Characterization of 1,2-Diacylglycerol Hydrolysis in Human Platelets

DEMONSTRATION OF AN ARACHIDONOYL-MONOACYLGLYCEROL INTERMEDIATE*

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When platelets are stimulated by thrombin, a phosphatidylinositol-specific phospholipase C produces a transient rise in 1,2-diacylglycerol. We have now characterized the hydrolysis of diacylglycerol by platelet membranes using doubly isotopically labeled substrates of defined fatty acid composition. We find that the fatty acid at sn-1 is hydrolyzed faster than that at sn-2 thereby producing a 2-monoacylglycerol intermediate.

If hydrolysis had occurred at either position randomly, 1-monoacylglycerol would also be produced. That none was detected indicates that either the sn-1 fatty acid must be cleaved first or that 1-monoacylglycerol is hydrolyzed by monoacylglycerol lipase much faster than 2-monoacylglycerol. The latter possibility was excluded by the finding that 1-monoacylglycerol and 2-monoacylglycerol are hydrolyzed at equal rates by platelet membranes.

The diacylglycerol lipase cleaves diacylglycerols with sn-1 palmitate as rapidly as those with sn-1 stearate. Arachidonate at sn-2 is cleaved twice as fast as sn-2 oleate by monoacylglycerol lipase. The two activities probably represent discrete enzymes since monoacylglycerol lipase activity can be separated from diacylglycerol lipase by fractionation on DEAE-Sepharose, although both are contained in the membrane fraction of platelets.

That the sequential breakdown of 1,2-diacylglycerol also occurs in intact platelets is indicated by our finding of a transient rise in arachidonoyl-monoacylglycerol in thrombin-stimulated platelets. This provides further evidence for a role of the phospholipase C-diacylglycerol lipase pathway in the release of arachidonic acid.

We have described diacylglycerol lipase activity in human platelets (1) and proposed that the combined activities of a phosphatidylinositol-specific phospholipase C, which generates an arachidonate-rich diacylglycerol (2), and the diacylglycerol lipase represent an alternative to phospholipase A2 as a mechanism for arachidonate release from membrane phospholipids. The phospholipase C-diacylglycerol lipase pathway, although less direct than a phospholipase A2, has several attractive features: 1) platelets contain adequate activity, as measured in vitro, to account for the rapid release of arachidonate observed in stimulated platelets; 2) phosphatidylinositol contains 80% arachidonate at sn-2 (3, 4) which explains why arachidonate is the major unsaturated fatty acid released; 3) this pathway links the phosphatidylinositol effect, which has been described in many secretory tissues (5), to the production of arachidonate metabolites. There now is evidence for the phospholipase C-diacylglycerol lipase pathway in human placental membranes (6, 7), porcine thyroid (8, 9), murine fibrosarcoma cells (10), rat mast cells (11), 3T3 cells (12), ram seminal vesicles (13), and rabbit (14), sheep (13), and human platelets.

The diacylglycerol lipase from human platelets has not been purified or extensively characterized. It is not known whether the fatty acids are hydrolyzed at sn-1 and sn-2 randomly or in an ordered, stepwise reaction. In retrospect, an ordered reaction, with sn-1 hydrolyzed first, was suggested by our experiment in which fatty acid was released simultaneously with glycerol from a substrate labeled in the glycerol and in the sn-2 fatty acid (1). Further, Okazaki et al. (7) have shown that palmitate is hydrolyzed faster than oleate from a mixture of labeled palmitoyl and oleoyl diacylglycerols using fetal membranes and decidua vera as an enzyme source. Chau and Tai (15) reached the same conclusion using microsomes from human platelets when they showed that 2-monoacylglycerol, but not 1-monoacylglycerol, forms during hydrolysis of a mixture of diacylglycerols, of undefined fatty acid composition, with isotopic label at sn-1 and sn-2. Neither of these previous studies excluded the possibility that different rates of hydrolysis of possible intermediate 1- and 2-monoacylglycerols could account for the observed effect. Okazaki et al. (7) found that arachidonate is hydrolyzed faster than oleate at sn-2. Chau and Tai (15) found that incubation with platelet microsomes altered the ratio of arachidonate to oleate in substrate diacylglycerols which had been doubly isotopically labeled suggesting preferential hydrolysis of arachidonate. However, in the latter study, it is also possible that the observed results depended on different fatty acids at sn-1 rather than specificity at sn-2 since their substrates did not have a defined fatty acid composition.

