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DIGLYCERIDE/MONOGLYCERIDE LIPASES PATHWAY IS NOT ESSENTIAL FOR ARACHIDONATE RELEASE IN THROMBIN-ACTIVATED HUMAN PLATELETS

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Summary: Human platelets prelabeled with arachidonate exhibited a rapid and transient rise in arachidonoyl monoglyceride in addition to arachidonoyl diglyceride following thrombin stimulation. Substantial release of arachidonate and its metabolites also occurred at the early phase. Preincubation of labeled platelets with RHC 80267, a potent inhibitor of diglyceride lipase, prior to thrombin stimulation abolished the transient rise in monoglyceride but not the increase in diglyceride and the release of arachidonate and its metabolites. These results suggest that diglyceride does metabolize to monoglyceride and release arachidonate in intact platelets. However, the diglyceride/monoglyceride lipases pathway does not appear to be essential in releasing arachidonate during thrombin stimulation.

Rapid turnover of phosphatidylinositol in human platelets following thrombin stimulation is known to be accompanied by a transient rise in arachidonoyl diglyceride (1). Degradation of phosphatidylinositol to diglyceride is thought to be catalyzed by a phosphatidylinositol-specific phospholipase C (2,3). Further metabolism of diglyceride to arachidonate was shown by Bell et al. who demonstrated the presence of a diglyceride lipase in platelet membrane fraction which catalyzed effectively the deacylation of diglyceride (4). Accordingly, a novel pathway for arachidonate release which combines the action of a phosphatidylinositol-specific phospholipase C and a diglyceride lipase was proposed (4). Using mixed positional labeled diglyceride as substrates, we found that release of arachidonate from diglyceride by platelet membranes actually required the sequential action of a diglyceride lipase and a monoglyceride lipase (5). Diglyceride lipase catalyzes first the deacylation of diglyceride at sn-1, and monoglyceride lipase then catalyzes the hydrolysis of the resulting 2-arachidonyl monoglyceride to arachidonate and glycerol (5,6). Although this novel pathway combining phospholipase C and two lipases appears to be attractive, evidence for the existence of this pathway and its significance in releasing arachidonate in intact platelets are lacking. We now demonstrate the transient accumulation of labeled

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arachidonoyl monoglyceride in addition to labeled arachidonoyl diglyceride in prelabeled human platelets following thrombin stimulation. We further show that addition of a specific inhibitor for diglyceride lipase to prelabeled platelets abolishes the transient rise of labeled monoglyceride but not arachidonate release.

MATERIALS AND METHODS

Materials: [1-^{14}C]Arachidonic acid (57.6 mCi/mmol) was purchased from Amersham. Human thrombin and essentially fatty acid free bovine albumin were from Sigma. 1,2-Diglyceride and 2-monoolein were purchased from Serdary Research Laboratories, Inc., London, Ontario, Canada. RHC-80267 (1,6-di(O-carbamoyl)cyclohexanone oxime)hexane was kindly supplied by Dr. C. Sutherland of Revlon Health Group. Precoated silica gel G plates were products of MCB Manufacturing Chemist, Inc., Cincinnati, OH. Fresh human platelet concentrates were obtained from the Central Kentucky Blood Bank, Lexington, KY.

Preparation of ^{14}C-arachidonate labeled platelets: Fresh human platelet concentrates from one unit of blood were provided by the blood bank within 15 hr of withdrawal from normal donor. Platelet concentrates were centrifuged at 1000 xg for 10 min at 4°C. Platelets were resuspended gently in 25 ml of Tris-citrate-bicarbonate buffer, pH 7.0, containing 10 mM citrate-dextrose and 1% fatty acid free bovine albumin (7) and then incubated at 37°C for one hour with 7.5 μCi [1-^{14}C]arachidonate which had been bound to albumin (8). After centrifugation at 1000 xg for 7 min at 4°C, platelet pellet was washed once with the same buffer described above and finally resuspended in buffer without citrate and albumin to a concentration of 4 x 10^9 platelets/ml. Plastics and siliconized glassware were used throughout.

Stimulation of platelets by thrombin: Labeled platelet suspension was warmed to room temperature before running experiments. For studying effect of RHC 80267 platelets were preincubated with 10 μM RHC 80267 for 15 min prior to thrombin stimulation. Reaction was initiated by adding 8 units of thrombin (in 40 μl of deionized water) to 1 ml of platelet suspension which was kept stirring at room temperature. After incubation with thrombin for the indicated time, 3.75 ml of $CHCl_3$/MeOH (1:2) was poured into reaction mixture and platelet lipids were extracted as described by Cohen et al. (9). For complete recovery of thromboxin B_2 and prostaglandins, methanol-water phase was acidified by adding 50 μl of acetic acid, and re-extracted twice with 2 ml of $CHCl_3$/MeOH (5:1). The lower $CHCl_3$ phase was combined and evaporated under nitrogen at 37°C.

