-arachidonoyl-GPC were prepared as described in Materials and Methods. Aliquots of homogenates were incubated for various times with 5 mM Ca^{2+} in the absence or presence of 5 μM unlabeled 1-alkyl-2-lyso-GPC. The lipids were extracted and a portion of the sample used to measure 1-[^{3}H]alkyl-2-lyso-GPC (A) and the balance to measure arachidonic acid (B) as described in Materials and Methods. Each point represents a sample of 10^8 neutrophils, and these results are representative of three similar experiments.

CoA-IT activity in the assay in which the activity was originally described. The two activities, 1-[^{3}H]-alkyl-2-lyso-GPC production and acylation of 1-[^{3}H]alkyl-2-lyso-GPC, had similar characteristics as indicated by the fact that both were: independent of Ca^{2+}, Mg^{2+}, CoA and CoA fatty acids; inactivated by heating the enzymes at 57° for 2 min; and inhibited by detergents such as β-octyl glucoside and deoxycholate [30, 38]. Table 1 illustrates that both activities could be localized to the microsomal fraction of neutrophil homogenates. In addition, both activities were stable in 10 mM dithiothreitol (DTT) and 2-mercaptoethanol (2ME), indicating that they can be differentiated from a typical PLA_2-catalyzed reaction [38].

Arachidonic acid release from membrane phospholipids: Phospholipase A_2

Theoretically, the major difference between the products formed in a CoA-IT reaction and a PLA_2 reaction is that free arachidonic is formed only in the PLA_2 reaction. Therefore, the next series of experiments were performed to define an assay which measures the release of free arachidonic acid from neutrophil homogenates. Recently, Ramesha and Taylor [39] described a simple assay for measuring Ca^{2+}-dependent cleavage of arachidonic acid from endogenous membrane phospholipids, presumably by PLA_2. In the present study, we have further characterized this activity and utilized it to

