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Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production 1–9

Phorbol Diester Enhances Calcium Ionophore A23187-Induced [^{3}H]Acetate Incorporation Into Platelet-Activating Factor in Murine Macrophages: Predominant Incorporation Into 1-O-Acyl-2-Acetyl-sn-Glycero-3-Phosphocholine

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Pretreatment of macrophages with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has been shown to enhance the release of arachidonic acid from cell phospholipids in response to agonist stimulation. This study describes the ability of TPA to also alter calcium ionophore A23187-induced incorporation of [^{3}H]acetate into platelet activating factor (PAF). Cultured murine peritoneal macrophages were preincubated with [^{3}H]acetate (25 μCi) and TPA (10 ng/ml) for 10 min, and subsequently incubated with 0.1 μM A23187 for 0.5–10 min. Buffer and cells were then extracted and PAF resolved by normal-phase HPLC. Sequential exposure to TPA and A23187 resulted in a greatly enhanced incorporation (11,861 dpm/10^6 cells) of [^{3}H]acetate into PAF compared to TPA alone, which did not significantly influence [^{3}H]acetate incorporation into PAF, and 0.1 μM A23187, which induced minimal incorporation (688 dpm/10^6 cells). Macrophage-produced [^{3}H]PAF was resolved by HPLC, extracted, treated with phospholipase-C, and acetylated to

Abbreviations: acylPAF, 1-*O*-acyl-2-acetyl-glycero-3-phosphocholine; $C_{18:1}$PAF,1-*O*-octadec-9-cis-enyl-2-acetyl-glycero-3-phosphocholine; GPC, glycero-3-phosphocholine; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HETE, hydroxyeicosatetraenoic acid; HPLC, high performance liquid chromatography, LT, leutotriene; Me_2SO, dimethylsulfoxide; PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-glycero-3-phosphocholine); TLC, thin layer chromatography; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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facilitate quantitation of 1-*O*-alkyl-2-acetyl-GPC (PAF) from 1-*O*-acyl-2-acetyl-GPC (acylPAF). A23187 alone (1 μM) produced 72% 1-*O*-acyl-2-[^{3}H]acetyl-GPC, and A23187 (0.1 μM) following TPA pretreatment produced 81% 1-*O*-acyl-2-[^{3}H]acetyl-GPC. Less than 2% of the radioactivity of acylPAF was in the acyl moiety. These data support a role for protein kinase C in modulating agonist-induced PAF synthesis. The results also suggest that acetyltransferase of murine macrophages does not possess specificity for 1-*O*-alkyl-2-lyso-GPC, and that availability of specific species of lyso-phospholipid may determine the type of PAF produced.

Key words: phospholipase A_2, murine peritoneal macrophages, calcium ionophore A23187, 12-*O*-tetradecanoylphorbol-13-acetate

Several investigations have suggested a close link between the biosynthesis of platelet-activating factor (PAF) and eicosanoid metabolites of arachidonic acid in neutrophils [1,2] and macrophages [3]. These cells have been shown to contain relatively high amounts of 1-*O*-alkyl-2-arachidonoyl-sn-glycero-3-phosphocholine (alkyl-arachidonoyl-GPC) [19,20], and the action of phospholipase A_2 on this phospholipid yields alkyl-lyso-GPC (lyso-PAF) and free arachidonic acid, the precursors for PAF and eicosanoids, respectively [1,18]. This [4] and other laboratories [5–10] have shown that pretreatment of leukocytes and platelets with the tumor promoter and protein kinase C activator, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), synergistically enhances the release of arachidonic acid and biosynthesis of eicosanoids in response to calcium ionophore A23187. An enhanced release of arachidonic acid should coincide with elevated levels of lyso-PAF and the potential for enhanced PAF production. The purpose of this study was therefore to determine if TPA pretreatment of murine peritoneal macrophages would enhance agonist-induced incorporation of [^{3}H]acetate into PAF.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]Acetate, 1-*O*-[^{14}C]palmitoyl-2-lyso-GPC, and 1-*O*-palmitoyl-2-[^{14}C]arachidonoyl-GPC were obtained from DuPont-New England Nuclear (Wilmington, DE), and 1-*O*-[^{3}H]hexadecyl-2-lyso-GPC was from Amersham (Arlington Heights, IL). Pyridine and acetic anhydride were reagent grade, and all other solvents were spectroquality or HPLC grade; all were obtained from Fisher Scientific Co. (Cincinnati, OH). Phospholipase C (Bacillus cereus, Type XI), calcium ionophore A23187 (free acid), 1-*O*-octadec-9-cis-enyl-2-acetyl-GPC ($C_{18:1}$PAF), 1,2-dioleoyl-GPC, 1-oleoyl-2-acetyl-sn-glycerol, Tris base, and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI-1640 culture medium was obtained from GIBCO (Grand Island, NY). Fetal bovine serum was obtained from Hyclone Laboratories (Logan, UT), and endotoxin levels were less than 0.1 ng/ml. TPA was obtained from Chemicals for Cancer Research (Eden Prairie, MN). Silica gel G TLC plates (250 μm) were obtained from Analabs (Norwalk, CT). All other chemicals were reagent grade or better and were obtained from Fisher Scientific Co.

