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MOBILIZATION OF ARACHIDONIC ACID FROM PHOSPHATIDYLETHANOLAMINE FRACTION TO PHOSPHATIDYLCHOLINE FRACTION IN PLATELETS

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SUMMARY: Rabbit platelets rapidly incorporated methyl groups of [^{3}H] methionine to phosphatidylcholine (PC). Rabbit platelets also incorporated [^{3}H]choline to PC, but the rate of incorporation was far lower than that of [^{3}H]methionine. Further fractionation of labeled PC revealed that a considerable amount of arachidonyl PC was synthesized *via* the *N*-methylation pathway. Thrombin stimulation resulted in a release of arachidonic acid from PC, and not from phosphatidylethanolamine (PE). These observations suggest that the *N*-methylation pathway plays an important role in the intracellular mobilization of arachidonic acid from the PE fraction to the PC fraction, this fraction being more sensitive to the hydrolysis with phospholipase A_2 during platelet activation.

INTRODUCTION

Platelet phospholipids containing arachidonyl chains serve as the source of free arachidonic acid (AA) liberated by various stimuli (1-7). It is reported that phosphatidylcholine (PC) and phosphatidylinositol (PI) are more preferentially hydrolyzed during platelet activation by phospholipase A_2 than phosphatidylethanolamine (PE) (3,4), although the PE fraction of platelets ordinarily contains much more AA than does the PC fraction (8,9). In this context, it was of interest to study the phospholipid metabolism in platelets with special reference to their acyl chain composition, especially, AA composition (10). Since PC fraction, one of the major sources

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Abbreviations used in this paper: AA, arachidonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyldimethylethanolamine; TBS, Tris-buffered saline.

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of free AA released during platelet activation, can be synthesized either by the choline pathway (11) or by N-methylation of PE (12,13), we attempted to investigate the synthesis of PC in platelets.

MATERIALS AND METHODS

Washed platelets of New Zealand white rabbits were obtained from platelet rich plasma as described previously (6,7). [1-^{14}C]AA (55.5 Ci/mol), L-[*methyl*-^{3}H]methionine (80.0 mCi/μmol) and [*methyl*-^{3}H] choline (80.0 mCi/μmol) were obtained from New England Nuclear, Mass, USA, and Radiochemical Center, Amersham, U.K. Platelets (4-8 x 10^{8}/ml) were incubated with 2 μCi of either [*methyl*-^{3}H]choline or 1 μCi of [1-^{14}C]AA in the medium containing 0.134 M NaCl, 15 mM Tris-HCl, pH 7.4, 5 mM glucose (TBS) and 0.25 % essentially fatty acid free bovine serum albumin (Sigma, St. Louis. Miss. USA), at 37 °C for 1 hr. After the incubation, the cells were washed twice and cellular lipids were extracted, according to the method of Folch, et al. (14). The separation and identification of phospholipid were carried out by thin-layer chromatography with a solvent system of chloroform/methanol/water (100:40:6, v/v/v). The lipids were visualized with β-camera (LB 290, Berthold, F.R.G.), and the radioactivity of the spots was measured using a Nuclear Chicago Liquid scintillation counter (Model Mark II). In some experiments, PC from labeled platelets was further fractioned by TLC on silver nitrate-impregnated Silica gel H (Merck, F.R.G.) according to the modified method of Arvidson (15) developed with a solvent system of chloroform/methanol/water (100:60:8, v/v/v). The spots corresponded to mono + dienoic PC and tetraenoic PC were eluted and assayed for radioactivity. Stimulation of platelets with thrombin was carried out after further incubation of [^{14}C]AA labeled platelets with 50 μM aspirin for 20 min at room temperature. The platelets were then resuspended in TBS containing 0.25 % essentially fatty acid free bovine serum albumin and 4 mM $CaCl_2$, and challenged by thrombin (5 units/ml, Dade Inc., Miami, Fl., U.S.A.).

RESULTS

When 4 x 10^{8}/ml washed rabbit platelets were incubated with [*methyl*-^{3}H]methionine under the conditions described in Materials and Methods, about 6-9 x 10^{4} dpm (8.5-12.8 pmoles/10^{10} cells) of radioactivity was incorporated into the platelet lipid fraction. As shown in Fig. 1, the ratio of PC:PDME:PMME was 49:42:9 on the base of radioactivity and apparently there was a stepwise N-methylation of PE. When platelets were incubated under the same conditions with [*methyl*-^{3}H]choline having the same specific radioactivity as methionine, about 0.9-1.3 x 10^{4} dpm (1.3-1.8 pmoles/10^{10} cells) of radioactivity was incorporated to the lipid fraction. The major reaction product was PC, and less than 6 % of radioactivity was detected in the other lipid fractions.

