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T K Bills, ... , J B Smith, M J Silver

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Research Article

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Selective Release of Arachidonic Acid from the Phospholipids of Human Platelets in Response to Thrombin

T. K. BILLS, J. B. SMITH, and M. J. SILVER

From the Cardeza Foundation and Department of Pharmacology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

ABSTRACT Arachidonic acid is unique amongst human platelet fatty acids in that it is the precursor of prostaglandins and thromboxanes. Since a number of these oxygenated products of arachidonic acid have potent effects on platelet function, an understanding of the metabolism of their precursor is important. Human platelets have a mechanism for incorporating arachidonic acid from plasma into their phospholipids and, in response to thrombin, they reveal mechanisms for hydrolyzing this arachidonic acid from platelet phosphatidylcholine and phosphatidylinositol. This report deals with the specificity of these mechanisms. The present studies show that human platelets contain phospholipase A₂ activities that preferentially release arachidonic acid. One of these activities specifically utilizes 1-acyl-2-arachidonoyl-phosphatidylcholine. Another utilizes platelet phosphatidylinositol and/or phosphatidylserine, both of which are highly enriched with arachidonic acid.

INTRODUCTION

During platelet aggregation, arachidonic acid is transformed into several oxygenated products now considered to play an important role in hemostasis. These products include prostaglandins G₂, H₂, and D₂ (1-4) as well as the newly discovered thromboxane A₂ (5). Since only trace amounts of free arachidonic acid are found in platelets (6), the arachidonic acid that is oxygenated during platelet aggregation must be released from complex lipids, and these appear to be

platelet phosphatidylcholine (PC)¹ and phosphatidylinositol (PI). We have shown that radioactive arachidonic acid is readily incorporated from plasma into the phospholipids of human platelets (7). When these platelets are washed and treated with thrombin, this radioactive arachidonic acid is released from PC and PI and oxygenated by platelet lipoxygenase and cyclooxygenase, giving rise to radioactive prostaglandins, thromboxane B₂ and hydroxy-fatty acids (7).

The release of arachidonic acid from platelet PC and PI is the result of an endogenous phospholipase activity. The stimulation of this activity must be an early event in the induction of platelet aggregation and the platelet release reaction. We now report on the substrate specificity of this phospholipase activity.

We present evidence here that platelets may have two different phospholipase A₂ activities which are stimulated by thrombin, one specifically releases arachidonic acid from PC; the other releases mainly arachidonic acid and trace amounts of other fatty acids from PI and/or phosphatidylserine (PS). The net effect is a specific release of arachidonic acid in response to thrombin.

METHODS

Fatty acid incorporation-comparative studies. Citrated platelet-rich plasma (40 ml) prepared as described previously (7) was cooled to 4°C and EDTA² was added to give a final concentration of 1 mM. The platelet-rich plasma was centrifuged at 2,000 g for 20 min at 4°C. The supernate was discarded and the platelet pellet was resuspended in an equal volume of buffered saline containing EDTA (1

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¹Abbreviations used in this paper: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

²EDTA was added to aid in resuspension of platelets.

mM EDTA, 5 mM D-glucose, 134 mM NaCl, 15 mM Tris-HCl, pH 7.4, 1.0% fatty acid-poor albumin). The platelets were recentrifuged at 2,000 g for 15 min at 4°C and finally resuspended in an equal volume of the above solution without EDTA. These platelet suspensions, containing about 3.3×10^9 platelets/ml, were warmed to 37°C for 2 min and poured into a vessel containing a radioactive fatty acid (27,000 dpm/ml of platelet suspension) under nitrogen.³ Only one radioactive fatty acid was incubated with a given sample of platelet suspension and in all cases, all of the fatty acid was found to be evenly distributed in the suspension medium after a 5-min incubation. Incubations were continued in open vessels for 60 min at 37°C. Thus, the incorporation of radioactive arachidonic, 8,11,14-eicosatrienoic, linoleic, or oleic acids was compared under identical conditions.

