Metabolism of Platelet-activating Factor in Human Platelets

TRANSCACYLASE-MEDIATED SYNTHESIS OF 1-O-ALKYL-2-ARACHIDONYL-sn-GLYCERO-3-PHOSPHOCHOLINE*

Ruth M. Kramer, George M. Patton, Carole R. Pritzker, and Daniel Deykin

From the Department of Biochemistry, Boston University School of Medicine and the Boston Veterans Administration Medical Center, Boston, Massachusetts 02130

The present study demonstrates that inactivation of exogenous 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (alkylacyl-GPC; platelet-activating factor) by human platelets is mediated by the sequential action of two enzymes, 1) a Ca2+-independent acetylhydrolase recovered in the cytosolic fraction of platelets that deacylates alkylacyl-GPC forming alkyllyso-GPC and 2) a CoA-independent, N-ethylmaleimide-sensitive transacylase associated with platelet membranes that incorporates a long-chain fatty acid into alkyllyso-GPC to produce alkylacyl-GPC. Separation of platelet phospholipids and subsequent resolution into individual molecular species by high-performance liquid chromatography revealed that the newly formed alkylacyl-GPC was exclusively arachidonoylcholine and that the arachidonoyl group for acylation of alkyllyso-GPC was provided by phosphatidylcholine. We conclude that the newly described platelet arachidonoyl transacylase (Kramer, R. M., and Deykin, D. (1983) J. Biol. Chem. 258, 13806-13811) may play an important role in the metabolism of platelet-activating factor.

Platelet-activating factor, identified as 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (1), is synthesized and released by a variety of cells and tissues upon stimulation (2, 3). Among the many biological activities described (2, 3), alkylacyl-GPC has a potent effect on platelets (19). Arachidonic acid (56.9 mCi/mmol, New England Nuclear) was bound to bovine serum albumin (essentially fatty acid free), CoA, and N-ethylmaleimide were from Sigma. All solvents were HPLC grade or spectrograde quality from Fisher.

Labeling and Preparation of Platelets—Blood was obtained from healthy volunteers and processed as described previously (16). The platelet-rich plasma from four different donors were pooled and washed platelets prepared as described (13). For labeling of platelets the platelet-rich plasma was incubated with [3H]arachidonate (1 mcI/10 ml) for 30 min at 37 °C resulting in approximately 10,000 cpm of esterified [3H]arachidonate/108 platelets. Platelets suspended to 1.0 X 108/ml in 120 mM NaCl, 2 mM EGTA, 30 mM Tris/HC1, pH 7.5 (buffer B), were frozen in solid CO2/acetone, and stored at -70 °C. After thawing, platelets were sonicated for 6 X 15 s at 4 °C using a microprobe sonicator (Heat Systems-Ultrasound Inc., Plainview, NY) at an output setting of 3. The resulting lysate (containing less than 4% unbroken platelets (13)) was centrifuged at 100,000 X g for 60 min at 4 °C. The supernatant containing the platelet-cytosolic fraction was collected, and the pellet enriched in platelet membranes was resuspended in buffer B by brief sonication in a waterbath type sonicator (RAI Research Corp.). Platelet cytosolic fractions and membranes could be stored at -70 °C for several months without loss of enzymatic activity.

Incubations—Lysate, cytosolic, or membrane fractions derived from 2 X 108 platelets were incubated at 37 °C with 0.06 mcI (1 pmol) of [3H]alkylacytly-GPC or [3H]alkyllyso-GPC (added in 25 μl of buffer B containing 1 mg/ml of bovine serum albumin) in a total volume of 250 μl of buffer B as indicated in figure legends. Incubations with heat-inactivated enzyme preparations (preincubated for 15 min at 60 °C) served as controls. The reaction was stopped by adding 20 volumes of chloroform/methanol (2:1), and the lipids extracted according to Folch et al. (15).
Platelet Arachidonoyl Transacylase