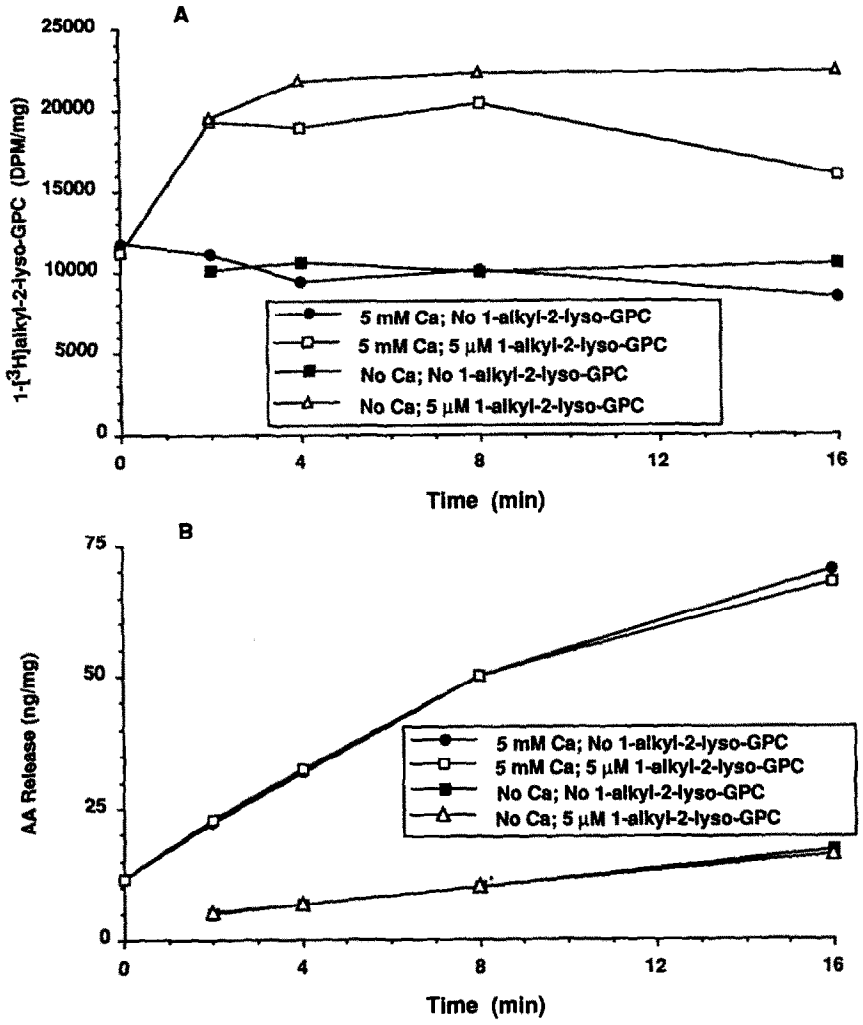

Fig. 2. Effect of Ca^{2+} on the production of 1-[^{3}H]alkyl-2-lyso-GPC and the release of arachidonic acid. Human neutrophil homogenates containing 1-[^{3}H]alkyl-2-arachidonoyl-GPC were prepared as described in Materials and Methods. Aliquots of homogenates were incubated for various times in the absence of Ca^{2+} with 1 mM EGTA or in the presence of 5 mM Ca^{2+} with or without 5 μM unlabeled 1-alkyl-2-lyso-GPC. The lipids were extracted and a portion of the sample was used to measure 1-[^{3}H]alkyl-2-lyso-GPC (A) and the balance to measure arachidonic acid (B) as described in Materials and Methods. Each point represents a sample of 10^8 neutrophils, and these results are representative of two similar experiments.

measure the formation of free arachidonic acid from membrane phospholipids.

Experiments were also performed examining the capacity of neutrophil homogenates to incorporate arachidonic acid into membrane phospholipids. This control experiment was crucial to determine if the arachidonic acid measured in this assay represented most of the arachidonic acid released from phospholipids or merely the balance between arachidonic acid that had been released and that had been reincorporated into complex lipids. These experiments indicated that neutrophil homogenates (alone) had minimal capacity to incorporate free arachidonic acid into membrane phospholipids (data not shown). Based on these data, this model appeared to be a viable one to measure arachidonic acid release and approximate PLA_2 activity in these studies.

Comparison of arachidonic acid release and 1-[^{3}H]-*alkyl-2-lyso-GPC formation*