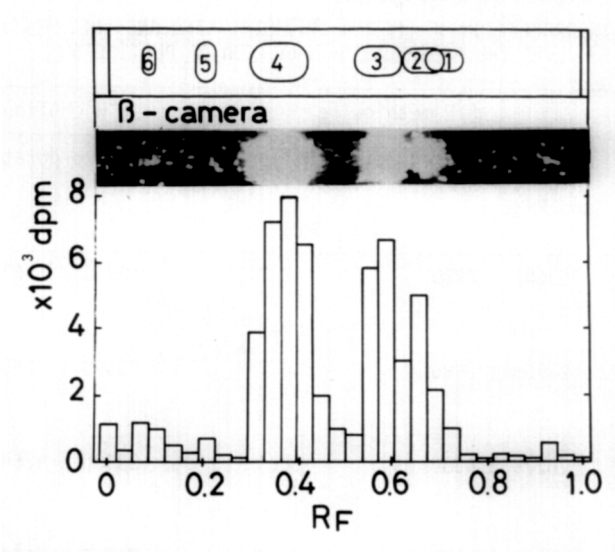

Fig. 1: Thin-layer chromatogram of the [^{3}H]methylated lipid reaction products. Platelet lipids were extracted, applied to a pre-coated silica gel plate (Silica gel G. Merck, F.R.G.) and chromatographed with a solvent system of chloroform/methanol/water (100:40:6, v/v/v). Regional identification: 1, PE; 2, PMME; 3, PDME; 4, PC; 5, sphingomyelin; 6, lyso PC.

To compare the rate of PC synthesis *via* the transmethylation pathway and the choline pathway, the washed platelet suspension was put into two tubes and incubated with either [*methyl*-^{3}H]methionine or [*methyl*-^{3}H] choline having the same specific radioactivity (Table 1). The molar ratio of platelet glycerophospholipid fractions incorporated radioactivity from [^{3}H]methionine:that from [^{3}H]choline was 74:26, whereas the ratios of 12:88, 10:90 and 32:68 were obtained with murine liver homogenate, murine macrophages (both derived from C3H/He inbred strain) and rabbit erythrocytes, respectively. The results show that the N-methylation pathway is relatively more active that the choline pathway in PC synthesis of platelets in comparison with other cells.

The results of the silver nitrate fractionation of platelet PC prelabeled with either [*methyl*-^{3}H]methionine or [*methyl*-^{3}H]choline are shown in Table 2. Mono- and dienoic type of PC is predominantly labeled with [*methyl*-^{3}H]choline, whereas the tetraenoic type has a relatively higher specific activity with respect to [*methyl*-^{3}H] groups derived from methionine. Gas chromatographic

Table 1: INCORPORATION OF [METHYL-^{3}H]METHIONINE AND [METHYL-^{3}H]CHOLINE INTO THE PHOSPHOLIPID FRACTION OF PLATELETS.

Cells	[^{3}H]methionine		[^{3}H]choline	Molar ratio of phospholipid incorporated [^{3}H]methionine /[^{3}H]choline.
	dpm incorporated into PC, PDME and PMME.	distribution of radioactivity (%) among PC, PDME and PMME.	dpm incorporated	
Rabbit Platelets[a]	63602 ± 2320	48.7 42.4 8.9	10340 ± 1162[a]	2.85[b]
Murine Macrophages	21703 ± 686	46.7 41.9 11.4	93590 ± 2235	0.11
Rabbit Erythrocyte	26276 ± 4380	50.1 38.9 10.6	23380 ± 6264	0.53
Murine Liver Homogenates	23552 ± 1121	55.9 34.7 9.4	74768 ± 4620	0.14

a. Values are mean ± S.D. of triplicate experiments.
b. Amount of phospholipid incorporated [^{3}H-methyl]methionine was calculated as follows; (dpm in PC x 1/3 + dpm in PDME x 1/2 + dpm in PMME)/specific radioactivity of [methyl-^{3}H]methionine.

analysis of fatty acid composition of PC and PE of platelets used in these experiments showed that PC and PE contain 6±2 % and 36±4 % of AA, respectively. Thus it can be calculated that 64-82 % of PC synthesized through N-methylation pathway should be of the tetraenoic type, but our results showed that apparently only 40 % of PC synthesized *via* transmethylation pathway is tetraenoic. This discrepancy may indicate that another mechanism which

Table 2: DISTRIBUTION OF RADIOACTIVITY (%) AMONG THE MOLECULAR CLASSES OF PLATELET PHOSPHATIDYLCHOLINE PRELABELED WITH EITHER [METHYL-^{3}H]METHIONINE OR [METHYL-^{3}H]CHOLINE. The data shown are means of two separate experiments.