RESULTS

Conversion of $[^3H]$Alkylacetyl-GPC by Platelets—In initial studies the metabolism of $[^3H]$alkylacetyl-GPC was examined in lysate, cytosolic and membrane fractions of platelets. In agreement with results of similar studies using intact platelets (10, 11) we found that incubation of $[^3H]$alkylacetyl-GPC with platelet lysate resulted in hydrolysis of $[^3H]$alkylacyl-GPC and formation of two radiolabeled products migrating with alkyllyso-GPC and alkylacyl-GPC, respectively, when separated by TLC. As demonstrated in a time course (Fig. 1A) the decrease in $[^3H]$alkylacyl-GPC was accompanied by an increase in $[^3H]$alkylacyl-GPC, while the amount of $[^3H]$alkyllyso-GPC never exceeded 5% of the total radioactivity. When $[^3H]$alkylacyl-GPC was added to platelet cytosol, on the other hand, its hydrolysis was paralleled by appearance of $[^3H]$alkyllyso-GPC and less than 20% of the total radioactivity. When $[^3H]$alkylacyl-GPC was added to platelet membranes, according to Bradford (18) using the dye reagent from Bio-Rad.

Acylation of $[^3H]$Alkyllyso-GPC by Platelet Membranes—Incubation of platelet membranes with $[^3H]$alkyllyso-GPC resulted in 68% conversion to $[^3H]$alkylacyl-GPC within 30 min (Fig. 1C). Addition of platelet cytosolic fraction to membranes, however, fully restored hydrolysis of $[^3H]$alkylacyl-GPC and resulted in formation of 12% $[^3H]$alkyllyso-GPC and 48% $[^3H]$alkylacyl-GPC after 30 min. In all preparations assayed, hydrolysis of $[^3H]$alkylacyl-GPC and formation of $[^3H]$alkyllyso-GPC were not affected by the addition of CoA (50 $\mu$M).

Acylation of $[^3H]$Alkylacyl-GPC by Platelet Membranes—Incubation of platelet membranes with $[^3H]$alkylacyl-GPC resulted in 68% conversion to $[^3H]$alkylacyl-GPC within 30 min (Fig. 1C). This reaction was not affected by preincubation of membranes with 5 $\mu$m disopropyl fluorophosphate. To determine whether formation of alkylacyl-GPC may be catalyzed by platelet transacylase, acylation of alkyllyso-GPC was further characterized in the presence of Ca$^{2+}$, cofactors for fatty acid activation or the sulfhydryl reagent N-ethyl-

Fig. 1. Time course of $[^3H]$alkylacetyl-GPC conversion by human platelets. Lysate (A), cytosolic (B), or membrane fractions (C) equivalent to 2 x 10^9 platelets were incubated at 37 °C with $[^3H]$alkylacetyl-GPC (0.05 $\mu$Ci, 1 pmol) in 250 $\mu$l of buffer B. After the indicated time, lipids were extracted and separated by TLC as detailed under “Experimental Procedures.” The data are given as percentage of the total radioactivity recovered in alkylacyl-GPC (D), alkyllyso-GPC (O), and alkylacyl-GPC (O) (66-78% of added label) and are representative of two experiments, each performed in duplicate.

Fig. 2. Time course of acylation of $[^3H]$alkyllyso-GPC by human platelet membranes. Membranes from 2 x 10^9 platelets were incubated with $[^3H]$alkyllyso-GPC (0.05 $\mu$Ci, 1 pmol) under standard conditions. At the times indicated, lipids were extracted and analyzed by TLC as described under “Experimental Procedures.” The data are given as percentage of the total radioactivity recovered in alkyllyso-GPC and alkylacyl-GPC (72-84% of added label) and are representative of two experiments performed in duplicate.