The next series of experiments were performed to determine if there were conditions which would influence 1-[^{3}H]alkyl-2-lyso-GPC production and not arachidonic acid release or vice versa. Initial experiments examined the influence of unlabeled lyso phospholipids on the formation of 1-[^{3}H]alkyl-2-lyso-GPC from 1-[^{3}H]alkyl-2-acyl-GPC and the release of arachidonic acid. These experiments were performed in the presence of Ca^{2+} (5 mM). As

Table 2. Effect of 2.5 nM 1-[^{3}H]alkyl-2-lyso-GPC on the turnover of 1-alkyl-2-acyl-[^{14}C]GPC and 1-alkyl-2-lyso-[^{14}C]GPC in neutrophil homogenates

Time (min)	Addition of 1-Alkyl-2-lyso-GPC	Label	1-Alkyl-2-acyl-GPC	1-Alkyl-2-lyso-GPC
(A) 0	None	[^{14}C]	35,600 ± 910 (73%)	13,200 ± 2,400 (27%)
(B) 16	None	[^{14}C]	41,000 ± 140 (85%)	7,420 ± 260 (15%)
(C) 16	2.5 nM	[^{14}C]	39,500 ± 420 (85%)	6,830 ± 200 (15%)
		[^{3}H]	7,130 ± 150 (77%)	2,100 ± 00 (23%)
(D) 16	5 μM	[^{14}C]	32,800 ± 1,100 (72%)	12,900 ± 60 (28%)
		[^{3}H]	2,090 ± 90 (26%)	6,060 ± 310 (74%)

Human neutrophils were incubated with 1-alkyl-2-lyso-[^{14}C]GPC for 60 min, washed and membranes prepared as described in Materials and Methods. The homogenate was incubated for 0 min (A) or for 16 min at 37° with 1 mM EGTA with either no addition (B), addition of 1-[^{3}H]alkyl-2-lyso-GPC (2.5 nM final) (C) or addition of 1-[^{3}H]alkyl-2-lyso-GPC plus unlabeled 1-alkyl-2-lyso-GPC (5 μM final) (D). After the incubation the lipids were extracted and separated, and the amounts of ^{3}H- and ^{14}C-labeled material corresponding to 1-alkyl-2-arachidonoyl-GPC and 1-alkyl-2-lyso-GPC were determined. The data are cpm using narrow windows for dual-labeled material, and the spill over of [^{14}C] activity into the [^{3}H] window was calculated and corrected. Data are the means ± SD of triplicate determinations and represent three experiments with similar results.

