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Transacylase-Mediated Alkylacyl-GPC Synthesis and Its Hydrolysis by Phospholipase D Occur in Separate Cell Compartments in the Human Neutrophil

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Abstract Subcellular localizations of CoA-independent transacylase and phospholipase D enzymes have been investigated in human neutrophils performing a two-step gradient system to separate plasma membranes from internal membranes and from the bulk of granules. The internal membranes were constituted by endoplasmic reticulum and by a subpopulation of specific and tertiary granules. The enzymes activities were assayed in vitro on gradient fractions using exogenous substrates. Following cell prelabelling with [^{3}H]alkyllyso-GPC, we also analyzed the in situ localization of labelled products involving the action of both enzymes. The CoA-independent transacylase activity, together with the CoA-dependent transacylase and acyltransferase activities were only located in the internal membranes. Following 15 min cell labelling, part of the [^{3}H]alkylacyl-GPC was recovered in plasma membranes indicating a rapid redistribution of the acylated compound. Very high contents in arachidonate containing [^{3}H]alkylacyl-GPC were recovered both in plasma membranes and internal membranes. Phospholipase D activity being assayed in the presence of cytosol, GTPγS and gradient fractions, only the plasma membrane fractions from resting or stimulated cells allowed the enzyme to be active. The [^{3}H]alkylacyl-GP and [^{3}H]alkylacyl-GPethanol, phospholipase D breakdown products from [^{3}H]alkylacyl-GPC, obtained after neutrophil prelabelling and activation by phorbol myristate acetate, were exclusively present in the plasma membranes. In contrast, the secondary generated [^{3}H]alkylacylglycerols were equally distributed between plasma and internal membranes. No labelled product was recovered on azurophil granules. These data demonstrate that internal membranes are the site of action of the CoA-independent transacylase and plasma membranes are the site of action of the phospholipase D. This topographical separation between CoA-independent transacylase which generated substrate and phospholipase D which degraded it, suggested that subcellular localisation and traffic of substrates within the cell can be important to regulate the enzymes.

Key words: CoA-independent transacylase, phospholipase D, subcellular localization, neutrophils

INTRODUCTION

The alkylacyl-GPC constitute an abundant subclass in the human neutrophils since they represent about half the whole phosphatidylcholine class [Mueller et al., 1984; Tencé et al., 1985]. Alkylacyl-GPC first retained attention as precursor of the lipid mediator alkylacetyl-GPC (Platelet-Activating Factor or PAF). We have shown that synthesis of this mediator either by the de-novo or the remodelling pathway occur in the endoplasmic reticulum [Ribbes et al., 1985; Vallari et al., 1990]. The human PMN contain a PAF-specific transport system able to carry the mediator from its site of synthesis to the plasma membrane [Ribbes et al., 1991]. The acylation of lyso-PAF into alkylarachidonyl-GPC is mediated by CoA-independent transacylase [Kramer et al., 1984; Robinson et al., 1985], this enzymatic step being the most common way to label endogenous phosphatidylcholine in the PMN [Chilton et al., 1983]. Conversely, the generation of lyso-PAF necessary to PAF synthesis has

Abbreviations used: Con-A, concanavalin A; PMN, polymorphonuclear leukocytes; LysoPAF, alkyllyso-GPC, lyso-platelet activating factor; GP, *sn*-glycero-3-phosphate; GPC, *sn*-glycero-3-phosphocholine; fMLP, N-formylmethionylleucylphenylalanine; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethylsulfoxide; TLC, thin layer chromatography; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethansulfonic acid.

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recently been shown to result from the action of CoA-independent transacylase on alkyl-arachidonyl-GPC [Uemura et al., 1991; Venable et al., 1991; Sugiura et al., 1990; Colard et al., 1993]. Arachidonate transfered to alkyl-arachidonyl-GPC and to alkenyl-arachidonyl-GPE is initially incorporated into diacyl-GPC as demonstrated in various cells [Colard et al., 1984; Sugiura et al., 1984 and reviewed in Snyder et al., 1992]. Then the CoA-independent transacylase, in mobilizing arachidonate to or from alkyl-arachidonyl-GPC, might control the availability of lyso-PAF for PAF synthesis [Breton and Colard, 1991; Ninio et al., 1991; Colard et al., 1993].

Alkylacyl-GPC has been also established to be the source of intracellular second messengers: phosphatidic acid and diglycerides. Several groups [Gelas et al., 1989; Agwu et al., 1989; Billah et al., 1989] have demonstrated the hydrolysis of phosphatidylcholine in human neutrophils by a phospholipase D triggered by the chemotactic peptide fMLP, and by PMA. Relationship between PAF synthesis and phosphatidic acid has been recently suggested [Garcia et al., 1993], the latter being involved in the shutdown of the lipid mediator production.

The tumor promoters phorbol esters, are known to stimulate phosphatidylcholine hydrolysis via phospholipase D activity [Billah et al., 1989; Mullmann et al., 1990], without phosphoinositide hydrolysis and calcium mobilisation [Tyagi et al., 1988]. At variance with fMLP [Bauldry et al., 1992], PMA-induced hydrolysis of phosphatidylcholine in human neutrophils leads to the generation of both phosphatidic acid and diglycerides [Gelas et al., 1989; Mullmann et al., 1990]. Phosphatidylcholine-derived diglycerides have been assigned a role similar to that of phosphatidylinositol-derived diglycerides in relation to protein kinase C. However, phosphatidylcholine-derived diglycerides do not appear to be involved in protein kinase C activation in vivo [Leach et al., 1991], although they are able to stimulate the kinase in vitro [Ford et al., 1989]. In this respect, it has been shown that, in TRH-stimulated cells, the sustained phase of diglyceride production is limited to intracellular membranes and is not accompanied by activation of protein kinase C [Martin et al., 1990]. These observations suggest that the phosphatidylcholine-derived diglycerides, might not be accessible in the cell to some isoforms of protein kinase C, and emphasise a regulation of signal transduction pathways via the topographical distribution of second messengers.

Therefore we investigated the subcellular distribution of enzymes, substrates, and breakdown products related to CoA-independent transacylase and phospholipase D reactions. In short, synthesis of alkylarachidonyl-GPC takes place in the internal membranes whereas its degradation by phospholipase D and production of second messengers (phosphatidic acid) is localised in the plasma membrane.