Incorporation of [^{3}H]Acetate Into Platelet-Activating Factor

Resident peritoneal macrophages were obtained from female CD-1 mice (8–12 weeks old) as previously described [4], and maintained in culture (35 × 15-mm

dishes) in RPMI-1640 medium supplemented with 10% fetal bovine serum and gentamycin (50 μg/ml) at 37°C in an atmosphere of 5% CO_2/95% air for 16–24 h. Cells (4–7 × 10^5/dish) were rinsed 3 times with HBSS and preincubated in 2 ml Krebs buffer with 25 mM HEPES containing 25 μCi [^{3}H]acetate and TPA (10 ng/ml) or ME_2SO (0.25% or 0.5%) for 10 min at 37°C. Calcium ionophore was then added to yield final concentrations of 0.1–5 μM. Final concentrations of Me_2SO were 0.5% or 1% and had no effect on [^{3}H]acetate incorporation into PAF. Incubations were continued at 37°C for 0.5% 10 minutes, and terminated by pipetting the incubation buffer into a glass extraction tube containing 1.79 ml chloroform/methanol/acetic acid (0.5:1.25:0.04, v/v/v) and addition of 1 ml ice-cold methanol to the dish. The dish was scraped with a rubber-policeman, rinsed with cold methanol, and all methanol fractions pooled with the buffer in the extraction tube; C_{18}:PAF (20 μg) was added as carrier and lipids extracted by the method of Bligh and Dyer [11]. The lower choroform phase was washed once with methanol/water (10:9, v/v), evaporated under N_2, and stored in chloroform at −20°C. The number of cells per dish was determined in two dishes from each experiment by exposing adherent cells to 2% lidocaine in calcium-magnesium-free HBSS followed by gentle scraping with a rubber-policeman and enumeration of the suspended cells in a hemocytometer.

Separation of Radiolabeled PAF

An aliquot of the lipid extract was used to determine the incorporation of [^{3}H]acetate into PAF following resolution by normal phase HPLC. The separation of PAF was carried out using a LiChrospher Si100 column (25 cm × 4.6 mm, 10 μm) from EM Sciences (Cherry Hill, NJ) by a modification of the method of Jackson et al. [12]. The eluting solvent was acetonitrile/methanol/sulfuric acid (130:5:0.05, v/v/v) at a flow rate of 0.8 ml per minute, and ultraviolet absorption was monitored at 205 nm. Fractions of eluate were collected in scintillation vials, Aquasol-2 was added, and radioactivity was determined by liquid scintillation counting. The identity of peaks was determined on the basis of co-chromatography with authentic standards. Counting efficiency was determined by the external standard method and used to correct for quenching. Peak radioactivity was calculated by subtraction of baseline counts from each peak fraction prior to their summation, and the result normalized to the number of cells per dish.

Derivatizations and TLC Procedures

1-*O*-[^{3}H]hexadecyl-2-acetyl-GPC and 1-*O*-[^{14}C]palmitoyl-2-acetyl-GPC were prepared by acetylation of the corresponding lyso-GPC as described by Mueller et al. [13], and used to assess extraction efficiency. Briefly, lyso-GPC, an equimolar amount of 4-dimethylaminopyridine and 100-fold molar excess of both acetic anhydride and triethylamine were dissolved in methylene chloride and stirred at room temperature for 24 h. The 1-*O*-radyl-2-acetyl-GPC produced by the above procedure and biosynthesized [^{3}H]PAF (isolated on the HPLC efluent) was extracted as described by Jackson et al. [12] by addition of one volume of chloroform, one volume of methanol, and two volumes of water. The upper phase was discarded and lower phase washed with two volumes of the upper phase of mixed chloroform-methanol-water (1:1:0.9, v/v/v). The extraction efficiency for 1-*O*-[^{3}H]hexadecyl- versus 1-*O*-[^{14}C]palmitoyl-2-acetyl-GPC was found to be 81.7% ± 0.4% (mean ± SEM, n=4) and 74.2% ± 0.2% (n=4), respectively. Biosynthesized [^{3}H]PAF was further analyzed by treat-