Chromatographic separation of lipid extracts: Lipid extract was fractionated by silicic acid column chromatography (10). Neutral lipid fraction was eluted by $CHCl_3$ and analyzed by two dimensional thin layer chromatography. Lipid samples with standards were applied to silica G plates (7 x 10 m). The plates were developed to 5 cm in the first solvent system of $CHCl_3$/MeOH/NH_4OH (95:2:1). After heating in an oven (60°C) for 15 min, plates were run in second dimension for 8 cm with the solvent system of petroleum ether/ethyl ether/acetic acid (60:40:1). Diglyceride, monoglyceride, 12-HETE and arachidonate were well separated. The lipids were localized by exposure to iodine vapor and scraped into scintillation vial for counting. Thromboxane B_2 and other prostaglandins eluted by $CHCl_3$/MeOH (95:5) from silicic acid column were analyzed by thin layer chromatography using the solvent system of benzene/dioxane/acetic acid (20:10:1). Prostaglandin standards were added during TLC for identification.

Diglyceride lipase and diglyceride kinase assays: Diglyceride lipase activity was assayed as described previously (5). For diglyceride kinase activity, the

reaction mixture contained: 50 μM 1-[^{14}C]stearoyl diglyceride, 5 mM ATP, 10 mM $MgCl_2$, and 100 μg of platelet microsomal protein in a total volume of 0.5 ml of 50 mM HEPES buffer, pH 7.0. The reaction was initiated by adding microsomal protein and terminated by adding 0.5 ml of $CHCl_3$/MeOH/ 1 N HCl (1:2:0.5, v/v) followed by 0.1 ml of 1 N HCl and 0.5 ml of $CHCl_3$. The lower $CHCl_3$ layer was removed after centrifugation and the MeOH-H_2O phase was extracted once more with 0.5 ml $CHCl_3$. The combined $CHCl_3$ layer was then dried under N_2. Lipid residue was redissolved in $CHCl_3$/MeOH (1:2) and spotted on a silica gel plate (2 x 10 m) with phosphatidic acid as standard. After development in a solvent system of $CHCl_3$/MeOH/acetic acid (90:10:12) for 7 cm, the phosphatidic acid region was localized by exposure to I_2 vapor and scraped off the plate for counting. For studying inhibitory effect of RHC 80267 on both diglyceride lipase and kinase activities, RHC 80267 was firstly dissolved in dimethyl sulfoxide and then diluted 100 fold into the reaction mixture. One percent of DMSO showed no inhibitory effect on both enzymes.

RESULTS AND DISCUSSION

Rittenhouse-Simmons (1) first reported a rapid and transient rise of labeled diglyceride in arachidonate-prelabeled human platelets following thrombin stimulation. This observation was subsequently confirmed in several laboratories (4,11,12). The transient nature of diglyceride existence suggests that metabolism of this substance occurs rapidly in activated platelets. Three metabolic routes for diglyceride have been described. The first one is the acylation of diglyceride to triglyceride. The acylation does not appear to be favorable in activated platelets since labeled triglyceride was not found to increase in arachidonate-prelabeled platelets following stimulation (11). The second one is the phosphorylation of diglyceride to phosphatidic acid catalyzed by a diglyceride kinase (9). This pathway was obviously functioning since phosphatidic acid was shown to increase in activated platelets (14,15). The third pathway is the deacylation of diglyceride to 2-monoglyceride catalyzed by a diglyceride lipase. Although the existence of this enzyme has been demonstrated in human platelet membrane fraction (4,5), the function of this enzyme in intact platelets has yet to be demonstrated. We have prelabeled human platelets with [^{14}C]arachidonate and stimulated them with thrombin. A transient rise of [^{14}C]arachidonoyl diglyceride at 15 seconds was observed as shown in Fig. 1. The time course of diglyceride formation was consistent with those described by other laboratories (1,12). Interestingly, [^{14}C]arachidonoyl monoglyceride was also found to accumulate peaking at 30 seconds following thrombin stimulation. Labeled arachidonate continued to rise immediately following stimulation and leveled off at about 2 minutes as shown in the same figure. Labeled metabolites of arachidonate such as thromboxane B_2 and 12-hydroxyeicosatetraenoic acid (12-HETE) also increased steadily and represented approximately 20% and 10% respectively of arachidonate released at 2 minutes following stimulation (data not shown). These results suggest that diglyceride does metabolize to monoglyceride in intact platelets. The transient labeled arachidonoyl monoglyceride was presumably further metabolized