Chromatography—For TLC, the lipid extracts were spotted on 0.25-mm silica sheets (aluminum, Silica Gel 60, Merck, Darmstadt, prerun in chloroform/methanol (1:1), air-dried and stored in a dessicator) and chromatographed in chloroform/methanol/acetic acid/water, 78:45:8:2, (17) or 50:25:8:4. The latter system resolved glycerophospholipids as follows: alkyllyso-GPC (RF = 0.12), alkylacetyl-GPC (0.29), phosphatidylcholine (0.55), phosphatidylserine + phosphatidylglycerol (0.69), and phosphatidylethanolamine (0.82). The lipids were visualized with iodine vapors, and selected areas cut out with scissors and transferred to counting vials. After addition of 10 ml of Hydrofluor (General Diagnostics)/methanol/water (20:1.5:1) samples were incubated overnight at room temperature (for complete elution of lipids from the silica) and then counted for radioactivity using a Packard scintillation counter.

For HPLC, the lipid extracts (from membranes derived from 1 x 10^8 platelets, approximately 250 $\mu$g of phospholipid) were redissolved in hexane/2-propanol/water (40:34:6) and separated into phospholipid classes by chromatography on a Hibar II column packed with Lichrospher Si-100/11 (5 $\mu$m) (EM Laboratories Inc., Elmsford, NY) using hexane, 2-propanol, 25 mM potassium phosphate, pH 7, ethanol, acetic acid (367:490:62:100:0.6) as eluting solvent (14). The phosphatidylethanolamine fraction was collected, dried under nitrogen, and redissolved in ethanol. Phosphatidylethanolamine was then further separated into molecular species on an Altex Ultrasphere ODS column using methanol/water/acetic acid (90.5:7.2:5.2) containing 20 mM choline chloride as eluting solvent (1.5 ml/min) (14). Fractions were collected into scintillation vials, dried under nitrogen, and radioactivity determined by liquid scintillation spectrometry as detailed above.

Other Methods—Protein was determined, after adding 1 mg/ml of dodecyl sulfate to samples, according to Bradford (18) using the dye reagent from Bio-Rad.

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maleimide (see Ref. 13). We found that conversion of \(^{3}H\) alkyllyso-GPC to \(^{3}H\)alkylacyl-GPC was not influenced by CaCl\(_2\) (1–10 mM) and was not enhanced in the presence of CoA (50 \(\mu\)M) and/or ATP (5 mM) and MgCl\(_2\) (10 mM). Furthermore, acylation of \(^{3}H\)alkyllyso-GPC was inhibited >90% after preincubation of membranes with 0.5 mM \(N\)-ethylmaleimide for 10 min at 37 °C. The enzymatic activity responsible for formation of \(^{3}H\)alkylacyl-GPC thus possesses the same properties as platelet transacylase (13).

Acylation of \(^{3}H\)alkyllyso-GPC was studied as a function of added platelet membranes. As indicated in Fig. 3 conversion of \(^{3}H\)alkyllyso-GPC to \(^{3}H\)alkylacyl-GPC was optimal in the presence of 0.5 mg/ml of membrane protein (equivalent to approximately 1 \(\times\) 10\(^9\) platelets/ml). Formation of alkylacyl-GPC as a function of alkyllyso-GPC concentration is presented in Fig. 4. Acylation of alkyllyso-GPC reached a maximum of 0.8 nmol/min/mg at 0.1 mM of added alkyllyso-GPC and decreased at higher concentrations (≥0.15 mM) of alkyllyso-GPC. The Lineweaver-Burk plot of the data gave apparent \(K_M\) and \(V_{max}\) values of 12 \(\mu\)M and 0.87 nmol/min/mg.

