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CONVERSION OF 1-ALKYL-2-ACETYL-sn-GLYCEROLS TO PLATELET ACTIVATING FACTOR AND RELATED PHOSPHOLIPIDS BY RABBIT PLATELETS

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SUMMARY — The metabolic pathway for 1-alkyl-2-acetyl-sn-glycerols, a recently discovered biologically active neutral lipid class, was elucidated in experiments conducted with rabbit platelets. The total lipid extract obtained from platelets incubated with 1-[1,2-^{3}H]alkyl-2-acetyl-sn-glycerols or 1-alkyl-2-[^{3}H]acetyl-sn-glycerols contained at least six metabolic products. The six metabolites, identified on the basis of chemical and enzymatic reactions combined with thin-layer or high-performance liquid chromatographic analyses, corresponded to 1-alkyl-sn-glycerols, 1-alkyl-2-acetyl-sn-glycero-3-phosphates, 1-alkyl-2-acyl(long-chain)-sn-glycero-3-phosphoethanolamines, 1-alkyl-2-acetyl-sn-glycero-3-phosphoethanolamines, 1-alkyl-2-acyl(long-chain)-sn-glycero-3-phosphocholines, and 1-alkyl-2-acetyl-sn-glycero-3-phosphocholines (platelet activating factor). These results indicate that the metabolic pathway for alkylacetylglycerols involves reaction steps catalyzed by the following enzymatic activities: choline- and ethanolamine- phosphotransferases, acetylhydrolase, an acyltransferase, and a phosphotransferase. The step responsible for the biosynthesis of platelet activating factor would appear to be the most important reaction in this pathway and this product could explain the hypotensive activities previously described for alkylacetyl-(or propionyl)-glycerols. Of particular interest was the preference exhibited for the utilization of the 1-hexadecyl-2-acetyl-sn-glycerol species in the formation of platelet activating factor.

Recently we reported that alkylacetylglycerols (and alkylpropionylglycerols) could elicit a hypotensive response in normotensive and hypertensive (SHR) WKY rats, whereas closely related analogs (e.g., acylacetylglycerols) were inactive (1). The mechanism for the hypotensive action of these neutral ether-linked lipids is unknown, but a likely possibility is that this biological activity is due to their conversion to PAF (alkylacetyl-GPC or PAF), a diverse cellular mediator that exhibits potent hypotensive properties (see 2-7 for reviews).

Alkylacetylglycerols are involved in PAF metabolism (8) in a manner analogous to the long chain diacylglycerols (9) and alkylacylglycerols (10) that serve as substrates for cholinephosphotransferase in the biosynthesis of

Abbreviations: alkylacetylglycerol, 1-alkyl-2-acetyl-sn-glycerol; GPC, sn-glycero-3-phosphocholine; GPE, sn-glycero-3-phosphoethanolamine; GP, sn-glycero-3-phosphate; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; PAF, platelet activating factor.

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phosphatidylcholine and alkylacyl(long-chain)-GPC, respectively. Unlike the cholinephosphotransferase (inhibited by dithiothreitol) that catalyzes the biosynthesis of phosphatidylcholine, a specific dithiothreitol-insensitive cholinephosphotransferase utilizes alkylacetylglycerols as substrates in the formation of PAF (8). There is also a possibility that the alkylacetylglycerols participate, as do the diacylglycerols, in the phosphorylation of specific cellular proteins by protein kinase C. Such a role is particularly intriguing since in human neutrophils (11) the acyl analog (acylacetylglycerols) can induce superoxide release via a protein kinase C activation, whereas the corresponding long chain diacylglycerols are ineffective. The importance of these acetylated ether-linked neutral lipids in cellular systems has been further emphasized by recent findings of McNamara et al. (12) who demonstrated that alkylacetylglycerols (but not the acyl analog) stimulated differentiation of HL-60 promyelocytic leukemia cells to macrophage-like cells.