ment with phospholipase C and acetylation as previously described [14] to generate the corresponding 1-*O*-radyl-2,3-diacetylglycerols. Standards were generated by PLC treatment and acetylation of $C_{18:1}$PAF, and acetylation of 1-oleoyl-2-acetyl-sn-glycerol. An aliquot of derivatized PAF was then subjected to TLC on silica gel G (250 μm) to separate 1-*O*-alkyl-2,3-diacetylglycerol from 1-*O*-acyl-2,3-diacetylglycerol. Samples and standards were first developed in hexane-diethyl ether (1:1) for 7–8 cm and then in toluene to within 2–3 cm of the top of the plate. Spots were visualized by brief exposure to iodine vapor, 2.5-mm section of gel scraped into scintillation vials for determination of radioactivity by liquid scintillation counting. Another aliquot of derivatized PAF was incubated with 2% concentrated sulfuric acid in methanol at 90°C for 2 h to transmethylate 1-*O*-acyl moieties to corresponding fatty acid methyl esters. 1-*O*-palmitoyl-2-[^{14}C]arachidonoyl-GPC was identically treated to assess efficiency of transmethylation and found to be 92%. The fatty acid methyl esters were extracted into diethyl ether, solvent evaporated under N_2, and redissolved in hexane. Samples and authentic standards for fatty acid methyl ester (methyl oleate) and free fatty acid (arachidonic acid) were separated by TLC on silica gel G using a mobile phase of petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v) [15].

RESULTS

Effect of TPA on Ionophore-Induced Incorporation of [^{3}H]Acetate Into PAF

Cultured macrophages exposed to calcium ionophore A23187 exhibited a time and concentration dependent incorporation of [^{3}H]acetate into PAF (Fig. 1). The amount of radioactivity in PAF increased rapidly for 2 min before steadily declining towards control levels. This pattern is consistent with the presence of acetylhydrolase activity and rapid conversion of newly synthesized PAF to lyso-PAF [16]. A peak cochromatographing with phosphatidylcholine (peak 1 in Fig. 2) contained minimal incorporated radioactivity. Preincubation of macrophages with TPA resulted in a large and selective enhancement in A23187-induced incorporation of [^{3}H]acetate into

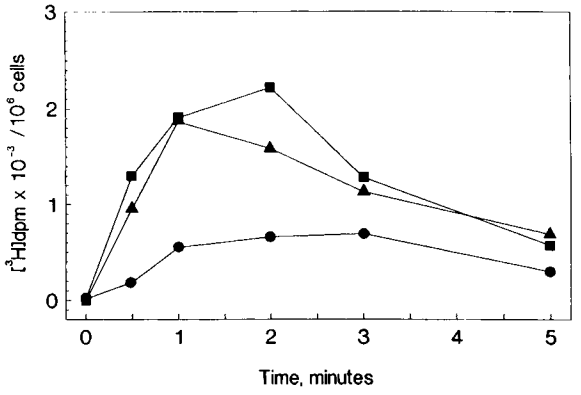

Fig. 1. Time course of calcium ionophore A23187-induced incorporation of [^{3}H]acetate into platelet-activating factor. Murine peritoneal macrophages were preincubated with 25 μCi [^{3}H]acetate for 10 min prior to exposure to 0.1 μM (-●-), 1 μM (-■-), or 5 μM (-▲-) A23187 for various times. The incubation buffer and cells were pooled and lipids extracted [11]; [^{3}H]PAF was separated by normal-phase HPLC and radioactivity quantitated. The points represent the mean of duplicate dishes from two separate experiments.