Monoacylglycerol lipase activity is also present in human platelets (16, 17). This could reflect a separate enzyme from diacylglycerol lipase or an additional activity of the same enzyme. Fielding (18) has proposed that monoacylglycerol lipase may have a physiological role in lipolysis of plasma glycerides.

We now report that the hydrolysis of fatty acids from 1,2-diacylglycerol is an ordered, two-step reaction with the sn-1 position released first. 2-Monoacylglycerol accumulates transiently in enzymatic assays and in thrombin-stimulated platelets. The latter point supports a physiological role for the proposed phospholipase C-diacylglycerol lipase pathway.

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There is a preference for arachidonate at sn-2, but no preference for stearate at sn-1 in assays of diacylglycerol lipase. Both monoacylglycerol and diacylglycerol lipase activities are found in the membrane fraction of platelets. A monoacylglycerol lipase can be separated from diacylglycerol lipase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fresh platelets were obtained from normal donors. Fresh, outdated concentrates of human platelets were a gift from the Blood Center of Southeastern Wisconsin, Inc. Bovine thrombin was a gift from Dr. Craig Jackson (Washington University, St. Louis, MO). [5,6,8,9,11,12,14,15-3H]Arachidonic acid (120 Ci/mmol), 1-[14C]palmitoyl lysophosphatidylcholine (57 mCi/mmol), and [1-14C]stearoyl lysophosphatidylcholine (90 mCi/mmol) were purchased from Amerchol. [1-14C]Arachidonic acid (50 mCi/mmol), [5,10-3H]oleic acid (5 Ci/mmol), and [9-10-3H]triolein (150 Ci/mmol) were purchased from New England Nuclear. [1-14C]triolein was purchased from Dhamon (Los Angeles, CA). Unlabeled fatty acids and neutral lipids were from NuChek. Hepes was purchased from Calbiochem. DEAE-Sepharose, unlabeled lysophospholipids, pancreatic lipase, and Tween 20 were from Sigma. The phospholiophosphate C was purified from Bacillus cereus (19). All solvents were reagent grade or better.

**Diacylglycerol and Monoacylglycerol Lipase Assays**—The assay for diacylglycerol lipase was performed as previously described (20). In experiments in which rates of release of different fatty acids were compared, the organic phase of the extraction mixture (21) had the appropriate unlabeled fatty acids added to a concentration of 5 mM as carrier. In some experiments, the reaction was stopped with 0.5 ml of chloroform:methanol:concentrated HCl (2:1:0.01 v/v/v) to precipitate free fatty acids and substrate. Reaction mixtures were separated by the addition of 0.15 ml of 1 N HCl containing 5 mM ethylene glycol bis-[beta-aminoethyl ether]-N,N,N',N'-tetraacetic acid, and the lower (organic) phase, which contained all the product and unreacted substrate, was analyzed by thin layer chromatography. Monoacylglycerol lipase was assayed just as the diacylglycerol lipase, except that the substrate was added as a solution in dimethyl sulfoxide instead of as a sonicated dispersion. Reaction mixtures contained up to 5 ml of dimethyl sulfoxide in a total assay volume of 100 ml. At this level, dimethyl sulfoxide was slightly stimulatory and did not alter the results of the extraction.

**Preparation of Substrates**—The 1,2-diacylglycerols used in these experiments were prepared as described (20, method 1). In double isotope experiments, two phosphatidylcholines with the appropriate composition were mixed and then incubated with phospholipase C, e.g., 1-[14C]palmitoyl, 2-oleoyl phosphatidylcholine + 1-palmitoyl, 2-oleoyl phosphatidylcholine + 1-[14C]palmitoyl, 2-[3H]oleoyl phosphatidylcholine. The hydrolysis of 1-[14C]palmitoyl, 2-[3H]oleoyl-sn-glycerol (240 pmol) was measured by scintillation spectrometry. 0, [1-14C]palmitate; 0, [3H]oleate.

