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1-O-ALKYL-2-ACYL-SN-GLYCERO-3-PHOSPHOCHOLINE: A NOVEL SOURCE OF ARACHIDONIC ACID IN NEUTROPHILS STIMULATED BY THE CALCIUM IONOPHORE A23187

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Summary. Rabbit peritoneal neutrophils incorporated [^{14}C]arachidonic acid into seven molecular species of choline-containing phosphoglycerides. These 2-[^{14}C]arachidonoyl species differed with respect to the alkyl ether or acyl residue bound at the sn-1 position; four of the seven were ether-linked. Stimulation with calcium ionophore A23187 induced a proportionate release of arachidonate from all seven molecular species: 40% of the released arachidonate came from alkyl ether species. Thus, 1-O-alkyl-2-arachidonoyl-sn-glycero-3-phosphocholine (GPC) is a significant source of metabolizable arachidonic acid. Since 1-O-alkyl-2-lyso-GPC is the metabolic precursor of platelet activating factor, these results further interrelate pathways forming arachidonate metabolites and platelet activating factor; they also supply a rationale for the observation that both classes of stimuli form concomitantly during cell activation.

Introduction. Stimulation of PMN[1] by phagocytosis or the calcium ionophore A23187 results in deacylation of arachidonate from membrane PC and PI via a calcium-dependent phospholipase A_2 (1). The exact source of metabolizable arachidonate in PC is of interest for two reasons. 1) Neutrophil PC has been assumed to be 1,2-diacyl-GPC, but Mueller and co-workers (2) have found that 45% of this fraction is composed of 1-O-alkyl-2-acyl-GPC containing 29% of the 20:4 found in PC. Alkyl ether-linked PC is not commonly differentiated from diacyl-linked PC using normal analytical techniques. Consequently, it is not

[1]The abbreviations used are: PMN, polymorphonuclear neutrophils; GPC, sn-glycero-3-phosphocholine; PC, choline-linked phosphoglyceride fraction; PI, inositol-linked phosphoglyceride fraction; DMSO, dimethylsulfoxide; HPLC, high performance liquid chromatography; PAF, platelet activating factor; 20:4, arachidonic acid; 18:0, stearic acid; 16:0, palmitic acid; 18:1, oleic acid; HETE, any hydroxylated eicosatetraenoic acid; 5-L-HETE, 5-L-hydroxy-6,8,11,14-eicosatetraenoic acid; LTB_4, 5,12-dihydroxyeicosatetraenoic acid or Leukotriene B_4; TLC, thin-layer chromatography.

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known if 20:4 is released from this fraction by cell stimulation. 2) In PMN and macrophages, alkyl ether-linked PC, when deacylated at the sn-2 position, is the immediate precursor for 1-O-alkyl-2-acetyl-GPC or platelet activating factor (3-6). Metabolites of arachidonate and PAF have overlapping spectra of bioactivities (7); are formed concomitantly in activated PMN as well as other cell types (1,7-10); and can act synergistically to promote cell function (11). Release of radiolabel from 1-O-alkyl-2-[^{14}C]arachidonoyl-GPC would supply a biochemical rationale for some of the observed interactions between two distinctly different classes of lipid stimuli. Accordingly, we have resolved neutrophil [^{14}C]arachidonoyl-containing PC into its component molecular species and have measured the A23187-induced hydrolysis of [^{14}C]arachidonate from both diacyl and alkyl ether-linked species of PC.