MATERIALS AND METHODS

Materials

1-O-[^{3}H]-Octadecyl-sn-glycero-3-phosphocholine (2.96–6.66 TBq/mmol [^{3}H]lyso PAF), [^{3}H]arachidonic acid (7.73), 1-[1-^{14}C]palmitoyl-lyso-GPC (2.07 GBq:mmol.), [^{57}Co] cyanocobalamin (6.7–11 MBq/μg) and N [acetyl-^{3}H]concanavalin A (1.1–3.3) were purchased from the Radiochemical Centre (Amersham International Amersham, UK) 1-O-[alkyl-1′2′-^{3}H]lyso-GPC (2.2.TBq/mmol) was from NEN, Du Pont de Nemours (France), [Acetyl-^{14}C]gelatine (37 KBq/mg) was a generous gift from Dr F. Morel, Grenoble, France. Plasmagel (R) was obtained from Laboratoires Roger Bellon, Neuilly, France. Percoll (R) was obtained from Pharmacia (Uppsala, Sweden). fMLP, PMA, oleoyl-CoA, fatty-acid free bovine serum albumin, phosphatidic acid and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell Isolation

Neutrophils were obtained from fresh human peripheral blood as previously described [Record et al., 1985; Ribbes et al., 1985]. Briefly, the leukocyte-rich supernatant was obtained after adding Plasmagel to total blood and further fractionated on Percoll gradient (15.4 ml Percoll, 6.6 ml of 400 mM NaCl buffered with 33 mM Tris-HCl, pH 7.4). Centrifugation was performed at 48,000 $\times$ g for 10 min. Remaining erythrocyte contamination of the neutrophil-enriched pellet was eliminated by suspension in cold isotonic NH_4Cl for 10 min.

Labelling of PMN with [^{3}H]LysoPAF

Briefly, [^{3}H]lysoPAF (7 to 14 nM final) was dried under nitrogen flux, resuspended in 5 μl of ethanol, and diluted in 1 ml of HEPES buffer (137 mM NaCl, 2.7 mM KCl, 5.5 mM glucose, 20 mM HEPES pH 7.4). [^{3}H]lysoPAF was added to

the PMN suspension (0.5 to 1 μCi/10^7 cells, in HEPES buffer), which was then incubated for 15 min at 37°C. The labelled cells were then washed twice with the HEPES buffer containing 2.5% (w/v) bovine serum albumin (fatty acid free). During this labelling period, approximately 60% of added [^{3}H]lysoPAF was taken up by the cells, and converted into phosphatidylcholine to a similar extend as previously reported [Gelas et al., 1989].

Subcellular Fractionation of PMN

As previously reported [Record et al., 1982], cells were suspended in 5 ml of an isotonic lysis buffer consisting of 100 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 25 mM Tris-HCl (pH 9.6), and containing PMSF (0.02 mM) as protease inhibitor. PMN were lysed at 4°C, by nitrogen cavitation under a pressure of 40 bars and an equilibrium time of 5 min. The cavitate was collected dropwise and nucleus and intact cells were removed by centrifugation at 1,000 × *g* for 15 min. The subcellular fractionation was performed on two successive Percoll gradients. For the first gradient, 4 ml of post-nuclear supernatant were layered on the top of a 61% final v/v Percoll preparation consisting of 11 ml Percoll, 2.2 ml distilled water, 4.8 ml of 400 mM KCl, 20 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.30. Centrifugation was performed at 160,000 × *g* with a ω^2t of 128 × 10^8 rad^2/sec and 11 fractions of 2 ml were collected from the top of the tube. Fractions N° 5 and 6 were pooled and mixed with a second Percoll preparation (Gradient 2) consisting of 11 ml Percoll, 3.2 ml distilled water, 4.8 ml of 400 mM KCl, 20 mM $MgCl_2$, 100 mM Tris-HCl pH 9.6. Centrifugation parameters and collecting procedures were the same as for the first gradient. In agreement with a previous report [Record et al., 1985], the position of the plasma membrane may vary by one fraction across the gradient, depending upon the batch of Percoll. Each 2 ml fraction of the gradient was added with two volumes of HEPES buffer pH 7.4 and was centrifuged at 40,000 rpm × 45 min. Protein pellets were recovered under 200 μl of buffer.

The following were assayed directly on aliquots of gradient fractions: [^{3}H]Concanavalin A for plasma membrane; marker enzymes for endoplasmic reticulum (CDP-choline phosphotransferase) and azurophil granules (β-D-glucuronidase); vitamin B12-binding protein, the marker enzyme for specific granules; and gelatinase activity for tertiary granules. Recoveries of markers across the subcellular fractionation procedure were in the range of those obtained in previous reports.

Analysis of Alkylacyl-GPC Breakdown Products Upon Cell Stimulation

Cells labelled with [^{3}H]lyso-PAF as reported above were then stimulated by PMA. A solution of PMA in dimethylsulfoxide (DMSO) was diluted in HEPES buffer to obtain a final concentration of 100 nM in the assay. DMSO never exceeded 0.1% in the incubation medium. Stimulation was performed for 5 min at 37°C in HEPES buffer containing 1 mM $MgCl_2$ and 1.3 mM $CaCl_2$. Parallel experiments were performed in the presence of 1% ethanol (v/v) which was added together with the agonist. The cell activation was stopped by adding a large volume of isotonic cold HEPES buffer. Ethanol concentration was varied from 0.25% to 4% v/v; we observed that phosphatidylethanol formation plateaued at 1% ethanol. In any case it was not possible to completely quench phosphatidic acid formation with ethanol concentrations compatible with cell viability.

Cells were then processed through the subcellular fractionation procedure detailed above. Lipids were directly extracted from the gradient fractions, and the products of phosphatidylcholine degradation were analysed with the solvent system of Cohen et al. [1971] on silicagel G plates sprayed with 0.25 M oxalic acid.

Measurement of CoA-Independent, CoA-Dependent Transacylase, and Acyltransferase

The acyltransferase was assayed by the acylation of 1-[1-^{14}C]palmitoyl-lyso-GPC in the presence of oleoyl-CoA. The CoA-dependent transacylase was assessed by the difference of acylation of [^{14}C]palmitoyl-lyso-GPC in the presence and in the absence of CoA, and the CoA-independent transacylase by the acylation of 1-O-[alkyl-1′2′-^{3}H]lyso-GPC in the absence of any added cofactors [Masrar et al., 1990]. The assay mixture contained the subcellular fraction diluted in 200 μl assay buffer containing 150 mM NaCl, 10 mM Na_2HPO_4, 1 mM EGTA, pH 7.4, and depending on the enzyme assayed, co-factors added were 50 μM CoA or 200 μM oleyl-CoA. The reaction was initiated by the addition of labelled lysoderivatives: [^{3}H]alkyl-lyso-GPC (1 μM, 0.1 μCi) in the CoA-independent transacylase, [^{14}C]palmitoyl-

lyso-GPC (1.6 μM, 0.025 μCi) in the CoA-dependent transacylase, and [^{14}C]palmitoyl-lyso-GPC, 32 μM, 0.025 μCi in the acyltransferase assay. The labelled lysoderivatives were added in 50 μl assay buffer containing 0.1% bovine serum albumin and continued for 10 min at 37°C. The reaction was stopped and the lipid extracted by the method of Bligh and Dyer [1959]. Samples in chloroform/methanol were separated by TLC in chloroform/methanol/H_2O, (100:40:6 v/v.). Labelled compounds were scrapped and counted by liquid scintillation counting.