**RESULTS**

**Order of Release of Fatty Acids from 1,2-Diacylglycerol**—The order of fatty acid release from 1-[14C]palmitoyl, 2-[3H]oleoyl-sn-glycerol is shown in Fig. 1. The sn-1 position is hydrolyzed first as shown by a greater accumulation of palmitate at each time. The pattern of release from sn-1 preceding that from sn-2 was observed in five experiments using membrane preparations from different donors (Table I). Although the total release varied by as much as 2-1/2-fold, palmitate release consistently exceeded oleate. In experiments where all of the products of the reaction were separated by TLC, up to 0.8 nmol of 2-[3H]monoolein accumulated in the assay, but no 1-[14C]monopalmitin was found. A little less than 0.1 nmol (~50 cpm) of [14C]monopalmitin could have been relia-

![Fig. 1](left). Order of release of fatty acids from 1,2-diacylglycerol.

![Fig. 2](right). Hydrolysis of 1-monooacylglycerol and 2-monooacylglycerol by platelet membranes. [1-14C]oleoyl-snglycerol and 2-[3H]oleoyl-sn-glycerol were mixed in equal proportions and added to a reaction mixture containing 25 mg of membrane protein. The substrate concentration was 500 pmol (250 pmol each substrate).  

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1 The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.
The fatty acid at sn-1 of 1,2-diacylglycerol is released before sn-2.

Five experiments were performed as described in the legend of Fig. 1. The ratio of palmitate (sn-1) to oleate (sn-2) released was calculated at each time point and an average from the same time point in different experiments was obtained. The points measured in three or more experiments are shown as are all points from the five experiments. A one-tailed "t"-test was used to evaluate the probability that the observed ratio was significantly different from 1:0.

| Time (min) | Released palmitate/oleate |
|------------|--------------------------|
| 2 (3)      | 1.8 ± 0.6                |
| 10 (4)     | 1.5 ± 0.3                |
| 20 (3)     | 1.4 ± 0.1                |
| 30 (5)     | 1.4 ± 0.2                |
| All points (20) | 1.51 ± 0.36                          |

* Not significant.

bly detected in the same experiment. The order of release of fatty acids was also determined in two experiments using 1-[14C]stearoyl, 2-[3H]arachidonoyl-sn-glycerol as substrate and the same pattern as that shown in Fig. 1 was obtained, i.e. 1.3-1.8-fold more stearate than arachidonate was found at all points from 5 to 30 min. These results suggest sequential release of the fatty acids with the sn-1 position of 1,2-diacylglycerol hydrolyzed first. An alternative explanation for the results is that the rate of release is identical from both positions of 1,2-diacylglycerol but that the resultant 1-monocacylglycerol is hydrolyzed faster than 2-monocacylglycerol. This hypothesis was excluded by our finding of an identical time course of hydrolysis of 1-[14C]stearoyl and 2-[3H]arachidonoyl-sn-glycerol as shown in Fig. 2. In other experiments performed with substrate concentrations from 100 μM to 1 mM, rates of hydrolysis ranged from 18 to 66 nmol/min/mg of microsomal protein and were equal with either substrate. The apparent Kₘ, obtained from Lineweaver-Burke plots is 530 μM for 1-monocacylglycerol and 750 μM for 2-monocacylglycerol. These values should be interpreted cautiously since the substrate is hydrophobic and a membrane preparation was the source of enzymatic activity. Nonetheless, the observed order of release from 1,2-diacylglycerol cannot be explained by different rates of hydrolysis of 1-monocacylglycerol versus 2-monocacylglycerol. A possible, but unlikely, exception to this conclusion is that a 1-monocacylglycerol containing a saturated fatty acid is preferred over a 2-monocacylglycerol containing an unsaturated fatty acid.