shown in Fig. 1A, 1-[^{3}H]alkyl-2-lyso-GPC produced by homogenates increased as a function of time after the addition of 5 μM 1-alkyl-2-lyso-GPC. Experiments in which the concentration of unlabeled 1-alkyl-2-lyso-GPC was varied demonstrated that 5 μM produced the maximal effect (data not shown). Arachidonic acid release was studied in the identical samples utilized for 1-[^{3}H]alkyl-2-lyso-GPC production. There was a time-dependent increase in arachidonic acid release in the absence of excess unlabeled 1-alkyl-2-lyso-GPC and the addition of unlabeled 1-alkyl-2-lyso-GPC had no effect on this release (Fig. 1B). In separate experiments (not shown), it was determined that the addition of unlabeled 1-alkyl-2-lyso-GPC had no effect on the reacylation of arachidonic acid, which remained essentially undetectable. Together, these results suggested that unlabeled 1-alkyl-2-lyso-GPC promoted the conversion of 1-[^{3}H]alkyl-2-arachidonoyl-GPC to 1-[^{3}H]alkyl-2-lyso-GPC but did not affect the release of arachidonic acid from membrane phospholipid.

Similar experiments were performed in the absence of Ca^{2+} with 1 mM EGTA. The absence of Ca^{2+} did not influence the conversion of 1-[^{3}H]alkyl-2-acyl-GPC to 1-[^{3}H]alkyl-2-lyso-GPC stimulated by exogenous lyso phospholipids and did not stimulate the conversion to 1-[^{3}H]alkyl-2-lyso-GPC in the absence of exogenous lyso phospholipids (Fig. 2A). In contrast, removal of Ca^{2+} caused a dramatic reduction in the quantity of arachidonic acid released from homogenates at all time points (Fig. 2B). However, it is important to note that there was a small but consistent release of arachidonic acid that occurred in the absence of Ca^{2+} (Fig. 2B), suggesting the presence of a Ca^{2+}-independent PLA_2 in the homogenate.

A fundamental question which arose from these experiments was whether the increase in 1-[^{3}H]alkyl-2-lyso-GPC was due to the CoA-IT-dependent removal of arachidonate from 1-[^{3}H]alkyl-2-acyl-GPC (catalyzed by the addition of unlabeled 1-alkyl-2-lyso-GPC) or to the addition of excess unlabeled 1-alkyl-2-lyso-GPC trapping 1-[^{3}H]alkyl-2-lyso-GPC as it is produced, preventing subsequent reacylation. For the latter to be true, it must be assumed that there is constant formation of 1-alkyl-2-lyso-GPC by a Ca^{2+}-independent PLA_2 and that 1-alkyl-2-lyso-GPC is acylated rapidly by an enzyme such as CoA-IT. The fact that there was a Ca^{2+}-independent release of arachidonic acid from the homogenate system (Fig. 2B) suggested that such a PLA_2 activity could act in concert with CoA-IT in the neutrophil homogenate. To address this question, a double label experiment was designed in which intact neutrophils were prelabeled with 1-alkyl-2-lyso-[^{14}C]-GPC to produce homogenates which contained predominantly 1-alkyl-2-acyl-[^{14}C]GPC with some 1-alkyl-2-lyso-[^{14}C]GPC (Table 2A). When these homogenates were incubated at 37° for 16 min, the ratio of 1-alkyl-2-acyl-[^{14}C]GPC (85%) to 1-alkyl-2-lyso-[^{14}C]GPC (15%) remained high (Table 2B). When a small quantity (2.5 nM) of 1-[^{3}H]alkyl-2-lyso-GPC was added to the reaction mixture, it was converted rapidly to 1-[^{3}H]alkyl-2-acyl-GPC and the ratio of 1-[^{3}H]alkyl-2-acyl-GPC to 1-[^{3}H]alkyl-2-lyso-GPC approached that of the [^{14}C]-labeled material in the homogenates (Table 2C). This concentration of 1-[^{3}H]alkyl-2-lyso-GPC is well below those concentrations (0.5 to 5 μM) found to stimulate transacylase activity in isolated microsomes [20, 27] and in this experiment did not appear to stimulate transacylation as the ratio of 1-alkyl-2-acyl-[^{14}C]GPC to 1-alkyl-2-lyso-[^{14}C]GPC was unchanged by addition of 2.5 nM 1-[^{3}H]alkyl-2-lyso-GPC (Table 2C). The fact that newly added 1-[^{3}H]alkyl-2-lyso-GPC was acylated rapidly and that the 1-alkyl-2-lyso-[^{14}C]GPC levels remained constant suggest that 1-alkyl-2-lyso-GPC was constantly being formed and reacylated in the homogenate membranes. Addition of a large amount (5 μM) of unlabeled 1-alkyl-2-lyso-GPC along with the 1-[^{3}H]alkyl-2-lyso-GPC