In Vitro Phospholipase D Measurement

Substrate preparation. Phospholipase D activity was measured using a liposomal substrate of [^{3}H]alkylacyl-GPC prepared from neutrophils. Briefly, [^{3}H]alkyllysophosphatidylcholine (100 μCi) conditioned as mentioned above, was added to 20 ml of cell suspension (2×10^9 cells) which was then incubated for 30 min at 37°C. Cells were washed twice in HEPES saline buffer containing 2.5% fatty acid-free bovine serum albumin. Cells were resuspended in 5 ml of 150 mM NaCl and lipids extracted according to Bligh and Dyer [1959] except that 2% acetic acid was included in the methanol phase. The chloroform phase was removed, dried, and the residue was redissolved in 500 μl of chloroform/methanol (1:1, v/v). The sample was deposited on a Silica Gel G TLC plate which was developed using the solvent system of Skipsky et al. [1964]. The spot of [^{3}H]alkylacyl-GPC, also detected using a radioscanner (Berthold), was scrapped and eluted with chloroform/methanol/distilled water (60/40/10, v/v/v). Specific radioactivity of the different batches of substrates, calculated from the radioactivity and the phosphate content of total phosphatidylcholine, varied from 30059 to 40361 dpm/nmol. Since neutrophil phosphatidylcholines contained 50.2 ± 7.1% of alkylacyl-GPC [Tencé et al., 1985], the actual specific activity ranged from 59878 to 80400 dpm/nmol.

Measurement of enzyme activity. Phospholipase D was measured with membranes (50 μg proteins) coming from each gradient fraction and incubated with cytosol (100 μg proteins) prepared separately. [^{3}H]alkylacyl-GPC (10 nmoles) was sonicated for 3 times 30 sec and mixed in 500 μl of incubation buffer. Briefly, after a pre-incubation time of 5 min at 37°C, 1% ethanol v/v was added and the incubation was continued for another 5 min before the addition of either 100 nM PMA and 0.5 mM ATP, or 10 μM GTPγS. After an incubation time of 20 min the reaction was terminated by acidic-modified extraction of Bligh and Dyer. The chloroform phase was analysed for [^{3}H]phosphatidic acid and [^{3}H]phosphatidylethanol using the solvent system of Olson et al. [1991].

Analysis of Phospholipids

The alkylacyl, alkenylacyl, and diacyl subclasses of phosphatidylcholine and phosphatidyl ethanolamine were separated after their conversion to glycerobenzoate derivatives as described by Blank et al. [1984]. The choline and ethanolamine containing phospholipids were separated by TLC and extracted from the silica gel by the extraction procedure of Bligh and Dyer. They were hydrolysed by phospholipase C from *Bacillus Cereus* and converted to benzoate derivatives which were separated by TLC in the solvent system of benzene/hexane/diethyl ether (50/45/4 by vol.) and visualised after spraying with dichlorofluorescein. Molecular species of alkylacyl-GPC were analysed by reverse phase HPLC using acetonitrile/propanol 2-ol (75/25, v/v) at a flow rate of 1 ml/min as already described [Masrar et al., 1990]. Radioactivity was counted on-line using a Radiometric Flow-one scintillation counter.

Other Methods

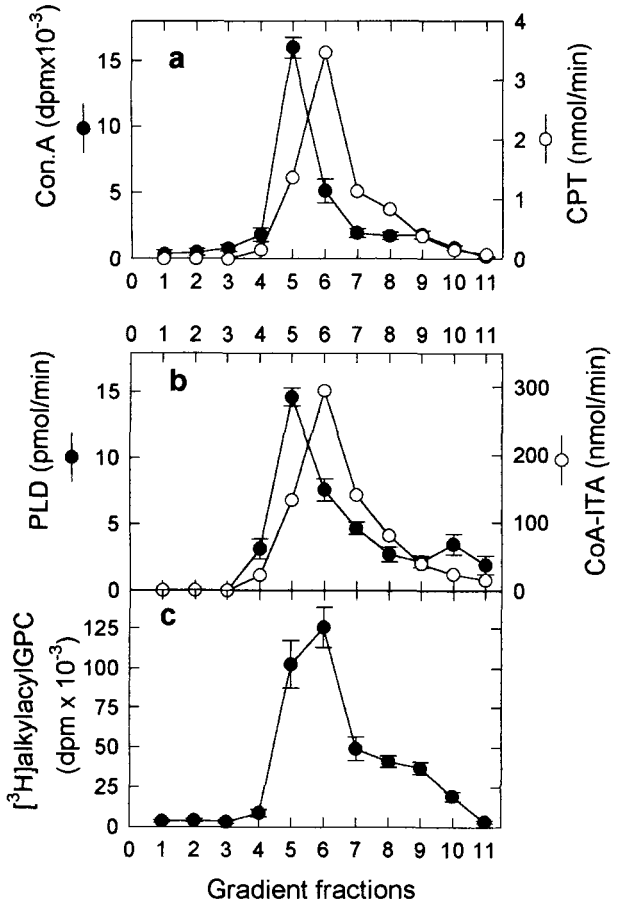
Localization of labelled lipids on TLC was performed by scanning plates with an automatic TLC linear analyser (LB2848, Berthold, Wildbad, Germany). Radioactive spots were scraped off and counted in a Packard Tricarb 4530 counter, with automatic quenching correction (Packard Instrument Co., Downers Grove, IL, USA), using Picofluor 15 as scintillation fluid. Proteins were determined according to Lowry et al. [1951] and phosphorus according to Böttcher et al. [1961].