In view of the potential physiological significance of alkylacetylglycerols as a new type of cellular mediator in relation to PAF, protein kinase-dependent, and cellular differentiation functions, we have examined the metabolism of this neutral lipid in an intact cell system. Rabbit platelets were selected for these studies since they not only produce PAF, but also are a target cell for this phospholipid mediator; furthermore, as cited above it appears that these acetylated neutral lipids (acylacetylglycerols) can activate a specific protein kinase in platelets (13). Our experiments show that radiolabeled alkylacetylglycerols are not only converted to PAF by platelets, but that at least five other labeled phospholipid products are also formed. The formation of PAF probably explains the hypotensive response previously described for alkylacetylglycerols (1). Moreover, these experiments emphasize that the specific dithiothreitol-insensitive cholinephosphotransferase (8) in PAF biosynthesis has been underestimated as an important pathway for PAF.

MATERIALS AND METHODS

[1,2-^{3}H]Alkylacetylglycerol was prepared by phospholipase C (*Bacillus cereus*) hydrolysis (14) of [1,2-^{3}H]alkylacetyl-GPC (New England Nuclear, Boston, MA) and its specific activity adjusted to 250 μCi/μmol by addition of unlabeled alkylacetylglycerols, which was prepared as previously described (1). Alkyl[^{3}H]acetyl-GPC (250 μCi/μmol), synthesized as reported earlier (15) was treated with phospholipase C to produce alkyl[^{3}H]acetylglycerol. Analysis of radioactivity in both substrates by TLC (8) demonstrated that they were >99% alkylacetylglycerols and contained <10% of the 1,3-isomers.

Platelets were isolated from blood of adult New Zealand white rabbits by a modification (16) of the method of Henson (17). Washed platelets were finally suspended in Tyrodes-gel solution, without Ca^{2+}, at a concentration of 1.2 x 10^{9} platelets/ml. [1,2-^{3}H]Alkylacetylglycerol or alkyl[^{3}H]acetylglycerol (2.5 μCi, 10^{-8} moles), dissolved in 10 μl of acetone, was added per ml of platelet suspension, duplicate samples for each substrate were then incubated at 37°C for 20 min. Lipids were extracted from the total platelet suspensions by the

method of Bligh and Dyer (18), except that the methanol contained 2% glacial acetic acid. The average recovery of radioactivity from the samples incubated with [1,2-^{3}H]alkylacetylglycerol or alkyl[^{3}H]acetylglycerol was 85% and 65%, respectively. Initial TLC analyses of the total labeled lipids were done on Silica Gel H layers developed in solvent system A (chloroform:methanol:glacial acetic acid:water - 50:30:8:3, v/v) for the phospholipids and Silica Gel G layers developed in solvent system B (chloroform:methanol:hexane:glacial acetic acid - 100:2:2:1, v/v) for the neutral lipids. Duplicate samples were combined and the labeled components isolated by preparative TLC in the acid solvent systems mentioned above. The fraction migrating between phosphatidylcholine and phosphatidylethanolamine was separated into two components on Silica Gel H layers developed in solvent system C (chloroform:methanol:concentrated ammonium hydroxide - 65:35:8, v/v). Each of the isolated phospholipid fractions were radioassayed by zonal scanning (19).

Alkylacetyl-GPC (Sigma Chemical Co., St. Louis, MO) was treated with phospholipase D (cabbage from Sigma Chemical Co.) in the absence and presence of ethanolamine to produce alkylacetyl-GP and alkylacetyl-GPE, respectively (20), for use as standards. Part of the alkylacetyl-GP was treated with a diethyl ether solution of diazomethane (21) to methylate the free phosphate group. The methylated alkylacetyl-GP standard had a TLC R_f of $\sim$0.5 while the unmethylated alkylacetyl-GP remained at the origin in solvent system D (chloroform:concentrated ammonium hydroxide - 100:1, v/v).

Details for the preparation of the various lipid benzoate derivatives analyzed by reverse-phase HPLC, has been described (22,23). The preparation of isopropylidene (24) and dinitrobenzene (21) derivatives and the deacylation of lipids with the monomethylamine reagent (25) were done according to established methods. All reactions used for the identification of metabolites were carried out to at least 80% completion.