Transient Accumulation of Arachidonoyl Monocacylglycerol in Thrombin-stimulated Platelets—When washed human platelets, which have been labeled with [3H]arachidonic acid, are stimulated with thrombin, there is a transient rise in 1,2-diacylglycerol (Fig. 3) as previously reported by Rittenhouse-Simmons (2). In approximately the same time course (Fig. 3, Table II), an accumulation and then loss of monocacylglycerol also occurs indicating the presence of 2-arachidonoyl-sn-glycerol as an intermediate product of the diacylglycerol lipase. Neither diacylglycerol nor monoacylglycerol accumulated in the absence of thrombin. In other incubations with identical labeling techniques and the same total incorporation of arachidonate, we measured the specific activity of [3H]arachidonate in phosphatidylglycerol and released arachidonate. Values ranged between 2500 and 3500 cpm/nmol. Based on these results, we estimate the peak of 1,2-diacylglycerol to be approximately 0.6 nmol/10⁶ platelets which agrees with the results obtained by Rittenhouse-Simmons (2). In the experiments shown in Table II, 2-arachidonoyl-sn-glycerol accumulated transiently to a maximum of approximately 75% of the diacylglycerol peak. The observation of this intermediate supports an ordered release from 1,2-diacylglycerol in intact, thrombin-stimulated platelets.

Substrate Specificity of Diacylglycerol Lipase—Since the predominant species of diacylglycerol derived from phosphatidylglycerol is 1-stearoyl, 2-arachidonoyl-sn-glycerol, we investigated whether this is a preferred substrate for hydrolysis. In each of two separate experiments, we measured rates of hydrolysis of arachidonate versus oleate from diacylglycerols with palmitate at sn-1 and find that there is a 2-fold greater release of arachidonate (Fig. 4) in agreement with observations in placental membranes (7). We have also examined the effect of the fatty acid at sn-1 or diacylglycerol breakdown using substrates with stearate or palmitate in the sn-1 position and arachidonate at sn-2 (Fig. 5) and find that palmitate-containing substrate is hydrolyzed at the same rate or even slightly faster than that containing stearate.

The Relationship of Monoacylglycerol Lipase and Diacylglycerol Lipase Activities—Subcellular fractionation of

![Diacylglycerol Hydrolysis in Platelets](image)
Diacylglycerol Hydrolysis in Platelets

Fig. 5. The effect of the fatty acid at sn-1 on the rate of hydrolysis of 1,2-diacylglycerol. Assays for diacylglycerol lipase activity in crude membranes were performed with substrates which were identical at sn-2 but had different fatty acids at sn-1. In the experiment illustrated in A, an equimolar mixture of 1-palmitoyl, 2-[14C]arachidonoyl-sn-glycerol and 1-stearoyl, 2-[3H]arachidonoyl-sn-glycerol (concentration of each in assay, 125 μM) was employed as the substrate. △, [14C]arachidonate (palmitate); ○, [3H]arachidonate (stearate). B, in another experiment, 1-[14C]palmitoyl, 2-arachidonoyl-sn-glycerol, and 1-[14C]stearoyl, 2-arachidonoyl-sn-glycerol were used as the substrates (250 μM) in separate, simultaneous assays. ○, stearate; △, palmitate.

Fig. 6. Diacylglycerol and monoacylglycerol lipase activities in subcellular fractions of human platelets. Platelets were disrupted by nitrogen decompression and fractionated by density gradient centrifugation. The contents of the fractions were: I, cytoplasm; 2-4, membranes (including microsomes); 4-5, mitochondria and lysosomes; 6-7, α granules; 8, dense bodies. Open bars, monoacylglycerol substrate; shaded bars, 1,2-diacylglycerol substrate.

Platelets was performed and each of the fractions were assayed for both monoacylglycerol and diacylglycerol lipase activities (Fig. 6). Both activities have the same distribution and are found in the membrane-containing fractions. In our respective assays, there consistently was 20 to 50 times as much monoacylglycerol lipase activity as diacylglycerol lipase although the differences decreased when the activities were measured at early time points at high substrate and low enzyme concentrations. Both activities are lost in parallel by heating at 50 °C or exposure to N-ethylmaleimide, diisopropylfluorophosphate, or phenylmethylsulfonyl fluoride (data not shown). We have attempted to determine whether the two activities can be separated from each other. As we have described (20), solubilization and a 3- to 4-fold purification can be accomplished by sonication in a buffer containing low concentrations of detergent followed by fractionation with ammonium sulfate. Monoacylglycerol and diacylglycerol lipase activities are not separated by these procedures. Attempts to purify the enzyme(s) by ion exchange, hydrophobic dye matrix, and gel filtration chromatography under a variety of conditions have been largely unsuccessful. In most cases, little activity binds to the column suggesting that although the activities are present in a 100,000 × g supernatant they are associated with lipid and/or detergent micelles. Monoacylglycerol lipase activ-