RESULTS

Separation of Granules, Plasma Membranes, and Internal Membranes Through Two Successive Gradients

The subcellular fractionation technique we used was a slight modification of our initial procedure [Record et al., 1982]. We first carefully loaded the $1000 \times g$ supernatant onto the Percoll medium before centrifugation. This first

gradient allowed us to separate the bulk of membranes from granules as depicted in Fig. 1. Plasma membranes were monitored by [^{3}H] Concanavalin A, and endoplasmic reticulum by choline phosphotransferase. This enzyme catalyzes the terminal reaction of phosphatidylcholine denovo pathway by condensing diglycerides and CDP-choline, a pathway also operating in a highly differenciated cell such as the neutrophil [Garcia-Gil et al., 1982]. Concanavalin A together with choline phosphotransferase were recovered in the light density region (fractions 5 + 6, Fig. 1a), whereas markers for azurophil granules and tertiary granules were rejected to high densities of the gradient (Fig. 1d). The specific granules displayed a broader peak, but were also enriched in the densest part of the gradient (Fig. 1e). The profiles of phospholipase D and transacylase activities paralleled those of [3H]Con-A and cholinephosphotransferase respectively (Fig. 1b), and [3H]alkyl-labelled phosphatidylcholine mostly coincided with both markers (Fig. 1c). To separate plasma membrane from endoplasmic reticulum, another gradient system was performed. Membrane-containing fractions from gradient 1 (n° 5 + 6) were harvested and mixed with another Percoll medium having a higher pH. Whereas plasma membrane remained at the same position i.e., in the light density part, the pH induced a shift of the endoplasmic reticulum marker to the dense region of the gradient (Fig. 2a). This is the major difference between the procedure reported herein and other techniques using Percoll gradient for



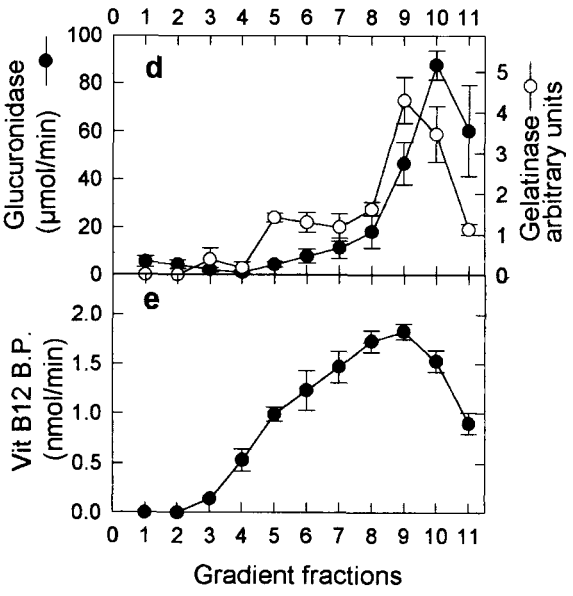


Fig. 1. Subcellular distribution of markers enzymes, phospholipase D, CoA-independent transacylase, and [^{3}H]alkylacyl-GPC on gradient system 1. After cell labelling with [^{3}H]alkyl-lyso-GPC, stimulation of the PMN (4 × 10^8) by PMA (100 nM) and cellular lysis, the post-nuclear supernatant was layered on the top of the Percoll preparation. Centrifugation parameters and markers for membranes and cellular organelles are described under Materials and Methods. Results are expressed as dpm or enzymatic units in 2 ml gradient fractions and are means ± S.E.M. of four determinations. **a:** Membranes markers: Con A = [^{3}H]Concanavalin A; CPT = choline phosphotransferase. **b:** CoA-ITA = CoA-independent transacylase and PLD = phospholipase D. **c:** [^{3}H]alkylacyl-GPC coming from [^{3}H]alkyllyso-GPC. **d:** Azurophil granule marker (glucuronidase) and tertiary granule marker (gelatinase). **e:** Specific granule marker Vit. B12BP = vitamin B 12 binding protein.

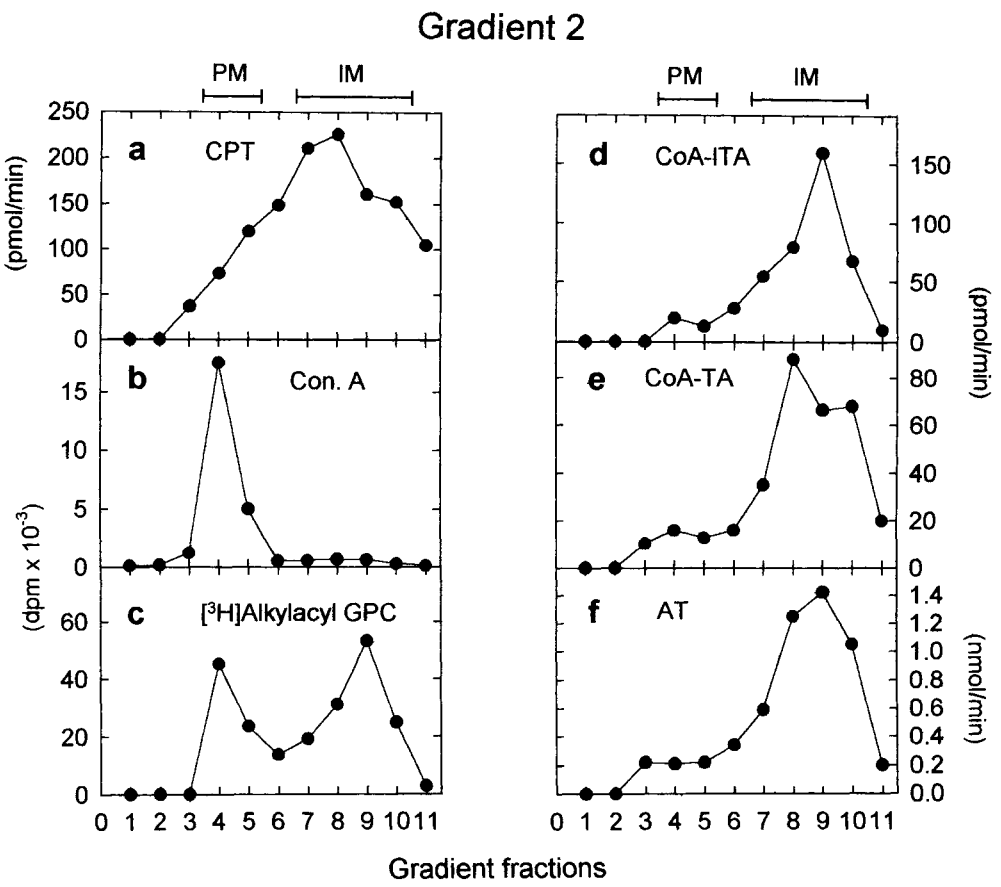


Fig. 2. Distribution of acyltransferase and transacylase activities and [^{3}H]alkylacyl-GPC, across gradient 2. Pooled fractions 5 and 6 from gradient 1 were mixed with the Percoll preparation of gradient 2. The centrifugation was carried out as indicated under Materials and Methods. Results are expressed as dpm or enzymatic units. Results are representatives of four experiments, except for profiles plotted in "f" (two independent determinations). **a:** CPT = choline phosphotransferase. **b:** Con A = [^{3}H]Concanavalin A. **c:** [^{3}H]Alkylacyl-GPC = phosphatidylcholine (1-O-[^{3}H]alkyl). **d:** CoA-ITA = Co A-independent transacylase. **e:** CoA-TA = CoA-dependent transacylase. **f:** AT = acyl-transferase. The gradient fractions corresponding to plasma membranes (PM) and to internal membranes (IM) are indicated.