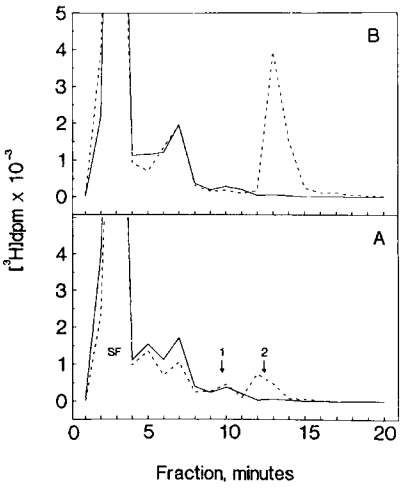

Fig. 2. Separation of platelet-activating factor by HPLC: Pattern of incorporation of [^{3}H]acetate into lipid fractions. Murine peritoneal macrophages were preincubated with 25 μCi [^{3}H]acetate and Me_2SO (0.5%) or TPA (10 ng/ml) for 10 min. Calcium ionophore A23187 (0.1 or 1 μM) or Me_2SO) (0.5%) were then added and cells incubated for an additional 3 min. Both before (**solid line**) and after (**dashed line**) the second incubation, buffer and cells were pooled and lipids extracted [11]. Equal aliquots of lipid extract for each treatment were subjected to normal-phase HPLC for separation and quantitation of [^{3}H]PAF. **A:** Preincubation with Me_2SO followed by incubation with ionophore (1 μM). **B:** Preincubation with TPA followed by incubation with ionophore (0.1 μM). Peaks were identified by coelution with authentic standards: peak 1, phosphatidylcholine (bovine braine); peak 2, 1-*O*-octadec-9-cis-enyl-2-acetyl-glycero-3-phosphocholine (C_{18}:PAF); SF, solvent front. Each line represents the average profile of duplicate dishes from a representative experiment.

PAF (Fig. 2b). This enhanced incorporation followed a similar, but delayed, biphasic temporal pattern to that observed for ionophore alone (Fig. 3). [^{3}H]PAF was still greatly elevated 10 min after initiation of ionophore exposure when cells were pretreated with TPA, whereas it had returned to baseline in the absence of TPA pretreatment. No effect of TPA on incorporation of radiolabel into phosphatidylcholine was observed (peak 1 in Fig. 2).

Characterization of [^{3}H]PAF

The chemical nature of the [^{3}H]material co-eluting with authentic PAF was further characterized for several reasons: 1) to verify further that the [^{3}H] peak was PAF; 2) to investigate the possible presence of 1-*O*-acyl-2-[^{3}H]acetyl-GPC, since its concurrent synthesis with 1-*O*-alkyl-acetyl-GPC has been described [13,17]; and 3) to investigate the potential incorporation of [^{3}H]acetate into the 1-*O*-acyl portion of 1-*O*-acyl-2-acetyl-GPC. Lipid extracts from four to six cultures exposed to A23187 (1 μM) alone or following pretreatment with TPA (10 ng/ml TPA and 0.1 μM A23187) were pooled, the [^{3}H]PAF peak was collected following HPLC separation and extracted as described in the Experimental Design section. An aliquot of extracted [^{3}H]PAF peak was then resubjected to HPLC, and 90% of the radioactivity again coeluted with authentic PAF. The purified material was treated with phospholipase C to generate the corresponding 1-*O*-radyl-2-acetyl-sn-glycerol and acetylated to pro-

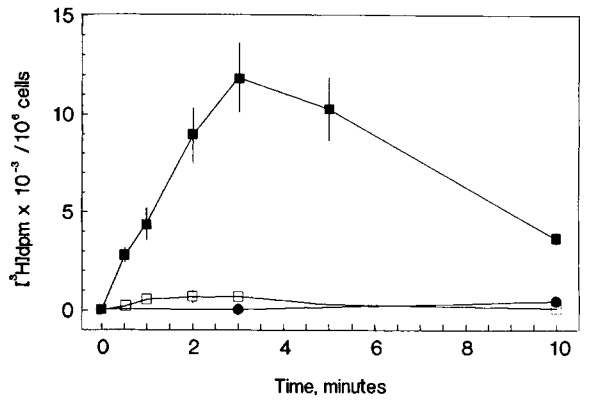

Fig. 3. Effect of TPA on the time course of calcium ionophore A23187-induced [^{3}H]acetate incorporation into PAF. Murine peritoneal macrophages were treated as follows: pretreatment with TPA (10 ng/ml) for 10 min followed by exposure to either calcium ionophore A23187 (0.1 μM, ■) or Me_2SO (0.5%, ●); pretreatment with Me_2SO (0.5%) for 10 min followed by calcium ionophore A23187 (0.1 μM, □) or Me_2SO (0.5%, not shown). Zero time was the beginning of exposure to A23187 and at the various times indicated both incubation buffer and cells were pooled and lipids extracted [11]; [^{3}H]PAF was separated by normal-phase HPLC and radioactivity quantitated. Exposure to Me_2SO alone had no measurable effect on [^{3}H]acetate incorporation into PAF. The points represent the mean of duplicate dishes from three separate experiments, and the vertical lines represent the SEM.