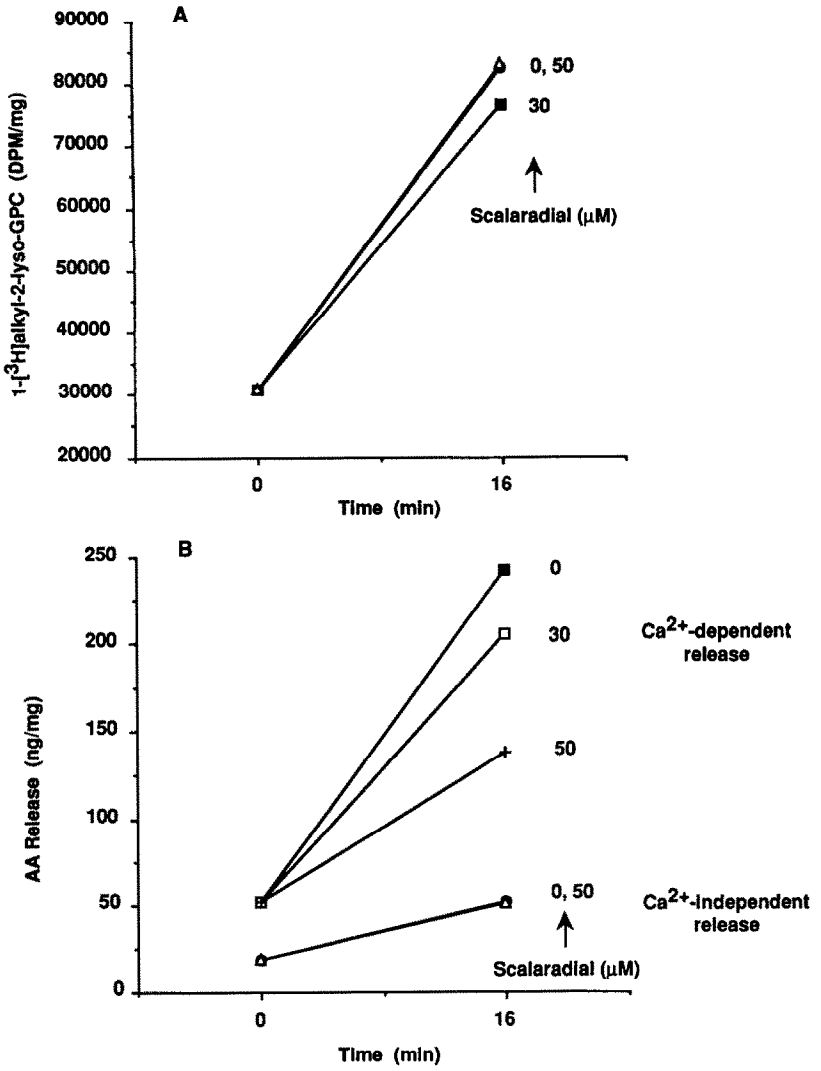

Fig. 3. Effect of scalaradial on the production of 1-[^{3}H]alkyl-2-lyso-GPC and the Ca^{2+}-dependent and -independent release of arachidonic acid. Human neutrophil homogenates containing 1-[^{3}H]alkyl-2-arachidonoyl-GPC were prepared as described in Materials and Methods. Aliquots of homogenates were incubated for 0 or 16 min with the indicated concentrations of scalaradial in the presence of 5 μM unlabeled 1-alkyl-2-lyso-GPC and of either 5 mM Ca^{2+} (Ca^{2+}-dependent release and 1-[^{3}H]alkyl-2-lyso-GPC production) or in the absence of Ca^{2+} with 1 mM EGTA (Ca^{2+}-independent release). The lipids were extracted and a portion of the sample was used to measure 1-[^{3}H]alkyl-2-lyso-GPC (A) and the balance to measure arachidonic acid (B) as described in Materials and Methods. Each point represents a sample of 10^8 neutrophils, and these results are representative of two similar experiments.

inhibited the acylation of 1-[^{3}H]alkyl-2-lyso-GPC and increased the amount of 1-alkyl-2-lyso-[^{14}C]GPC (Table 2D).