Experimental Procedures

Materials: Phospholipid standards were purchased from Serdary Research Laboratories, London, Ontario, Canada, as were 1-16:0-2-lyso-GPC and 1-18:0-2-lyso-GPC. 1-O-hexadecyl-2-lyso-GPC and 1-O-octadecenyl-2-lyso-GPC were synthesized in collaboration with Dr. Claude Piantadosi and Jefferson R. Surles, University of North Carolina at Chapel Hill. A23187 was purchased from Calbiochem-Behring Corporation, La Jolla, CA. Choline chloride was from Aldrich Chemical Company. Preparation of standards: 1-O-hexadecyl-2-lyso-GPC, 1-O-octadecenyl-2-lyso-GPC and 1-acyl-2-lyso-GPC were acylated by reaction with a 2-molar excess of the desired acyl anhydride (NuChek Prep Inc., Elysian, MN) in a solvent consisting of 3:1 dry chloroform/dry pyridine containing a 4-molar excess of 4-dimethylaminopyridine. After stirring 18 h at ambient temperature, the resulting phospholipid was recovered by preparative TLC and purified to homogeneity by reverse-phase HPLC. The composition of the standards was confirmed by mass spectrometry and by fatty acid analysis employing gas-liquid chromatography. Radiolabeled fatty acids: [9,10-^{3}H(N)]oleic acid, 9.5 Ci/mM, and 1-[^{14}C]arachidonic acid, 58.4 mCi/mM, were purchased from New England Nuclear. [9,10-^{3}H(N)]stearate, 9.5 Ci/mM, was prepared from radiolabeled oleic acid by catalytic hydrogenation. The methyl ester of the resulting radiolabeled product migrated with standard methyl stearate by argentation TLC (12) and was confirmed to be radiolabeled stearate by gas-liquid chromatography utilizing an in-line proportional counter. The [^{3}H]stearate was diluted with unlabeled stearate to a specific activity equal to that of the 1-[^{14}C]arachidonate, 58.4 mCi/mM.

Preparation and labeling of PMN: PMN were obtained from rabbit peritoneal exudates 4 h after an intraperitoneal injection of 1% shellfish glycogen as previously described (13). These preparations were 95 to 98% pure and contained fewer than 20 platelets/100 PMN. PMN were suspended in TRIS-buffered Hanks' balanced salt solution, pH 7.4, to 3.5 x 10^{7}/ml. Radiolabeled fatty acids were added in a maximum volume of 1 µl ethanol per ml of cell suspension (0.03 µCi/ml, final concentration of 0.5 µM for each fatty acid) and the mixture incubated with gentle shaking at 37°C for 2 h (1). The labeled cells were washed free of unincorporated label and resuspended to 7 x 10^{7}/ml in Hanks' solution containing 2.8 mM calcium. The PMN were then challenged for 5 min at 37°C by the addition of an equal volume of Hanks'

solution containing 4 μM A23187 (added in DMSO; final concentration, 0.04%, v/v) or 0.04% DMSO alone.

Lipid extraction and analysis: The reaction was stopped by extracting the lipids from the entire mixture by the method of Bligh and Dyer (14) in which the methanol contained 2% acetic acid. Distribution of radiolabels in phospholipids was determined by TLC on silica gel H in a solvent system of chloroform/methanol/acetic acid/water (50:25:8:4, v/v). Areas migrating with standards were scraped and the amount of radioactivity determined by liquid scintillation spectrometry. The PC fraction was isolated by preparative TLC using the same solvent system as described above. The distribution of radiolabeled arachidonate in 1-O-alkyl-2-acyl-GPC and diacyl-GPC was determined in two ways. The first involved preparation of 1-radyl-2-acyl-3-acetylglycerols from purified PC by acetolysis (15). The purified 1-radyl-2-acyl-3-acetylglycerols were then resolved into their component 1-O-alkyl-2-acyl and diacyl species by TLC on silica gel H using a solvent system of hexane/ether (60:40, v/v). The distribution of label in these products was determined by zonal profile scanning (16). The second method utilized the system described by Patton and co-workers (17) for the separation of intact phospholipids into their individual species by reverse-phase HPLC. Details are given in the legends to the figures. Peaks containing radiolabel were identified by comparison to the elution of known standards and by fatty acid analysis. Recovery of radioactivity from the column was greater than 90%.

Results and Discussion. After 2 h of incubation with radiolabeled fatty acids, rabbit PMN incorporated 80% of the [^{14}C]20:4 and 70% of the [^{3}H]18:0 added to the system. Typically, the total radioactivity in lipids extracted from PMN and medium was approximately 450,000 dpm [^{3}H]18:0 and 400,000 dpm [^{14}C]20:4. Of this, approximately 87% of the [^{14}C]20:4 and 50% of the [^{3}H]18:0 was incorporated into phospholipids. As shown in Table 1, PC contained approximately 24% of the [^{14}C]20:4 and 16% of the [^{3}H]18:0 incorporated by the cell. When the PMN were stimulated by A23187, [^{14}C]20:4 was released from both PC and PI (Table 1). Concomitantly, there was a corresponding increase in [^{14}C] label in the neutral lipid fraction (see Table 1). In contrast, there was no significant change in [^{3}H]18:0 with ionophore stimulation. This observation is consistent with the interpretation that [^{14}C]20:4 is released by phospholipase A_2 (1,18).