neutrophil fractionation [O'Flaherty et al., 1989; Borregaard et al., 1983]. Whereas these techniques allow to separate the various types of granules but not the different membranes, our procedure leads to an opposite result: external membranes and internal membranes were well resolved on the second gradient whereas the granules were packed in the bottom of the first gradient. The fractions containing the endoplasmic reticulum marker (fractions 7 to 10) did not contain glucuronidase activity but contained some activity of specific and tertiary granules markers (Vit B12 binding protein and gelatinase). These markers represented about 6% of their respective activity in the 1000 × *g* supernatant (not shown). Recovery of the choline phosphotransferase was of the same order (Table I). However, the choline phosphotransferase activity appeared somewhat unstable during the fractionation procedure. As shown in Table I, the total choline phosphotransferase activity recovered across the first gradient accounted for 42% of the initial supernatant when recovery of CoA-independent transacylase and phospholipase D activities were 61 and 77 respectively. Thus it was not possible to estimate the respective amounts of endoplasmic reticulum and granules. Therefore, the dense membranes characterized in gradient 2 (Fig. 2, fractions 7 to 10) will be further referred as internal membranes.

Subcellular Distribution of Transacylases and Their Cellular Products

The first gradient indicates that the CoA-independent transacylase activity was not bound to granules (Fig. 1). Since activity of the enzyme

TABLE I. Recovery of Markers, CoA-Independent Transacylase, and Phospholipase D Through the Fractionation Procedure*

Marker	S1000	→ Gradient 1 Total	Fractions 5 + 6 →	Gradient 2
Concanavalin A				
dpm	34632	31286	21205	23961
% recovery	100	90.3	61.2	69.2
Choline phosphotransferase				
pmol/min	26902	11420	7308	1371
% recovery	100	42.4	27.0	5.1
Glucuronidase				
μmol/min	264	144	10.3	nd
% recovery	100	54.7	3.9	
Phospholipase D				
pmol/min	66.5	50.5	25.2	31.2
% recovery	100	76.7	37.9	46.9
Transacylase				
pmol/min	1211	735	448	810
% recovery	100	60.7	37.0	66.9

*The basis of the fractionation procedure was to obtain homogenate in which aggregates were minimized by alkaline pH. Thus the 1000 × *g* supernatant contained all the markers and enzymes presently investigated and was used as reference for yields. The sum of activities (total) across gradient 1 indicated the recovery of this first fractionation step. The activities in fractions 5 + 6 (which contained the bulk of membrane) are compared with the sum of activities across gradient 2 (since only fractions 5 + 6 of gradient 1 were further processed across gradient 2).

closely paralleled that of choline phosphotransferase, the CoA-independent transacylase appeared to be mostly located on the endoplasmic reticulum membrane. This was confirmed by the pattern observed on the second gradient. The CoA-independent transacylase sedimented in the dense part of the gradient and was completely separated from the plasma membrane fraction (Fig. 2). Surprisingly, we recovered 1.8 more CoA-independent transacylase activity across the second gradient than in fractions 5 + 6 of the first gradient (Table I). Since the enzyme activity was assayed using an acceptor system, one possible explanation could be an increased amount of arachidonate donor phospholipids in the related fractions of the second gradient. This final activity corresponded to a ten-fold enrichment as compared with the CoA-independent transacylase activity measured in the 1000 × *g* supernatant, from 152 to 1679 pmol/min/mg. The CoA-dependent transacylase and acyl transferase activities also sedimented in the dense part of the gradient corresponding to internal membranes (Fig. 2). Thus, acylation of lysoderivatives and acyl transfer between phospholipids can only take place in internal membranes. Identical patterns of the enzymes involved in phospholipids acylation were obtained when resting or PMA-stimulated neutrophils were fractionated.

Intracellular distribution of acylation products was investigated following neutrophils incubation in the presence of [^{3}H]alkyllyso-GPC. After 15 min incubation, [^{3}H]alkylacyl-GPC was recovered in internal membrane but also in plasma membrane (Fig. 2) whereas, as shown above, the acylation enzymes are not located in plasma membranes. Incubation time with [^{3}H]alkyllyso-GPC shorter than 15 min led to a higher content of acylated product in internal membranes and incubation time higher than 15 min led to higher content in plasma membrane (not shown). These data suggested an active transfer of phospholipids from their site of synthesis (internal membranes) to the plasma membrane. Molecular species of diradylglycerobenzoates, prepared from [^{3}H]alkylacyl-GPC extracted from plasma membranes or internal membranes displayed high similarities, and confirmed that alkyllyso-GPC was preferentially acylated by arachidonic acid (not shown).

Subcellular Distribution of Phospholipase D Activity and Products

Phospholipase D activity was measured with an in vitro assay using [^{3}H]alkylacyl-GPC as a liposomal substrate (Fig. 3). Optimum conditions were first investigated on mixed membranes (microsomes) in the presence of 1% ethanol. Results of Fig. 3 indicate that synthesis of [^{3}H]alkylacyl-GPethanol required membranes, cytosol, and GTPγS. Membranes and cytosol alone are devoided of activity even in the presence of GTPγS. Maximum activity was obtained with 60 μM lysosomal substrate and the reaction was linear up to 40 min (not shown). Since both membranes and cytosol were necessary for phospholipase D to be active, this assay does not allow to discriminate between a membranous enzyme activated by a cytosolic factor or the reverse. The recent discovery of arf, a small g protein activating phospholipase D [Cockcroft et al., 1994; Brown et al., 1993] rather support a membranous enzyme activated by a GTP-dependent cytosolic factor. However, the enzyme could also be activated by a membranous small g pro-