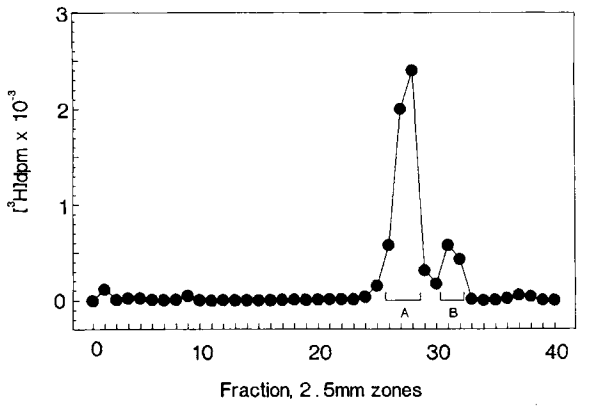

Fig. 4. Separation by TLC of 1-*O*-acyl-[2-^{3}H],3-diacetate and 1-*O*-alkyl-[2-^{3}H],3-diacetate. Murine peritoneal macrophages were preincubated with 25 μCi [^{3}H]acetate and TPA (10 ng/ml) for 10 min followed by calcium ionophore A23187 (0.1 μM) for an additional 3 min. Both incubation buffer and cells were pooled, lipids extracted [11], and [^{3}H]PAF separated by normal-phase HPLC. The eluting [^{3}H]PAF was recovered and derivatized to the corresponding 1-*O*-radyl-2-^{3}H],3-diacetate and subjected to TLC on silica gel G as previously described [14], to separate 1-*O*-acyl-[2-^{3}H],3-diacetate (peak **A**) from 1-*O*-alkyl-[2-^{3}H],3-diacetate (peak **B**).

duce the corresponding radiolabeled 1-*O*-acyl-2,3-diacetylglycerol or 1-*O*-alkyl-2,3-diacetylglycerol. Separation by TLC (Fig. 4) revealed a predominant incorporation of [^{3}H]acetate into acylPAF for both cultures treated with A23187 alone (71.8% $\pm$ 2.5%, n=3) and A23187 (0.1 μM) following TPA (10 ng/ml) pretreatment (81.4% $\pm$ 0.4%, n= 3). Transmethylation of derivatized [^{3}H]PAF revealed that less than 2%

of the radioactivity of acylPAF was in the acyl moiety. The incorporated radioactivity of [^{3}H]PAF was therefore primarily as 1-*O*-radyl-2-[^{3}H]acetyl-GPC.

DISCUSSION

The present study demonstates that TPA and calcium ionophore A23187 also interact synergistically to enhance the net incorporation of [^{3}H]acetate into PAF and acylPAF. Recently, an interaction of phorbol diester with calcium ionophore A23187 on [^{3}H]acetate incorporation into PAF was described for human neutrophils [21], but less than a twofold enhancement was observed. In contrast, a 17-fold enhancement was observed in the present study. Possible explanations for this difference are the use of human neutrophils versus murine macrophages, or possibly the use of high concentrations of A23187 (10 μM) in the former study. In another study using human neutrophils [22], the ability of lipoxygenase products of arachidonic acid (5-HETE and LT-B_4) to enhance A23187 (0.5 μM)-induced [^{3}H]acetate incorporation into PAF has been demonstrated. Again, the enhancement (approximately threefold) was much less than that observed in the present study, and lends support to possible differences in response between murine macrophages and human neutrophils.

The simultaneous enhancement of both PAF and eicosanoid biosynthesis suggests that a point of metabolic control, common to both pathways, has been modulated by TPA. An ability of TPA to alter the responsiveness of phospholipase A_2 to stimulatory factors is consistent with this possiblity. Studies by others using inhibitors of cyclooxygenase [8] or lipoxygenase [6] to prevent metabolism of released arachidonic acid, or high concentrations of fatty acid-free bovine serum albumin to prevent both metabolism and re-esterification of released arachidonic acid [7] have shown that TPA enhances agonist-induced release of free arachidonic acid, and therefore supports an effect of phorbol diesters on phospholipase A_2 activation. Phorbol diesters and diacylglycerols bind to protein kinase C and increase its activity, therefore implicating a phosphorylation event in the sensitization of phospholipase A_2. Increases in the activity of acetyltransferase (1-*O*-alkyl-sn-glycero-3-phosphocholine:acetyl-CoA acetyltransferase), which catalyzes the incorporation of acetate into PAF, may also be involved. Acetyltransferase activity has been shown to be increased by a phosphorylation process, but there is disagreement concerning the role of protein kinase C [23,24] versus other protein kinases [25,26].