Since 30 to 40% of PMN phospholipid has been shown to be PC as compared to between 1 and 7% for PI (2,19,20) and based on the 20:4 content of the PC and PI, it was apparent that PC is the major source of 20:4 in PMN stimulated with A23187. The contribution of the 1-O-alkyl-2-acyl-GPC to the free 20:4 pool was next assessed. As shown in Fig. 1, analysis of the diglyceride acetates derived from the labeled PC revealed that 50% of the [^{14}C]20:4 was

Table 1

Distribution of Radiolabeled Arachidonic Acid and Stearic Acid in Neutrophil Phospholipids Before and After Treatment With A23187

Phospholipid	Treatment	[^{14}C]Arachidonic Acid		[^{3}H]Stearic Acid	
		% Total dpm	% Change	% Total dpm	% Change
PC	Control	24.3 ± 3.4	-	16.1 ± 3.0	-
	A23187	13.7 ± 1.6	-12.5	14.9 ± 7.0	-2.4
PS/PI	Control	31.8 ± 5.4	-	16.1 ± 4.0	-
	A23187	20.6 ± 1.8	-12.7	16.4 ± 4.0	-1.6
PE	Control	20.4 ± 4.0	-	11.1 ± 1.0	-
	A23187	19.8 ± 6.0	-1.2	11.5 ± 2.0	+0.7
Neutral lipid	Control	13.0 ± 9.0	-	53.6 ± 6.0	-
	A23187	43.1 ± 8.8	+26.1	54.1 ± 12.0	+2.5

Radiolabeled PMN were suspended to 7 x 10^7 PMN/ml in Hanks' solution with 2.8 mM calcium and challenged with an equal volume of Hanks' solution (Control) or Hanks' solution containing 4 μM A23187 (A23187). Final challenge conditions were: 3.5 x 10^7 PMN/ml, 1.4 mM calcium and 2 μM A23187. The reaction was stopped after 5 min at 37°C and the lipids were extracted from the entire mixture. Lipid extracts were analyzed by thin-layer chromatography as described in Experimental Procedures. The amount of radioactivity in each phospholipid species is expressed as a percent of the total radioactivity recovered from the thin-layer plate with the percent change determined by the difference between corresponding control and challenged values. Total radioactivity in a typical extract was approximately 450,000 dpm [^{3}H]18:0 and 400,000 dpm [^{14}C]20:4. The mean values from four experiments are presented ± the standard error where indicated. PC, choline-linked phosphoglycerides; PS/PI combined inositol- and serine-linked phosphoglycerides; PE, ethanolamine-linked phosphoglycerides.

associated with the diacyl species whereas most of the remaining [^{14}C]20:4 (40%) was in the 1-O-alkyl-2-acyl species. On the other hand, > 95% of the [^{3}H]18:0 was found in the diacyl species. After stimulation by A23187 the [^{3}H]18:0 remained constant (Table 1), but there was an approximate 30% reduction of [^{14}C]20:4 in both the 1,2-diacyl and 1-alkyl-2-acyl species. Despite this loss, there was no significant change in the ratio of the radiolabel between the diacyl- and 1-O-alkyl-containing species (Fig. 1). This suggests that phospholipase A_2, upon stimulation with A23187, acts concomitantly on both 1-O-alkyl-2-acyl and diacyl species.

The release of [^{14}C]20:4 was examined in more detail by analyzing purified PC by reverse-phase HPLC (17). This procedure allows resolution of most of the major molecular species found in PC based on the chain length and extent of unsaturation of the acyl side chains. The system is also capable of