RESULTS AND DISCUSSION

Figure 1 is a representative TLC separation of the tritiated components from the total lipids extracted from the suspension of rabbit platelets after incubation with [1,2-^{3}H]alkylacetylglycerol. A similar labeling pattern was observed in platelet lipids after incubation with alkyl[^{3}H]acetylglycerol except that only traces of radioactivity were found in the TLC areas corresponding to peaks 2 and 5. The labeled components comigrated in both TLC solvent systems A and C with standards of alkylacetyl-GPC (peak 1), alkylacyl-(long-chain)-GPC (peak 2), alkylacetyl-GPE (peak 3), alkylacetyl-GP (peak 4), alkylacyl(long-chain)-GPE (peak 5), and neutral lipids consisting of [^{3}H]alkylglycerols and the unchanged labeled substrate of alkylacetylglycerols (peak 6). Lipids designated peaks 1, 2, 3 + 4, 5, and 6 (Fig. 1) were first isolated by preparative TLC in solvent system A; the lipids representing peaks 3 and 4 were then separated in solvent system C. Table I shows the distribution of tritium in platelet lipid classes after incubation with each substrate. Further TLC analysis (solvent system B) of the neutral lipid fraction (peak 6, Fig. 1) from incubations with the alkyl[^{3}H]acetylglycerol revealed that essentially all of the radioactivity represented a mixture of the 1,2- and 1,3-isomers of the substrate. However, in samples incubated with [1,2-^{3}H]alkylacetylglycerol, where the label is in the alkyl chain, 79% of the radioactivity associated with

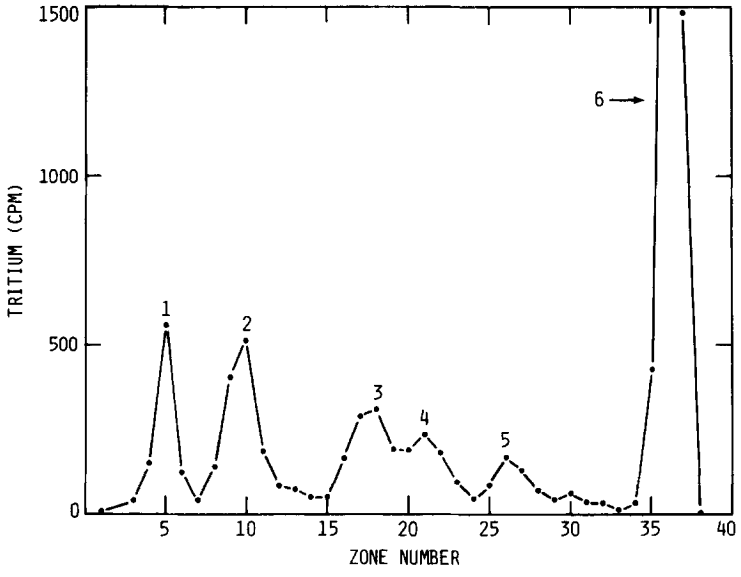

Fig. 1. TLC zonal scan profile of lipid metabolites produced by rabbit platelets from [1,2-^{3}H]alkylacetylglycerol. The developing solvent was chloroform:methanol:glacial acetic acid:water (50:30:8:3, v/v). Lipid components had TLC R_fs corresponding to standards of: peaks 1=alkylacetyl-GPC, 2=alkylacyl(long-chain)-GPC, 3=alkylacetyl-GPE, 4=alkylacetyl-GP, 5=alkylacyl(long-chain)-GPE, and 6=alkylacetylglycerols and alkylglycerols.

peak 6 (Fig. 1) chromatographed with alkylacetylglycerols and 21% with alkylglycerols. All of the purified fractions of metabolites were further identified on the basis of derivatives formed after chemical and enzymatic treatments as described below.

When alkyl[^{3}H]acetylglycerol was the precursor, as expected, no tritium was found in alkylglycerols, alkylacyl(long-chain)-GPC, or alkylacyl(long-chain)-

TABLE I

DISTRIBUTION OF METABOLIC PRODUCTS FORMED AFTER INCUBATION OF TRITIUM-LABELED ALKYLACETYLGLYCEROLS WITH RABBIT PLATELETS

Precursor	Lipid Fraction					
	1	2	3	4	5	6
	% of Total Tritium in Peak					
Alkyl[^{3}H]acetylglycerol	5.9	tr	8.5	4.6	tr	81.1
[1,2-^{3}H]Alkylacetylglycerol	7.5	12.6	8.3	6.4	5.4	59.8

Values represent the average from two separate incubations that varied by < ± 10% from the mean except for the value of peak 5, which had a variation of ± 26%. Lipid fraction numbers correspond to those peak numbers shown in Fig. 1, e.g., 1=alkylacetyl-GPC, 2=alkylacyl(long-chain)-GPC, 3=alkylacetyl-GPE, 4=alkylacetyl-GP, 5=alkylacyl(long-chain)-GPE, and 6=alkylacetylglycerols and alkylglycerols.