Influence of putative PLA_2 inhibitors on 1-[^{3}H]*alkyl-2-lyso-GPC production and arachidonic acid release*

In an attempt to further dissociate 1-[^{3}H]alkyl-2-lyso-GPC production from arachidonic acid release, three compounds were examined for their ability to selectively affect one process and not the other. Scalaradial is a potent inhibitor of PLA_2 in cell-free systems, with an IC_{50} for inhibiting PLA_2 of 800 nM [40]. When this compound was examined in the CoA-IT assay measuring the acylation of 1-[^{3}H]alkyl-2-lyso-GPC, it was much less potent for inhibition of CoA-IT, with an IC_{50} of 40 μM (data not shown). In homogenates of neutrophils scalaradial inhibited Ca^{2+}-dependent arachidonic acid release by 50% at 50 μM, but had no effect on Ca^{2+}-independent arachidonic acid release nor on the production of 1-[^{3}H]alkyl-2-lyso-GPC (Fig. 3). Aristolochic acid has no effect on CoA-IT activity in a cell-free system at concentrations up to 500 μM [27]. This compound has been reported to inhibit PLA_2 activity with IC_{50} values in the range of 300–

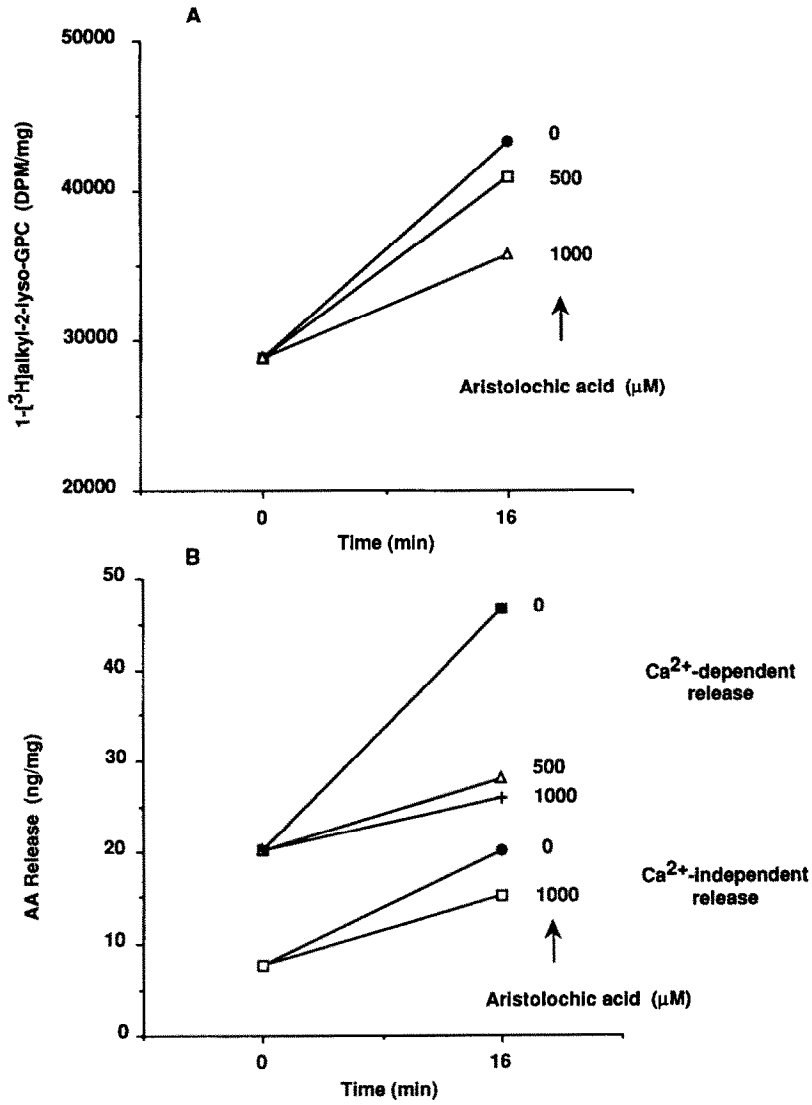

Fig. 4. Effect of aristolochic acid on the production of 1-[^{3}H]alkyl-2-lyso-GPC and the Ca^{2+}-dependent and -independent release of arachidonic acid. Homogenates of human neutrophils, prepared as in Fig. 1, were treated with 0, 500 or 1000 μM aristolochic acid, and then were incubated for 0 or 16 min in the presence of 5 μM unlabeled 1-alkyl-2-lyso-GPC and of either 5 mM Ca^{2+} (Ca^{2+}-dependent release and 1-[^{3}H]alkyl-2-lyso-GPC production) or in the absence of Ca^{2+} with 1 mM EGTA (Ca^{2+}-independent release). The lipids were extracted and a portion of the sample was used to measure 1-[^{3}H]alkyl-2-lyso-GPC (A) and the balance to measure arachidonic acid (B) as described in Materials and Methods. Each point represents a sample of 10^6 neutrophils, and these results are representative of two similar experiments.

500 μM [41]. In the present study, aristolochic acid inhibited the Ca^{2+}-dependent release of arachidonic acid almost completely at 500 μM, but had minimal effects on the production of 1-[^{3}H]alkyl-2-lyso-GPC and Ca^{2+}-independent release of arachidonic acid (Fig. 4). Bromophenacyl bromide inhibited cell-free CoA-IT activity in a concentration-dependent manner with an IC_{50} of approximately 6 μM (data not shown). This compound has also been shown to inhibit PLA_2 activity in cell-free systems with an IC_{50} of 40 μM and in intact cells at higher concentrations, with an IC_{50} of 550 μM [42, 43]. Figure 5 illustrates the effect of *p*-bromophenacyl bromide on 1-[^{3}H]-alkyl-2-lyso-GPC production and arachidonic acid release from neutrophil homogenates. *p*-Bromophenacyl bromide had almost equivalent effects on 1-[^{3}H]alkyl-2-lyso-GPC production and on Ca^{2+}-dependent and -independent arachidonic acid release, suggesting that it may nonselectively inhibit PLA_2 and CoA-IT enzymes, possibly due to covalent interaction with histidine sites on these enzymes [44].