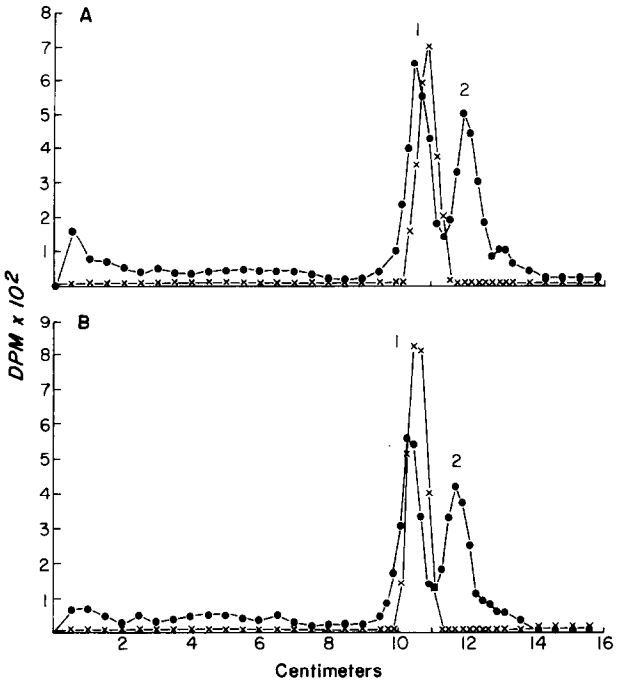

Figure 1. Thin-layer chromatography of acetylated diglycerides prepared from neutrophil phosphatidylcholine labeled with [^{3}H]stearic acid and [^{14}C]arachidonic acid. The acetylated derivatives of PC from control and A23187-stimulated PMN were prepared as described in Experimental Procedures. Distribution of the radiolabel in components of this fraction was determined by TLC on silica gel H, developed in hexane/ether (60:40, v/v), followed by zonal profile scanning. Panels A and B show the diglycerides prepared from control PMN and from A23187-stimulated PMN respectively. (×) represents [^{3}H]18:0. (•) represents [^{14}C]20:4. The labeled product in Peak 1 migrated with a standard diglyceride acetate prepared from diacyl-GPC (9.6 to 11 cm); Peak 2 migrated with a standard prepared from 1-O-alkyl-2-acyl-GPC (11.2 to 12.4 cm).

resolving 1-O-alkyl and plasmalogen species from their diacyl analogs[2]. For the experiments typified by Fig. 2, equivalent amounts of radiolabeled PC (as determined by [^{3}H]18:0 content which was not altered by A23187 challenge) from control or A23187-treated PMN were applied to the column and the loss of radiolabel from individual molecular species as a result of ionophore treatment was assessed. As shown in Fig. 2, the [^{14}C]20:4 was localized in seven species numbered in order of their elution from the column; the [^{3}H]18:0 species are not shown in Fig. 2. Six species have been characterized as indicated (Fig. 2) with four being identified as ether-linked. In general, the release of labeled

[2]Swendsen, C. L., Ellis, J. M., Chilton, F. H., O'Flaherty, J. T., and Wykle, R. L., manuscript in preparation.