Another possibility concerns the regulatory role of calcium in both phospholipase A_2 [27] and acetyltransferase activity [35]. TPA [28] and diacylglycerols [29,30] have both been shown to increase the affinity of protein kinase C for calcium, resulting in an activation of phosphotransferase activity. It is therefore reasonable to suggest that TPA may modify the responsiveness of phospholipase A_2 and acetyltransferase in the intact cell by similarly increasing their affinity for calcium, and thus lowering the threshold concentration of intracellular calcium necessary for activation. Consistent with this hypothesis is the reported ability of TPA to sensitize macrophages to calcium ionophore A23187-induced eicosanoid biosynthesis [4] and, as reported here, PAF biosynthesis.

Lastly, an effect of TPA on the conversion of [^{3}H]acetate to [^{3}H]acetyl-CoA must be considered as potentially contributing to the observed effect on [^{3}H]acetate incorporation into PAF. TPA exposure has been shown to result in increased incorporation (2–3 fold) of radiolabeled acetate into cholesterol esters [31] of human skin

fibroblast and phospholipids [31,32] of human fibroblasts and leukemic cells. However, these effects occurred only after relatively long incubations with TPA ($\geqslant$3 h). Preincubations with TPA in the present study were carried out for 10 min, and no effect on incorporation of [^{3}H]acetate into lipids other than PAF was observed. It is therefore considered unlikely that this effect would constitute a major contribution to the observed TPA-induced enhancement of ionophore-induced [^{3}H]acetate incorporation into PAF, but confirmation of this will require further experimentation.

Previous studies have shown that stimulated leukocytes produce PAF of two major chemical types characterized by the presence of an ether or ester at the 1-position of the glycerol backbone [13,17]. There was a wide descrepancy, however, in the reported distribution of these two PAF types. Mueller et al. [13], using methods similar to the present study, found 25.7% 1-*O*-acyl-2-[^{3}H]acetyl-GPC production by shellfish glycogen-ellicited rabbit leukocytes and 13% by human peripheral leukocytes following stimulation with ionophore A23187. Statouchi et al. [17] found as much as 50% 1-*O*-acyl-2-acetyl-GPC (measured by gas chromatography-mass spectrometry) produced by human leukocytes stimulated with ionophore A23187. The present study provides evidence, in murine peritoneal macrophages, for a greater incorporation of acetate into 1-*O*-acyl-2-acetyl-GPC than 1-*O*-alkyl-2-acetyl-GPC. The significance of this finding to cell function remains to be determined. Structure activity studies have demonstrated that the 1-*O*-acyl-2-acetyl-GPC possesses lower (300-fold) activity than 1-*O*-alkyl-2-acetyl-GPC [33] towards platelet aggregation, but still possessed activity at 10^{-8}–10^{-9} M.

Several studies on acetyltransferase have investigated its specificity for different lysophospholipids and demonstrated that 1-*O*-acyl-2-lyso-GPC was utilized, but that 1-*O*-alkyl-2-lyso-GPC was the preferred substrate [34–36]. Furthermore, 1-*O*-acyl-2-lyso-GPC was shown to be a competitive inhibitor (at constant acetyl-CoA concentration) of 1-*O*-alkyl-2-acetyl-GPC formation mediated by acetyltransferase in rat spleen microsomes [36]. This suggests that high amounts of 1-*O*-acyl-2-lyso-GPC relative to 1-*O*-alkyl-2-lyso-GPC released in stimulated cells could result in a preponderance of 1-*O*-acyl-2-acetyl-GPC production, and that this may be the case for murine peritoneal macrophages. There could also be species differences in the lyso-phospholidpid substrate specificity of acetyltransferase, although determination of this will require further study. An additional contribution to a specific distribution of chemical species of PAF could result from the substrate specificity of phospholipase A_2, and thus the availability of specific species of lyso-phospholipid for PAF biosynthesis. It is clear, however, that cells are capable of producing a variety of sn-2 acetylated phospholipids in response to stimulatory factors, and future studies of PAF biosynthesis should address their potential presence.

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