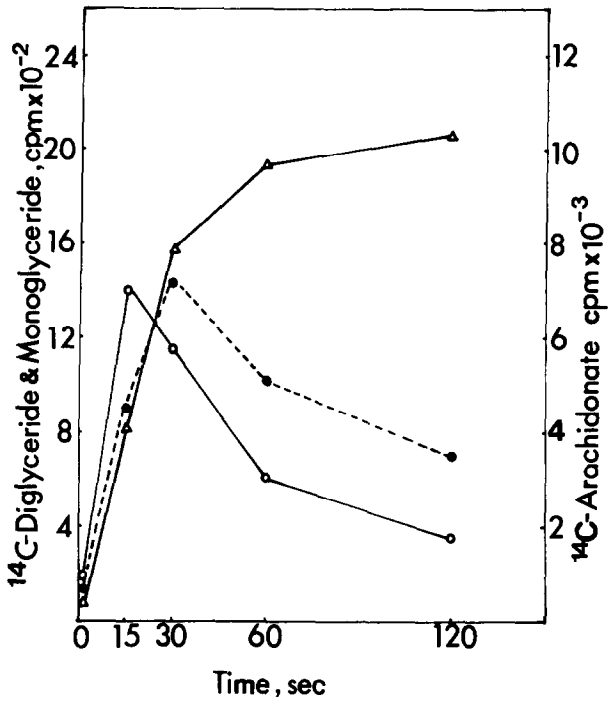

Fig. 1: Transient accumulations of diglyceride and monoglyceride in thrombin-stimulated human platelets.
[^{14}C]Arachidonate labelled platelets (4 x 10^9/ml) containing 30,000 cpm in PI were stimulated with thrombin (8 U/ml) for various times as indicated. Lipids were extracted and analyzed as described in Materials and Methods.

O—O, diglyceride; ●—●, monoglyceride; Δ—Δ, arachidonic acid

to labeled arachidonate since a very active monoglyceride lipase were shown to be present in platelets (5,6). This presumption was supported by the fact that platelet aggregation could be induced by 1-arachidonoyl monoglyceride and inhibited by further addition of aspirin and indomethacin (16).

If metabolism of arachidonoyl diglyceride to arachidonoyl monoglyceride and subsequently to arachidonate is essential in providing arachidonate, and metabolism of diglyceride to phosphatidate is only for resynthesis of phosphatidylinositol through phosphatidylinositol cycle (17), inhibition of diglyceride lipase alone should result in disappearance of transient rise in monoglyceride and decrease in overall arachidonate release. A specific inhibitor for diglyceride lipase will undoubtedly aid in assessing the role of diglyceride/monoglyceride lipases pathway in arachidonate release. Recently, Sutherland and Amin (18) reported a potent and selective inhibitor, RHC 80267, for canine platelet diglyceride lipase (I_{50} = 4 μM) which showed little inhibitory effect on phospholipases A_2 and C. We have found that this compound is also a potent inhibitor for human platelet diglyceride lipase and inhibits diglyceride

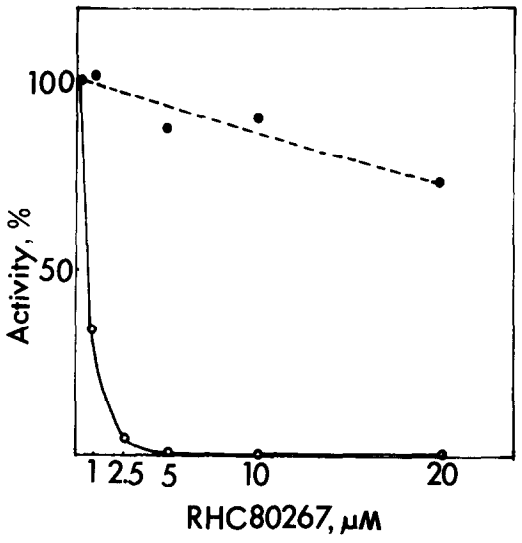

Fig. 2: Effect of RHC 80267 on diglyceride lipase and diglyceride kinase activities from human platelet microsomes.
Both lipase and kinase activities were assayed under conditions described in Materials and Methods with various concentrations of RHC 80267 as indicated.