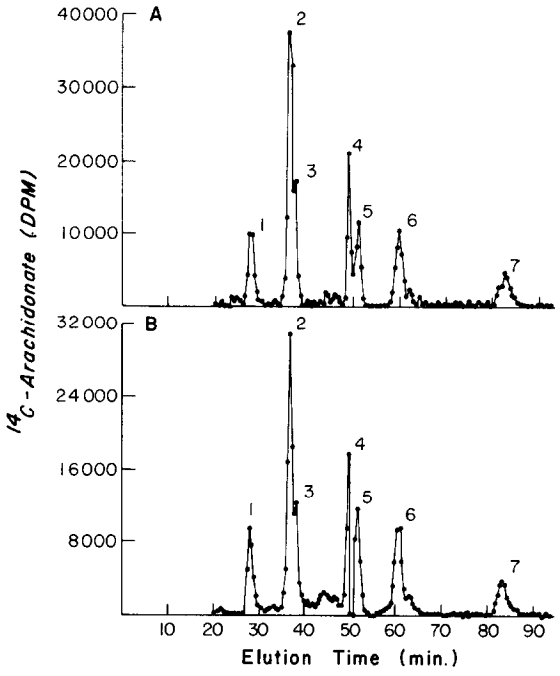

Figure 2. Reverse-phase HPLC of PC extracted from neutrophils labeled with [^{14}C]arachidonic acid. PC was isolated from control and A23187-stimulated PMN. Individual molecular species of PC were separated on an Altex Ultrasphere ODS column (0.45 x 25 cm) (Analab, Inc.) using a mobile phase of methanol/water/acetonitrile (90.5:70:25$_{\delta}$ v/v) containing 20 mM choline chloride. The flow rate was 2 ml/min (at 35°C) and absorbance was monitored at 206 nm. Samples, each containing 500,000 dpm [^{3}H]18:0 were mixed with standards and applied to the column in 40 µl methanol/methylene chloride (1:1, v/v). One-milliliter fractions were collected beginning 20 min after injection and the [^{14}C]20:4 determined in each fraction by liquid scintillation spectrometry. For reasons of clarity, the [^{3}H]18:0-labeled peaks are not shown in the figure. All the ^{14}C-labeled peaks contained 20:4 at the sn-2 position. At the sn-1 position they contained: Peak 1, 14:0-alkyl; Peak 2, 16:0-acyl; Peak 3, unknown; Peak 4, 16:0-alkyl; Peak 5, 18:1-alkyl; Peak 6, 18:0-acyl; Peak 7, 18:0-alkyl. Panels A and B depict PC from control and A23187-stimulated PMN, respectively. The percent distribution of [^{14}C]20:4 among the molecular species of PC from both control and stimulated PMN was: Peak 1, 11%; Peak 2, 33%; Peak 3, 7%; Peak 4, 14%; Peak 5, 8%; Peak 6, 12%; Peak 7, 6%.

20:4 from PC in response to A23187 as assessed by HPLC agreed with results obtained by TLC (Fig. 1). That is, approximately 40% of the released [^{14}C]20:4 came from four molecular species of 1-O-alkyl-2-[^{14}C]arachidonoyl-GPC and the percent distribution of [^{14}C]20:4 among the molecular species was not changed as a result of challenge with A23187. These data indicate that, in PMN, the A23187-stimulated arachidonate deacylation reaction does not discriminate among molecular species of PC on the basis of residues at the sn-1 position. Thus, by two independent methods we have shown alkyl ether-linked PC as well as diacyl-linked PC to be a significant source of 20:4 in PMN.

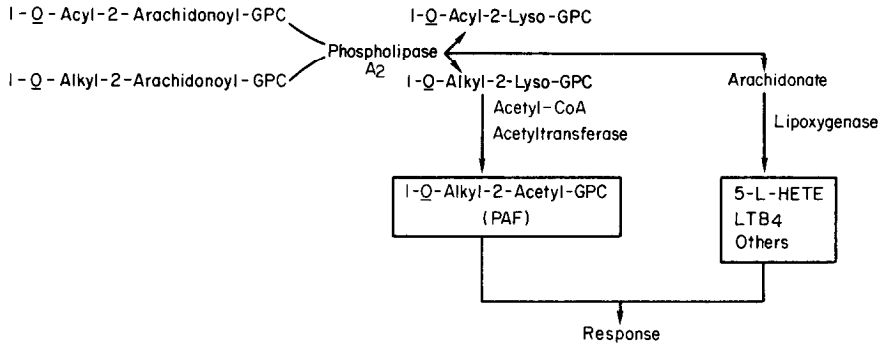

Figure 3. Hypothetical scheme showing the relationship between synthesis of PAF and arachidonate metabolites by stimulated neutrophils. Arachidonic acid is hydrolyzed from diacyl- and 1-O-alkyl-2-acyl-GPC by phospholipase A_2 and metabolized to bioactive eicosatetraenoates. The other product of the deacylation of 1-O-alkyl-2-arachidonoyl-GPC, 1-O-alkyl-2-lyso-GPC, is acetylated to form PAF. PAF and arachidonate metabolites may then act individually or in combination to mediate neutrophil responses.

Figure 3 summarizes the precursors and pathways involved in release of 20:4 from PC. We note that deacylation of 1-O-alkyl-2-arachidonoyl-GPC at once forms two products that can be further metabolized into distinctly different bioactive compounds: arachidonate and 1-O-alkyl-2-lyso-GPC. The former can be rapidly converted to HETEs, leukotrienes, or prostaglandins (7); the latter, to PAF (3,5,6). Thus, the concomitant production (1,7-10), and synergistic interactions (11) of the two classes of lipids may be related to a single mobilizing event wherein cell stimulation activates a phospholipase A_2-like reaction. The result is the generation of two classes of lipid mediators from a common precursor pool.

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