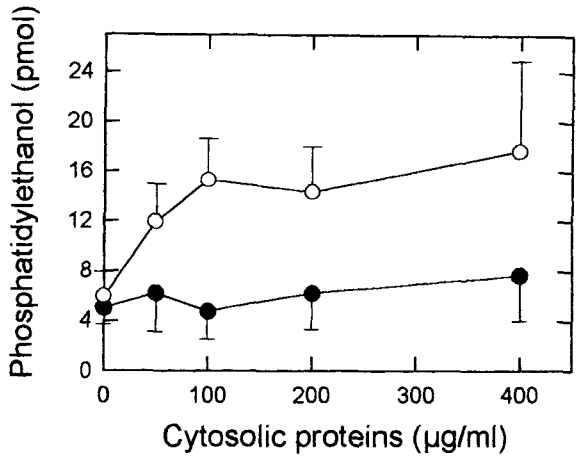


Fig. 3. In vitro dependence of phospholipase D activity upon cytosol and GTP. Neutrophil membranes (microsomes) and cytosol were obtained by a 200,000 × *g* centrifugation for 45 min of a "post-mitochondrial" supernatant (issued of a 8000 × *g* centrifugation for 30 min). Membranes (50 µg) were incubated with increasing amounts of cytosol in presence (-○-) or absence (-●-) of 10 µM GTPγS. The substrate consisted of liposomal phosphatidylcholines extracted from neutrophil after labeling with [^{3}H]alkyl-lyso-GPC. Final ethanol concentration was 1% v/v. Phosphatidylethanol was recovered as detailed in Materials and Methods.

tein related to the rho family [Bowman et al., 1993].

Which particulate fraction was necessary for phospholipase D to be active was investigated using cytosol, GTPγS, and the gradients fractions. There was no phospholipase D activity in the fractions corresponding to azurophil and tertiary granules whereas the membrane fraction of the first gradient contained phospholipase D activity (Fig. 1). When the two types of membranes were further separated on the second gradient, phospholipase D activity could be measured only with the plasma membrane fraction (Fig. 4). This fraction showed a 24-fold enrichment of enzyme activity related to S1000 supernatant from 2.4 to 57.9 pmol/min/mg (Table II).

To investigate the subcellular distribution of products of phospholipase D activity on [^{3}H]alkylacyl-GPC, cells were labelled with [^{3}H]alkyl-lyso-GPC, then stimulated with PMA in the presence of 1% ethanol before fractionation. Both [^{3}H]alkylacyl-GP (PA) and [^{3}H]alkylacyl-GPethanol (PEt) were generated in those conditions and focused only with the plasma membrane peak (Figs. 4c,d) whereas the substrate was also present in the internal membranes (Fig. 2c). In contrast, the alkylacylglycerol coming from alkylacyl-GP degradation by phosphatidate phosphatase, was also recovered in internal membranes (Fig. 4e) indicating redistribution of diglycerides from plasma membranes to internal membranes. In order to control that the profiles of phosphatidylcholine breakdown products across gradients were not a result of their redistribution during the cell fractionation, plasma membranes containing in situ generated [^{3}H]alkylacyl-GP (fractions 4 + 5 of gradient 2, Fig. 4d) and endoplasmic reticulum containing in situ generated [^{3}H]alkylacylglycerol (i.e., fractions 8 + 9 from gradient 2, Fig. 4e) were independently mixed with a cold 1000 × *g* supernatant, and processed through the gradient 2 protocol. Results reported in Figure 5 showed that the lipids were recovered in the expected part of the gradient, i.e., in the light fractions for phosphatidic acid and in the dense ones for diglycerides. The radioactivity background observed in the fractions distinct of the peaks could account for some redistribution, but was not specific of any cell structure, since the radioactivity was evenly spread.

In summary, upon neutrophil activation, the products of phospholipase D activity were recovered in the plasma membranes and this fraction was necessary for phospholipase D to be active in our in vitro assay. In contrast, acylase and transacylase activities were only present on internal membranes and their products were rapidly transfered to other membrane fractions.

DISCUSSION

In neutrophils as in other inflammatory cells, exogenous alkyllyso-GPC is rapidly taken up and specifically acylated by arachidonate, this reaction being catalysed by a CoA-independent transacylase [Kramer et al., 1984; Robinson et al., 1985; Chilton et al., 1983]. The alkyl-arachidonyl-GPC is likely to be the precursor of a number of lipid mediators when neutrophils are activated. In addition to PAF and arachidonate-derived mediators, this particular phospholipid can be the source of phosphatidic acid and diglyceride through the phospholipase D pathway [Gelas et al., 1989; Agwu et al., 1989; Billah et al., 1989]. It was then of interest to compare the subcellular localisation of CoA-independent transacylase inducing synthesis of alkyl-arachidonyl-GPC and of phospholipase D inducing its degradation. We also investigated the subcellular localisation of the products resulting from the action of both enzymes.