GPE; tritium appeared in these metabolites only when the alkylacetylglycerol precursor was labeled in the alkyl chain. Further analysis of these three labeled classes of compounds isolated by TLC were conducted in the following manner. [1,2-^{3}H]Alkylglycerols (Fig. 1, peak 6) had the same TLC migration before and after treatment with the monomethylamine reagent and formed both isopropylidene or dibenzoate derivatives; furthermore, 67.9% of the radiolabeled dibenzoate derivative eluted with standard hexadecyldibenzoylglycerol during HPLC analysis (22). Monomethylamine treatment of [1,2-^{3}H]alkyacyl(long-chain)-GPC (Fig. 1, peak 2) yielded a labeled compound having the same TLC R_f as the alkyllyso-GPC standard; labeled alkylacylglycerols were formed by phospholipase C hydrolysis and benzoylation of this product produced tritium-labeled alkylacylbenzoylglycerols, based on the TLC R_f of the corresponding standards. [1,2-^{3}H]Alkylacyl(long-chain)-GPE (Fig. 1, peak 5) formed alkyllyso-GPE, alkylacylglycerols, and alkylacylbenzoylglycerols after reacting with monomethylamine, phospholipase C, and benzoylation of the phospholipase C products, respectively. In addition, after derivatization of the tritiated alkylacyl-GPE with fluorodinitrobenzene, the labeled product cochromatographed on TLC (26) with the dinitrobenzene derivative of a standard diacyl-GPE.

The three other metabolites seen in Fig. 1 (alkylacetyl-GP, alkylacetyl-GPE, and alkylacetyl-GPC) were produced by rabbit platelets from both the [1,2-^{3}H]-alkylacetylglycerol and alkyl[^{3}H]acetylglycerol substrates. Treatment of [1,2-^{3}H]alkylacetyl-GP (Fig. 1, peak 4) and alkyl[^{3}H]acetyl-GP with diazomethane produced tritium-labeled compounds having the identical TLC R_f in solvent system D as the methylated alkylacetyl-GP standard. [1,2-^{3}H]Alkylacetyl-GPE (I) (Fig. 1, peak 3) and alkyl[^{3}H]acetyl-GPE (II) were identified as follows: -- a) monomethylamine deacylation produced tritium-labeled alkyllyso-GPE from I and a near complete loss of tritium from II, b) radiolabeled alkylacetylglycerols were obtained from both compounds after phospholipase C hydrolysis, c) benzoylation of the phospholipase C hydrolytic products derived from both I and II produced tritium-labeled derivatives having TLC R_f values identical to alkylacetylbenzoylglycerol standards, and d) treatment with fluorodinitrobenzene yielded the expected tritium-labeled dinitrobenzene derivatives of both I and II. [1,2-^{3}H]Alkylacetyl-GPC (Fig. 1, peak 1) and alkyl[^{3}H]acetyl-GPC were identified on the basis that deacylation of [1,2-^{3}H]alkylacetyl-GPC with monomethylamine produced radiolabeled alkyllyso-GPC, whereas tritium was lost when alkyl[^{3}H]acetyl-GPC was treated with this reagent; the same results were obtained as described in b and c above after phospholipase C and benzoylation of the phospholipase C-derived lipid product.