Identification of \(^{3}H\)Alkylacyl-GPC—Platelet transacylase was previously found to mediate selective transesterification of arachidonate from endogenous platelet phosphatidylcholine to lysoplasmynylethanolamine (13). In order to examine further the possible involvement of this enzyme in the for-
of alkylacyl-GPC, the nature and origin of the fatty acid(s) incorporated into [3H]alkyllyso-GPC were identified as follows. Platelet membranes were incubated with saturating amounts (see Fig. 4) of radiolabeled hexadecyllyso-GPC to obtain a maximal yield of hexadecylacyl-GPC. After extraction of lipids, the platelet PC fraction containing the newly formed [3H]hexadecylacyl-GPC was isolated and then further analyzed by HPLC, resolving PC into individual molecular species. As previously demonstrated (14), within any phospholipid class, molecular species elute from a reverse-phase column according to chain length and degree of unsaturation of the fatty acyl moieties and can be identified by their retention time. Fig. 5 shows that the generated [3H] hexadecylacyl-GPC eluted as one peak indicating the presence of only one molecular species. The newly formed hexadecylacyl-GPC could also be detected spectrophotometrically (Fig. 5) and its retention time, expected to be longer than for the corresponding 1-palmitoyl analog, suggested that it may be hexadecyllinoleoyl-GPC or hexadecylarachidonoyl-GPC (14). When membranes were preincubated with N-ethylmaleimide formation of hexadecylacyl-GPC as monitored by increased UV absorption and appearance of radioactivity in peak 16 was inhibited >95%.

To determine whether arachidonate was transferred from platelet phospholipids to hexadecyllyso-GPC, membranes from [14C]arachidonic acid-labeled platelets (1 × 10^9) were incubated with [3H]hexadecyllyso-GPC (100 nmol) for 30 min. Analysis of extracted platelet phosphatides by TLC revealed that although 5 nmol of hexadecylacyl-GPC were produced, the distribution of [14C]arachidonate among the major platelet lipids, including total PC, phosphatidylethanolamine, plasmalogen, phosphatidylserine, phosphatidylinositol, and neutral lipids remained unchanged and was 61, 12, 3, 9, 7, and 8%, respectively, in membranes incubated with or without hexadecyllyso-GPC. However, resolution of the total PC fraction into individual molecular species by HPLC (Fig. 6) demonstrated that the newly formed [3H]hexadecylacyl-GPC contained 14C radioactivity (5% of the total 14C radioactivity in PC) thus identifying the incorporated fatty acid as arachidonate.

The gain of 14C radioactivity in the newly formed [3H]hexadecyl-14C arachidonoyl-GPC was accompanied by a concomitant loss of radiolabel from arachidonate-containing species of PC, particularly palmitoylaraachidonoyl-GPC and stearoylarachidonoyl-GPC (Table I).
Further, the reaction involves a direct specific transfer of esterified arachidonate from endogenous PC to alkyllyso-GPC resulting in exclusive formation of alkylarachidonoyl-GPC. There is evidence to indicate that biosynthesis of alkylacetyl-GPC is achieved by deacylation of pre-existing alkylacyl-GPC by phospholipase A₂ (23, 24) and subsequent acetylation of the newly formed alkyllyso-GPC to biologically active alkylacyl-GPC by acetyl-CoA transferase (25-29). The enzymatic pathway for inactivation of alkylacyl-GPC likewise involves deacylation of alkylacyl-GPC followed by reconversion to alkylacyl-GPC (9-12) (leading to selective formation of alkylarachidonoyl-GPC in human neutrophils (12) and platelets). It thus appears that the metabolism of alkylacyl-GPC proceeds through deacylation-reacylation cycles (Fig. 7).

Human platelets contain significant amounts of alkylarachidonoyl-GPC (30). Stimulation of platelets induces release of arachidonic acid from platelet PC (31) with subsequent conversion to biologically active compounds (32) and also promotes synthesis of platelet-activating factor (26, 33). It can thus be speculated that alkylarachidonoyl-GPC may serve as a common precursor for both alkylacyl-GPC and arachidonic acid metabolites. In support of this hypothesis are recent findings of Swendson et al. (34) demonstrating that in stimulated rabbit neutrophils, 40% of the arachidonic acid released from choline-containing glycerophospholipids originated from alkyl ether-linked species.

We conclude that the enzyme arachidonoyl transacylase plays an important role in the metabolism of platelet-activating factor and is responsible for synthesis of alkylarachidonoyl-GPC, a likely precursor of platelet-activating factor.

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Fig. 7. Proposed pathways for metabolism of alkylacetyl-GPC in platelets. PAF, platelet-activating factor; FA, fatty acid; AA, arachidonic acid; LPC, lysophosphatidylethanolamine; AA-PC, arachidonoyl-PC.