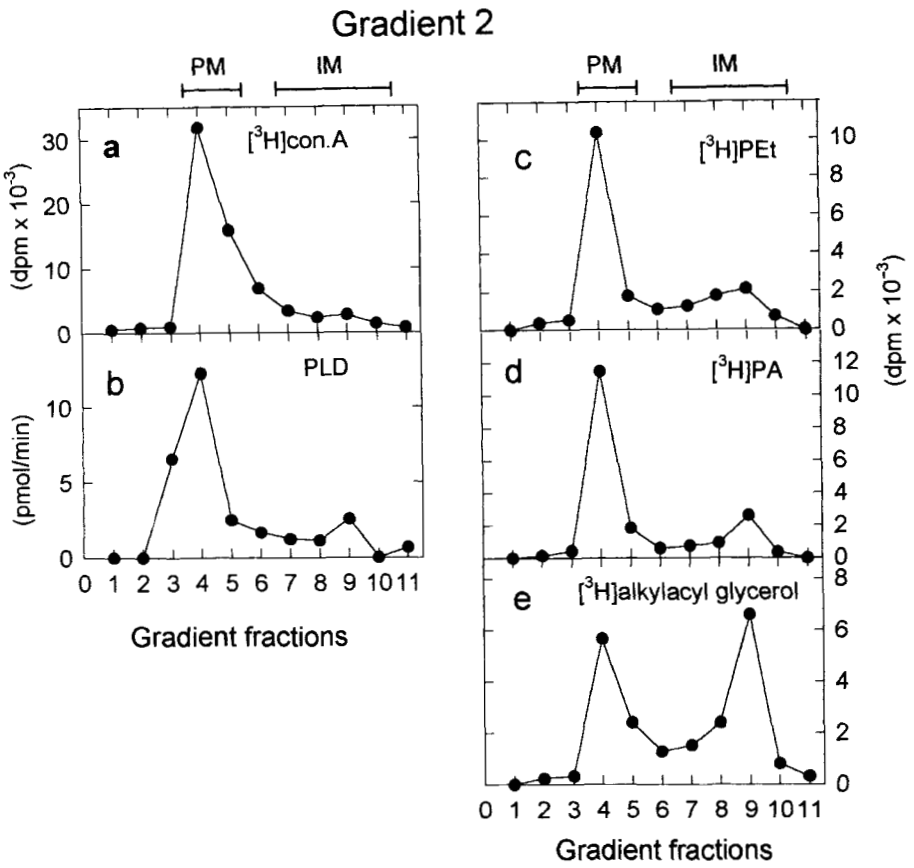


Fig. 4. Distribution of phospholipase D activity and breakdown products across gradient system 2. To analyze the distribution of phospholipase D breakdown products, PMN were prelabelled with [^{3}H]lyso-PAF. After washing, they were stimulated with 100 nM PMA in the presence of 1% ethanol for 5 min at 37°C. Pooled fractions 5 and 6 of gradient 1 were mixed with the Percoll preparation of gradient 2. Centrifugation, lipid extraction, and analysis were performed as described in Materials and Methods. [^{3}H]Con A and phospholipase D activity were analyzed on a different preparation. Results are expressed as dpm or enzymatic units and are the mean of two separate experiments. **a:** [^{3}H]Con A = [^{3}H]Concanavalin A. **b:** PLD = phospholipase D. **c:** [^{3}H]PEt = phosphatidylethanols (1-O-[^{3}H]alkyl). **d:** [^{3}H]PA = phosphatidic acids (1-O-[^{3}H]alkyl). **e:** [^{3}H]Alkylacyl glycerol = diglycerides (1-O-[^{3}H]alkyl).

The fractionation procedure developed herein allows discrimination between the bulk of granules and membranes (Fig. 1), in addition to separate plasma membranes from intracellular membranes (Figs. 2, 4). The localisation of enzymes of phospholipid metabolism (transacylases and phospholipase D) in separate cell compartments further strengthens the validation of the fractionation procedure. The bulk of membranes is separated from granules across a first gradient, performed by depositing a post-nuclear supernatant on the dense medium. The membranes are then resolved across a second gradient, in which the [^{3}H]Concanavalin A-labelled plasma membranes were clearly separated from membranes of higher density monitored by the choline phosphotransferase (Fig. 2). These latter fractions also contained some amount of secondary and tertiary granules markers, as well as the golgi marker galactosyltransferase (not shown). Therefore, this fraction has been referred as "intracellular or internal membranes."

The particulate fraction necessary to evidence phospholipase D activity in the presence of cytosol and GTPγS corresponded to plasma membranes whereas the CoA-independent transacylase was bound to internal membranes. In addition to the CoA-independent transacylase, the two other enzymes involved in arachidonate incorporation into phospholipids, the arachidonyl-CoA transferase, and the CoA-dependent transacylase, were also present in internal membranes. These results point to the question of how the product of the transacylase generated in the intracellular compartment can be the substrate of the phospholipase D acting in the plasma membrane. Therefore, we investigated the localisation of the reaction products of both

TABLE II. Specific Activities of CoA-Independent Transacylase and Phospholipase D Through the Subcellular Fractionation Procedure*

	CoA-independent transacylase activity (pmol × min^{-1} × mg^{-1})	phospholipase D activity (pmol × min^{-1} × mg^{-1})
Post nuclear supernatant	152 ± 19	2.4
Gradient 1 fractions 5 + 6	593 ± 135	11.3
Gradient 2 fractions 3 + 4	nd	57.9
Gradient 2 fractions 8 + 9	1679 ± 236	nd

*Resting neutrophils were disrupted and subfractionated as described in Methods. Determinations of transacylase and phospholipase D activities were performed in the respective peak of interest across gradient. Values are the mean ± SD of two experiments performed in duplicates.

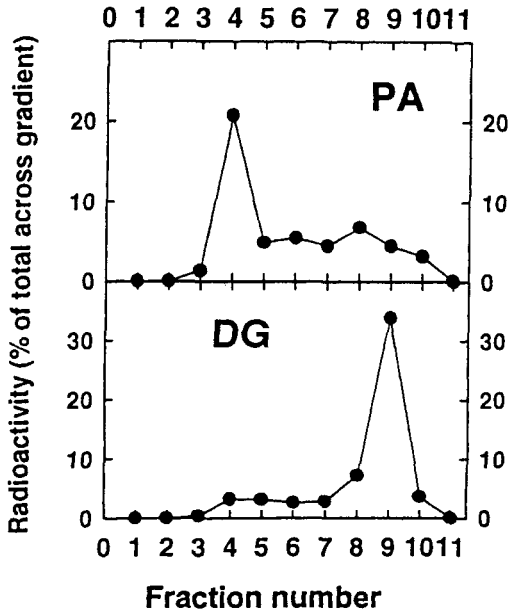


Fig. 5. Absence of lipid redistribution during the time course of cellular fractionation. Fraction 4 of gradient 2 (see Fig. 4) were mixed with a cold homogenate and further processed through the same type of gradient. Similarly, fraction 9 of gradient 2 (see Fig. 4) were mixed with another cold homogenate and processed also through gradient system 2. Lipids were extracted and phosphatidic acid and diglycerides were resolved on TLC. Results are expressed as percents of total 1-O-[^{3}H]alkyl-labelled phosphatidic acid or 1-O-[^{3}H]alkyl-labelled diglycerides across gradient.

enzymes. Following 15 min incorporation with [^{3}H]alkyllyso-GPC the labelling was recovered in both intracellular and plasma membrane fractions of neutrophils and the molecular species of the acylated compound were similarly highly acylated with arachidonate in both fractions. This observation suggests a rapid redistribution of newly synthesised phospholipids from their synthesis place, the intracellular membranes, to other membrane fractions. This redistribution is probably mediated by the phosphatidylcholine exchange protein we previously evidenced in these cells [Ribbes et al., 1991]. In that respect the phosphoinositide transfer protein has been shown to be an activator of the phosphoinositide phospholipase C [Thomas et al., 1993], known to be located in the plasma membrane. Similar to what we report herein for phosphatidylcholine, the basal synthesis of phosphatidylinositol has been shown to take place in internal membranes [Imai and Gershengorn, 1987].