O—O, diglyceride lipase; ●—●, diglyceride kinase

kinase only at relatively high concentrations as shown in Fig. 2. Preincubation of prelabeled platelets with 10 μM of RHC 80267 followed by stimulation with thrombin showed that a transient rise in labeled diglyceride but not in labeled monoglyceride was observed as shown in Fig. 3. Interestingly, labeled arachidonate increased progressively following stimulation. In fact, the amount of labeled arachidonate released was slightly higher in the presence than in the absence of RHC 80267. This was probably due to slight inhibition of cyclooxygenase or thromboxane synthetase by RHC 80267 since total amount of labeled arachidonate and its metabolites remained constant. This proposition was supported by the finding that arachidonate induced thromboxane synthesis in intact platelets was inhibited by the presence of this compound (data not shown). The fact that release of arachidonate and its metabolites remained unchanged in the presence of RHC 80267 suggests that diglyceride/monoglyceride lipases pathway is not essential in arachidonate release. Apparently, diglyceride diverted its metabolism entirely to phosphatidate if not to triglyceride in the presence of RHC 80267 since we showed that dichotomous metabolism of diglyceride to monoglyceride and to phosphatidate could occur concurrently based on kinetic properties of diglyceride lipase and diglyceride kinase (6).

In addition to diglyceride/monoglyceride lipases pathway, other pathways for arachidonate release have been proposed. Direct deacylation of phospholipids by phosphalipase A_2 has been recognized as a favorable pathway since

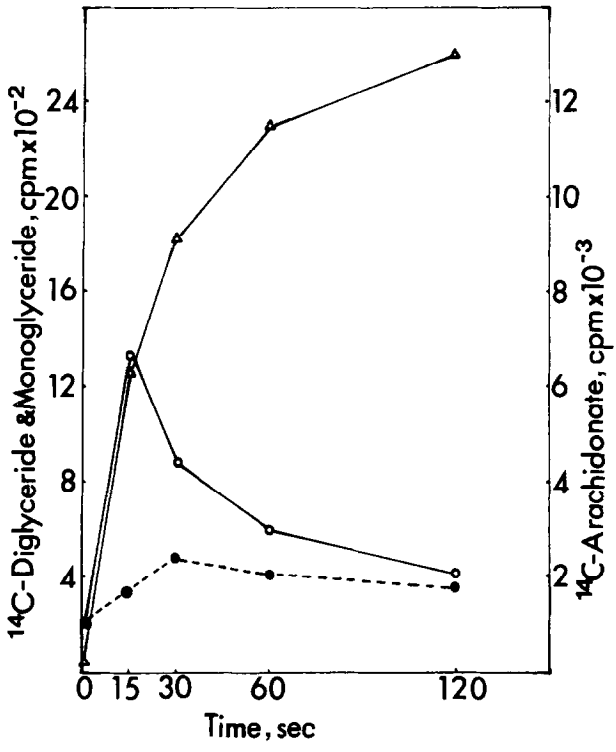

Fig. 3: Effect of RHC 80267 on the formation of diglyceride and monoglyceride in thrombin-stimulated human platelets. Labeled platelets were preincubated with 10 μM of RHC 80267 for 15 min prior to thrombin stimulation. Conditions of stimulation and methods of analysis are the same as indicated in Fig. 1.

O—O, diglyceride; ●—●, monoglyceride; Δ—Δ, arachidonic acid

this represents the simplest mode of release of arachidonate (8,19). However, Billah et al. (20) proposed a modified phospholipase A_2 pathway that diglyceride derived from phosphatidylinositol was converted entirely to phosphatidate, at least in activated horse platelets, which was effected to release arachidonate catalyzed by a phosphatidate specific phospholipase A_2. The resultant lysophosphatidate can be reacylated by arachidonate-containing phospholipids through transesterification process (21). Lysophospholipids found in stimulated platelets can be derived by this manner and/or by direct deacylation of phospholipids initiated by phosphatidate formed (22). Although our finding indicates that diglyceride/monoglyceride lipases pathway may not be essential in arachidonate release, it does not necessarily support phospholipase A_2 and/or modified phospholipase A_2 pathways play a major role in arachidonate release. The percent contribution by diglyceride/monoglyceride pathway to overall arachidonate release following thrombin stimulation still remains to be determined.

Just prior to submission of this paper for publication, we noted that Prescott and Majerus also demonstrated very recently the transient accumulation of an arachidonoyl-monoglyceride intermediate during thrombin stimulation (23).

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