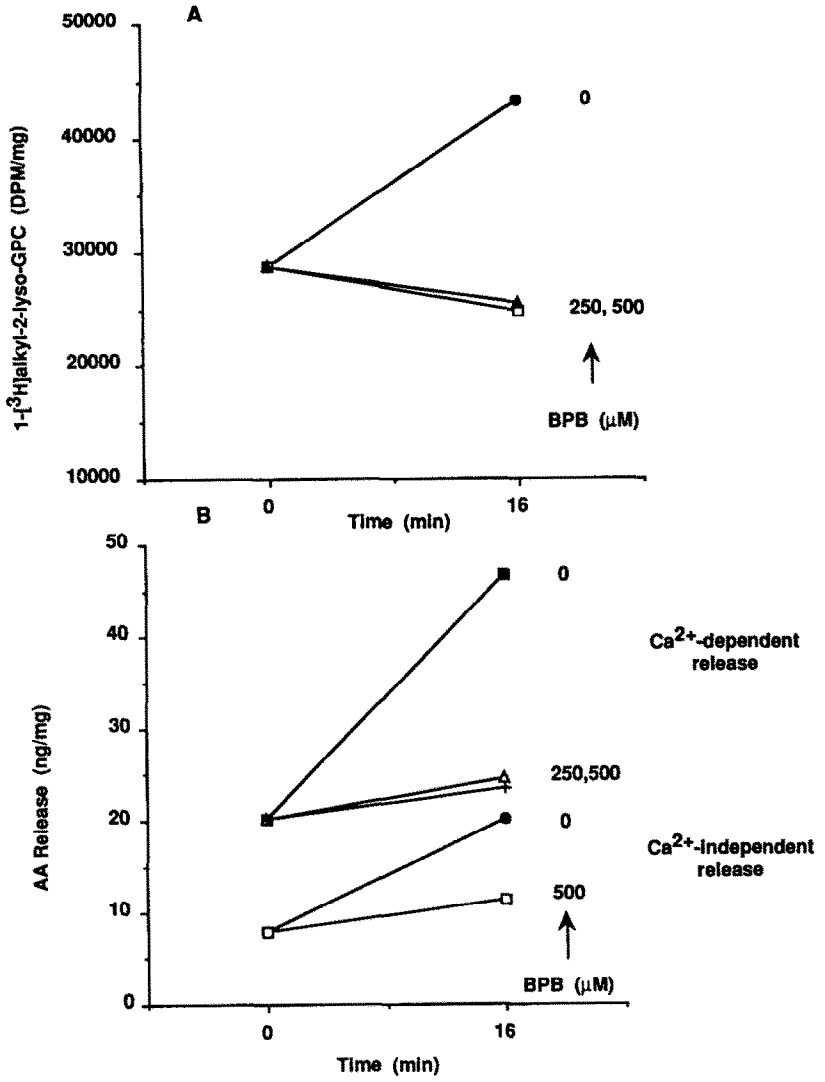

Fig. 5. Effect of *p*-bromophenacyl bromide (BPB) on the production of 1-[^{3}H]alkyl-2-lyso-GPC and the Ca^{2+}-dependent and -independent release of arachidonic acid. Homogenates of human neutrophils, prepared as in Fig. 1, were treated with 0, 250 or 500 μM BPB, then incubated for 0 or 16 min in the presence of 5 μM unlabeled 1-alkyl-2-lyso-GPC and of either 5 mM Ca^{2+} (Ca^{2+}-dependent release and 1-[^{3}H]alkyl-2-lyso-GPC production) or in the absence of Ca^{2+} with 1 mM EGTA (Ca^{2+}-independent release). The lipids were extracted and a portion of the sample was used to measure 1-[^{3}H]alkyl-2-lyso-GPC (A) and the balance to measure arachidonic acid (B) as described in Materials and Methods. Each point represents a sample of 10^6 neutrophils, and these results are representative of two similar experiments.

DISCUSSION

It has been inferred for several years that the initial enzymatic step in both eicosanoid and PAF biosynthesis is a PLA_2 reaction which catalyzes the removal of arachidonic acid from the *sn*-2 position of membrane phospholipids to form lyso phospholipids such as 1-alkyl-2-lyso-GPC. In addition, a number of studies have suggested that 1-alkyl-2-arachidonoyl-GPC is a phospholipid which is present in large quantities in inflammatory cells and could serve as a common precursor for PAF and eicosanoids in such a PLA_2 reaction [4, 6–12]. The present study further examines this hypothesis by concomitantly measuring 1-alkyl-2-lyso-GPC production and arachidonic acid release, addressing the question of whether these two mediator precursors are produced by one or more than one enzyme activity in neutrophil homogenates. The data presented in this study revealed that the production of 1-alkyl-2-lyso-GPC was enhanced by the presence of unlabeled lyso phospholipids, unaffected by Ca^{2+} and unaffected by aristolochic acid as well as scalaradial at concentrations which dramatically reduced Ca^{2+}-dependent arachidonic acid release. In contrast, the release of arachidonic acid from membrane

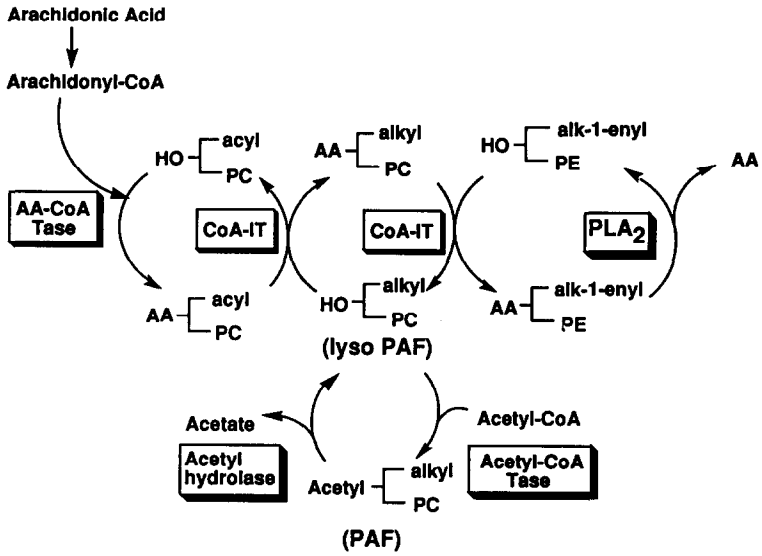

Fig. 6. Hypothetical scheme of the production of 1-alkyl-2-lyso-GPC (lyso PAF) and the release of arachidonic acid. Abbreviations: CoA-IT, CoA-independent transacylase; AA-CoA Tase, arachidonoyl-CoA transferase; acetyl-CoA Tase, acetyltransferase; PAF, platelet-activating factor; and PLA_2, phospholipase A_2.