Whatever the cytosolic or plasma membrane localisation of phospholipase D is, its action on the only [^{3}H]alkyl-arachidonyl-GPC present in the plasma membrane is accounted for by the recovery of [^{3}H]alkylacyl-GP and [^{3}H]alkylacyl-GPethanol only in plasma membranes when cells are stimulated (Fig. 4). In contrast, [^{3}H]alkylacylglycerol also generated when neutrophils are stimulated with PMA follow the same intracellular distribution as [^{3}H]alkyl-arachidonyl-GPC, and this is not due to a redistribution during the cell fractionation (Fig. 5).

We have shown that agonist-activated human neutrophils resplenish their phosphatidylcholine pool by diglyceride-mediated cytidylyltransferase translocation, subsequently to phospholipase D activation [Tronchère et al., 1995]. It is likely that cytidylyltransferase translocation occurs onto internal membranes [Tercé et al., 1988]. These membranes therefore contain enzymes of the de novo pathway (cytidylyltransferase) and of the remodelling pathway (transacylases). We propose that internal membranes are the site of phosphatidylcholine synthesis, and that their breakdown occurs only in plasma membranes.

The Figure 6 summarizes the results reported herein. Alkyl-lyso-GPC, substrate of CoA-independent transacylase has to cross the plasma membrane before reaching the enzyme on the internal membranes (pathway 1 of Fig. 6). Recently, we demonstrated that PAF was preferentially synthesised in mast cells through endogenous alkyl-arachidonyl-GPC despite a large excess of exogenous alkyllyso-GPC [Colard et

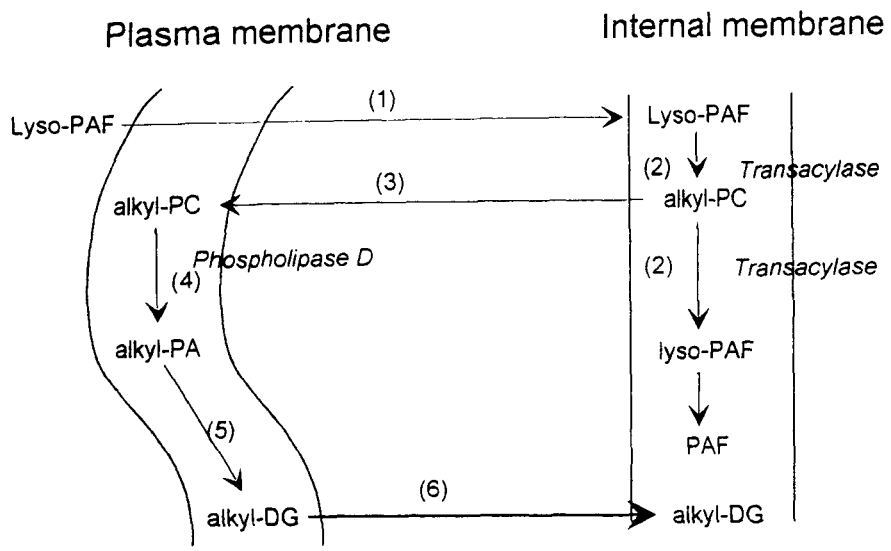


Fig. 6. Proposed intracellular metabolic fate of lyso-PAF in the human neutrophil. Exogenous lyso-PAF has to be carried to the internal membranes (*pathway 1*) to be acylated by CoA-independent transacylase (*2*) into alkylacyl-GPC, mainly alkyl-arachidonyl-GPC. This phospholipid is then hydrolyzed to lyso-PAF to generate PAF or redistributed to plasma membranes (*3*). Upon cell stimulation, alkylacyl-GPC will be hydrolysed into alkylacyl-GP (*4*), which remain in the plasma membrane, subsequently hydrolysed into diglycerides (*5*) that will equilibrate between membranes (*6*).

al., 1993]. This is consistent with the occurrence of different pools of alkyllyso-GPC, the precursor pool for PAF, being not mixed with the bulk of alkyllyso-GPC in the cell. The enzyme responsible for the last step in PAF synthesis, the acetyl transferase, is present in internal membranes and not in the plasma membrane [Ribbes et al., 1985], as the CoA-independent transacylase. Then it is tempting to assume that only the alkyllyso-GPC produced through the CoA-independent transacylase in internal membranes can be acetylated into PAF. A PAF exchange protein, distinct from the phosphatidylcholine exchange protein, accounts for its exit from the cell [Ribbes et al., 1991]. Since the cytosolic phospholipase A_2 ($cPLA_2$) is translocated to the plasma membrane in a Ca^{2+} dependent process [Channon and Leslie, 1990; Clark et al., 1991], the alkyllyso-GPC produced in the plasma membrane by $cPLA_2$ would have to follow the same intracellular processing as exogenous alkyllyso-GPC before being acetylated in internal membranes. Taken as a whole, our previous data on the topography of PAF metabolism [Record et al., 1989] together with the results reported herein on transacylases assess the internal membranes as the site of synthesis of both alkyl-arachidonyl-GPC (pathway 2 of Fig. 6) and PAF.

Upon cell stimulation, phospholipase D is activated (pathway 4). The enzyme could be activated by a membranous small g protein related to the *rho* family [Bowmann et al., 1993], or by a cytosolic one, *arf* [Cockcroft et al., 1994; Brown et al., 1993]. The location of pathway 5, i.e., conversion of phosphatidic acid into diglyceride, on the plasma membrane is consistent with the location of the phosphatidate phosphohydrolase involved in signal transduction in this cell compartement [Jamal et al., 1991]. We show that phosphatidic acid remain in the plasma membrane (Fig. 4), which suggests the lack of phosphatidic acid exchange protein, as observed in platelets [Laffont et al., 1981]. By contrast, the sterol carrier protein (non-specific Lipid Transfer Protein) which also carries diglycerides [Wirtz, 1991], is probably involved in the redistribution of phosphatidic acid-derived diglycerides to internal membranes (pathway 6). Thus the production of phosphatidylcholine-derived second messengers appears dependent upon the supply of alkylacyl-GPC to the plasma membrane (pathway 3). The phosphatidylcholine transfer protein [Ribbes et al., 1991] could play a key role in supplying substrate to the phospholipase D.

So far no tool such as specific antibodies are available for investigating the cellular localisation of either phospholipase D or transacylases. Using a resolutive fractionation procedure, we demonstrate a topographical separation between these two enzymes, which suggests additional regulatory mechanisms due to diverse

subcellular localizations of enzymes and substrate pools.

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