The tritium-labeled alkylacetylbenzoylglycerols derived from the [1,2-^{3}H]-alkylacetyl-GPC and alkyl[^{3}H]acetyl-GPC metabolites and benzoate derivatives of

TABLE II

ALKYL SELECTIVITY IN THE FORMATION OF PAF (ALKYLACETYL-GPC) FROM TRITIUM-LABELED ALKYLACETYLGLYCEROLS BY RABBIT PLATELETS

	[1,2-^{3}H]Alkylacetylglycerol		Alkyl[^{3}H]acetylglycerol	
Alkyl moieties*	Precursor	PAF Product ([1,2-^{3}H]Alkyl-acetyl-GPC)	Precursor	PAF Product (Alkyl-[^{3}H]acetyl-GPC)
	% of Tritium in Total Class			
14:0	3.8	3.9	13.4	13.5
15:0	12.8	11.9	3.6	4.6
16:0	60.8	73.8	69.8	80.6
17:0	11.4	6.5	3.0	tr
18:0	11.2	2.4	10.1	1.3

* The first number designates length of the alkyl chain and the second the number of double bonds in the alkyl chain. Some branched chains also appeared to be present in the substrate and products of [1,2-^{3}H]alkylacetylglycerol.

HPLC analyses of pooled samples were done as previously described (23) except a mobile phase of acetonitrile:2-propanol (75:25, v/v) was used.

the alkylacetylglycerol substrates used in the incubations were analyzed by reverse-phase HPLC (Table II). With both substrates, an enrichment of the hexadecylacetylglycerol species occurs in the alkylacetyl-GPC, i.e., PAF produced. This apparent selectivity of hexadecylacetylglycerol in the conversion of alkylacetylglycerols to the biologically active alkylacetyl-GPC by rabbit platelets occurred at the expense of the octadecyl- and heptadecyl-acetylglycerol molecular species.

Recently the conversion of alkylacetylglycerols to PAF by rabbit platelets has been described by Satouchi et al. (27) in studies where mass spectrometry in conjunction with a selected ion monitoring approach was used to identify the newly formed PAF. Both our results and those of Satouchi et al. (27) demonstrate the conversion of the neutral lipid to PAF is independent of extracellular calcium; however, if exogenous calcium is present when the PAF is synthesized, aggregation of the platelets then occurs (27).

Based on the identification of six metabolic products [alkylglycerols, alkylacetyl-GP, alkylacyl(long-chain)-GPE, alkylacetyl-GPE, alkylacyl(long-chain)-GPC, and alkylacetyl-GPC] formed from alkylacetylglycerols, we have formulated a pathway for the metabolism of alkylacetylglycerols in rabbit platelets as illustrated in Fig. 2. Although the pathway is unexpectedly

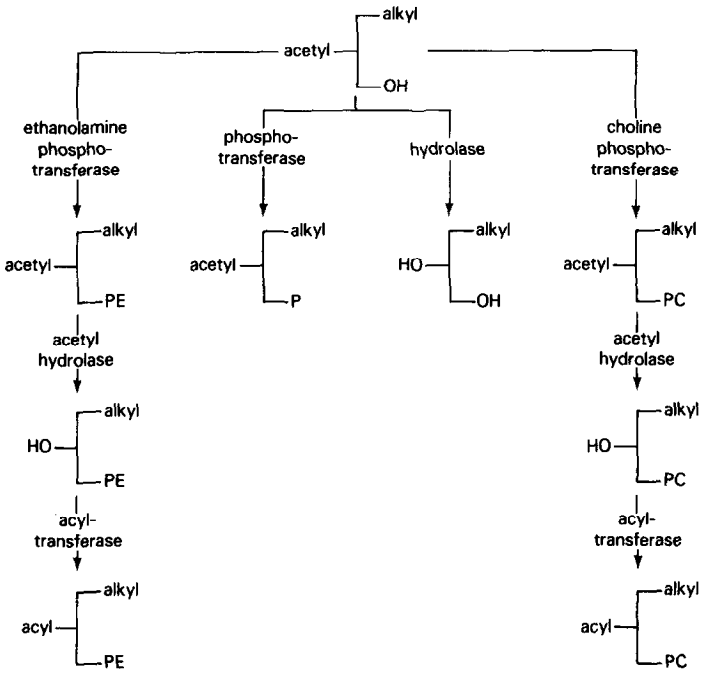

Fig. 2. Proposed scheme for the metabolism of alkylacetylglycerols by rabbit platelets.

complex, the most important of the reactions illustrated in this scheme would appear to be the conversion of alkylacetylglycerols to PAF, a known cell mediator with potent and diverse biological activities.

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