Fig. 7. DEAE-Sepharose chromatography of monoacylglycerol lipase. A solubilized preparation from platelet concentrates, containing 6.5 pmol/min of monoacylglycerol lipase activity (see text), was applied to a 32-ml column (2.5 × 6.5 cm) which had been equilibrated in the starting buffer: 20 mM Hepes, pH 7.0, 10 mM EDTA, 14 mM 2-mercaptoethanol, and 0.01% Tween 20. The column was washed at 60 ml/h with the same buffer and fractions (2.8 ml) were collected. Bound protein (peaks D-F) was eluted by adding 1 M NaCl to the buffer (fraction 56 on). The chromatography was performed at 4 °C. Fractions were pooled as shown (A-F). ●, lipase activity; - - - , A_{230}.
ity elutes in three peaks from a DEAE-Sephacel column as shown in Fig. 7. The first peak does not bind to the column, and the others elute with further washing and the addition of NaCl, respectively. When the pooled fractions were assayed for both monoacylglycerol and diacylglycerol activities, the intermediate peak of monoacylglycerol lipase activity (C) was found to be almost devoid of diacylglycerol lipase activity while the other peaks contained both (Fig. 8). Thus, it appears that there is a monoacylglycerol lipase that does not metabolize 1,2-diacylglycerol. This does not resolve the issue of whether a single enzyme that acts on 1,2-diacylglycerol can hydrolyze a monoacylglycerol as well.

**Discussion**

Okazaki et al. (7) concluded that diacylglycerol lipase from placental tissue cleaves the sn-1 position of 1,2-diacylglycerol prior to cleaving the sn-2 position. Chau and Tai (15), using platelet microsomes and diacylglycerol lipase of undefined fatty acid composition, which were labeled in either the sn-1 or sn-2 position, observed the accumulation of 2-monounsaturated fatty acid but not 1-monounsaturated fatty acid and also concluded that the sn-1 position of diacylglycerol is cleaved first. We have confirmed and extended these results using substrate diacylglycerols of defined fatty acid composition. We find that: 1) the sn-1 position is cleaved before the sn-2 position; 2) 2-monounsaturated fatty acid, but not 1-monounsaturated fatty acid, accumulates during the reaction; 3) 1- and 2-monounsaturated fatty acids are hydrolyzed at equal rates by platelet microsomes, thereby excluding the possibility that 1-monounsaturated fatty acid does not appear as an intermediate because of prefrential hydrolysis; 4) arachidonate at sn-2 is cleaved slightly faster (~2-fold) than oleate at the same position; and 5) either palmitate or stearate is readily hydrolyzed from sn-1 indicating a lack of specificity for the fatty acid in this position.

The fact that the sn-1 position of diacylglycerol is cleaved first and that platelet microsomes cleave palmityl- and stearoyl-diacylglycerol readily suggests that the specificity of fatty acid hydrolysis in intact platelets as well as in assays of membranes using exogenous diacylglycerol.

The monoacylglycerol and diacylglycerol lipase activities of human platelets may represent different enzymes. Chau and Tai (15) reached this conclusion based on differences in pH optimum and Okazaki, et al. (7) also favored two distinct enzymes in placental tissues based on different rates of inhibition and different percentages of the two activities in the supernatant following homogenization and centrifugation. In our experiments, both activities were found in the same subcellular fractions (Fig. 6), were inactivated equally well by several inhibitors and heat, were both solubilized with detergent, and were found in the same ammonium sulfate fractions. However, a portion of the monoacylglycerol lipase activity was separated from fractions containing both activities suggesting that monoacylglycerol lipase is a separate enzyme (Fig. 8). Whether there is another enzyme which has only diacylglycerol lipase activity or both activities remains undetermined in spite of our attempts at purification.

We conclude that most of the 1-stearoyl, 2-arachidonoyl-sn-glycerol which appears in thrombin-stimulated platelets is acted upon by a diacylglycerol lipase to yield 2-arachidonoyl-sn-glycerol. This monoacylglycerol undergoes hydrolysis catalyzed by a monoacylglycerol lipase, which is probably a separate enzyme, to release arachidonate for further metabolism.

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