Molecular class	[^{3}H]methionine	[^{3}H]choline
Mono- and Dienoic	48.0	80.8
Tetraenoic	40.2	13.6
Others[a]	9.4	5.7

a. Radioactivity detected at the origin of thin layer chromatography (Rf=0) is included.

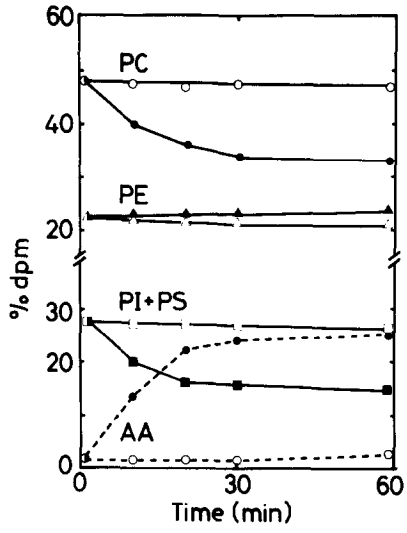

Fig. 2: Time course of thrombin-induced change in the radioactivity in phospholipid fractions of platelets prelabeled by [^{14}C]arachidonic acid. Percent radioactivity of each phospholipid fraction was calculated and plotted. (●—●, ○—○), PC fraction; (▲—▲, △—△), PE fraction; (■—■, □—□), PI + phosphatidylserine fraction; (●---●, ○---○), free arachidonic acid fraction. Closed symbols (●▲■), thrombin (5 units/ml) added; open symbols (○△□), control (saline added).

modifies the fatty acid composition of phospholipids; i.e., deacylation-reacylation cycle, is at work even under these artificial experimental conditions.

As shown in Fig. 2, addition of thrombin produced a mobilization of [^{14}C]AA from prelabeled platelets. The source of free AA was mainly the PC and PI (+PS) fractions, and essentially no AA was released from the PE fraction. In preliminary experiments, when platelets prelabeled with either [*methyl*-^{3}H]methionine or [*methyl*-^{3}H]choline were stimulated with thrombin under the same conditions, only a slight decrease of the radioactivities in both PC fractions was noted (statistically not significant), and the increase in the radioactivities in both lysophosphatidylcholine fractions was also not significant at the time of 10, 20, 30 and 60 min after the addition of thrombin. These results might imply that newly synthesized arachidonyl PC does not serve as substrate for phospholipase immediately after being N-methylated, or that lysophosphatidylcholine, the cytotoxic

substance produced by the action of phospholipase A_2 during thrombin activation, was quickly re-acylated. Detailed investigation on the role of N-methylation pathway in the actual activating process of platelets is now under way.

DISCUSSION

From the results obtained above, it can be concluded that the methylation pathway of PC synthesis is highly active in platelets, and seems to be one of the characteristic features of platelet phospholipid metabolism. Since the amount of PMME and PDME is low in platelets, the high rate of incorporation of [*methyl*-^{3}H] groups from methionine to PC seems to reflect the high rate of turnover of PC synthesized through this pathway.

The N-methylation pathway of PC synthesis was relatively more important than the incorporation of choline in supplying arachidonyl PC, whereas the choline pathway supplied mainly mono- or dienoic PC. Since PE contains more AA than PC, it is natural that PC synthesized through the N-methylation pathway contains much more AA than the average PC. On the other hand, fatty acid composition of PC synthesized through the choline pathway is probably affected by the acyl chain specificity of acyl CoA:lysophosphatidic acid acyltransferase activity, which has no prominent AA specificity (16). Thus, PC synthesized *via* the choline pathway has only a small amount of AA.

In platelets, thrombin stimulation results in the release of AA, the precursors of prostaglandins and thromboxanes, from phospholipids through the activation of phospholipase A_2 (1-7, 17). Among the various phospholipid classes, PC is assumed to be one of the major sources of released AA and PE is less accessible to thrombin stimulation (3-5, this paper, and Kannagi, et al. submitted for publication). Thus, the physiological role played by the N-methylation pathway would be the mobilization of AA from the PE fraction, a relatively inactive reservoir of cellular AA, to the

PC fraction, which serves as one of the direct precursors of AA released by phospholipase A_2 once platelets were activated by thrombin or other various stimuli.

Whether or not AA released from the PC fraction by thrombin stimulation was derived exclusively from the pool of PC synthesized through N-methylation pathway is not clear, since the mono- and dienoic PC synthesized *via* the choline pathway can also supply a significant amount of free AA after being reacylated with AA through the deacylation-reacylation cycle. The quantitative evaluation of the role played by the N-methylation pathway in thrombin-induced AA release awaits further investigation. At present, it can be concluded that the N-methylation pathway plays a role in the intracellular mobilization of AA in resting platelets from PE to PC, whereas deacylation-reacylation cycle seems to play a role in the incorporation of AA into cellular phospholipids from the surrounding external medium of cells.

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