phospholipids was enhanced by Ca^{2+} and was unaffected by lyso phospholipids. These data are therefore consistent with the concept that 1-alkyl-2-lyso-GPC and Ca^{2+}-dependent arachidonic acid production can be controlled independently by different enzyme activities.

While we have gone to great care in these studies to define our assays, there are a number of important points about these experiments which must be taken into consideration. For example, in the 1-alkyl-2-lyso-GPC production assay, lyso phospholipids must be added to the reaction mixture to catalyze the transacylase reaction. This raises the critical question of whether lyso phospholipids found in the whole cell ever accumulate to sufficient levels to induce such a reaction. Wykle and colleagues [45] have provided evidence recently that this maybe the case in the neutrophil by demonstrating high levels of 1-alk-1-enyl-2-lyso-GPE after cell activation.

Another potential concern is the possibility that the transacylase activity referred to in this study may actually be an activity working in concert with a Ca^{2+}-independent PLA_2 activity and that adding lyso phospholipids to the reaction mixture merely competes with labeled 1-alkyl-2-lyso-GPC being formed and rapidly reacylated. In fact, this study did identify a Ca^{2+}-independent PLA_2 activity which catalyzed the release of arachidonic acid, but was not affected by putative PLA_2 inhibitors. This Ca^{2+}-independent PLA_2 may work in concert with a CoA-IT to continuously provide lyso phospholipids which can then, in turn, be acylated.

A logical extension of these results in broken cell experiments is that during activation of intact cells there is a Ca^{2+}-dependent PLA_2 enzyme that is responsible for the release of arachidonic acid from membrane phospholipids while another activity, such as CoA-independent transacylase, is responsible for the production of 1-alkyl-2-lyso-GPC. This latter hypothesis has been proposed recently by Uemura *et al.* [31] and Venable *et al.* [30]. Based on these new findings and previous studies examining both CoA-IT and PLA_2, it is possible to envision a pathway in inflammatory cells where these two enzymes, CoA-IT and PLA_2, work in concert with one another. Phospholipase A_2 may drive the initial formation of lyso phospholipids which is necessary to catalyze the transacylase reaction. Several lines of direct and indirect evidence support such a pathway. For example, previous work from several laboratories indicate that PE, and 1-alk-1′enyl-2-arachidonoyl-GPE in particular, is the major source of the arachidonate released from membrane phospholipids in the neutrophil and mast cell during cell activation [7–10]. Taken together with the finding that lyso PE accumulates in the stimulated neutrophil [45], these studies suggest that there is a Ca^{2+}-dependent release of free arachidonate from PE of inflammatory cells which has characteristics of a PLA_2 reaction. Once lyso PE is formed in this reaction, it can then drive the transfer of arachidonate from 1-alkyl-2-arachidonoyl-GPC, forming 1-alkyl-2-lyso-GPC. A portion of this 1-alkyl-2-lyso-GPC can then be acetylated to produce PAF while the remaining 1-alkyl-2-lyso-GPC is reacylated with other fatty acids. Again, several other studies give credence to the latter portion of this pathway. For example, lyso PE has been demonstrated to be a preferred substrate for CoA-IT in a number of different assay systems [28]. In addition, a recent study by Sugiura and co-workers [29] demonstrated that the addition of lyso PE to whole neutrophils is sufficient to catalyze PAF production.

The major points of this discussion are depicted in the hypothetical scheme shown in Fig. 6. While this scheme represents an attractive hypothesis, it is important to realize that a major portion of the evidence to support these pathways has been derived from studies such as this one in broken cell preparations. Therefore, it is clear that studies must now be done in whole cells to better define these pathways. However, the present study does provide direct evidence in neutrophil homogenates that there may be different mechanisms involved in the formation of 1-alkyl-2-lyso-GPC and the calcium-dependent release of arachidonic acid. Further, these data implicate that these two distinct mechanisms may involve CoA-IT and PLA_2 reactions.

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