After incubation, the suspensions were cooled at 4°C and EDTA was added to a final concentration of 1 mM. The suspensions were centrifuged at 2,000 g for 20 min at 4°C to yield a pellet containing 98% of the platelets. The supernate, containing unincorporated fatty acids, was removed. The platelets were resuspended in an equal volume of the above buffered saline containing EDTA, but without albumin and then recentrifuged at 2,000 g for 15 min at 4°C to yield a pellet containing 98% of the platelets. The platelets were finally resuspended in one-half volume of buffered saline without EDTA or albumin. The lipids from 3-ml samples of these platelet suspensions (containing about 2.0×10^9 platelets) were extracted, separated chromatographically, and then assayed for radioactivity exactly as described elsewhere (7).

Thrombin-induced changes in ¹⁴C-fatty acid-labeled platelets. Suspensions of platelets (3 ml containing about 2.0×10^9 platelets that had incorporated one of the ¹⁴C-fatty acids exactly as described above) were treated with either saline or thrombin (5 U/ml) for 5 min at 37°C. The lipids were extracted, isolated, and assayed for radioactivity. Thin-layer chromatography of phospholipids did not separate PS from PI and material in this fraction is referred to as PS + PI.

Thrombin-induced changes in total free fatty acids. Suspensions of platelets, that had incorporated [¹⁴C]arachidonic acid as described above, were treated with either saline or thrombin (5 U/ml) for 5 min at 37°C. Samples that were to be treated with thrombin were preincubated with 30 μM 5,8,11,14-eicosatetraenoic acid for 5 min at 37°C to block the oxygenation of the released arachidonic acid (7). The lipids from these samples were extracted and separated chromatographically. (Docosanoic acid was added to each sample as an internal standard before extraction). The zones of silica gel from the thin-layer chromatographic plate containing free fatty acids were scraped into columns stoppered with glass wool and the fatty acids were eluted with 10 ml chloroform. A 2-ml sample of the eluent was transferred to a scintillation vial, evaporated, and then assayed for radioactivity. The remainder of the eluent was blown to dryness and the fatty acid methyl esters were prepared as previously described (8). These methyl esters were separated by gas-liquid chromatography using a Barber-Colman instrument (Barber-Colman Co., Rockford, Ill.) fitted with a column packed with 10% Silar-10C on Gas-Chrom Q. The column was programmed between 170 and 235°C with a rise in temperature of 3°C/min. Quantitation of fatty acid methyl esters was done using a Vidar integrator (Vidar Corp., Mountain View, Calif.).

³ Suspensions of washed platelets were used to remove the nonradioactive fatty acid present in plasma so that there was no dilution in the initial specific activity of the added ¹⁴C-fatty acid.

All of the other chromatographically isolated lipids (7) were assayed for radioactivity to confirm that the normally observed changes occurred after thrombin treatment.

Estimation of pool size of [¹⁴C]arachidonic acid-labeled PC. To determine the relationship between [¹⁴C]arachidonic acid-labeled PC and total PC, we compared changes in radioactive PC induced by thrombin with changes in total PC. Trace amounts of [¹⁴C]arachidonic acid were incubated with citrated platelet-rich plasma (7) for 60 min at 37°C. The platelets were isolated by centrifugation, washed, and resuspended in buffered saline as described previously (7). Samples of the platelet suspension (3 ml) containing about 4.0×10^9 platelets were incubated at 37°C for 5 min with either saline or thrombin (5 U/ml). Total lipids were extracted from the samples and phospholipids were isolated by column chromatography (7). The PC and phosphatidylethanolamine (PE) were resolved by thin-layer chromatography (7) and visualized by means of iodine vapor. Zones of silica gel containing these individual phospholipids were scraped into columns stoppered with glass wool and the phospholipids were eluted with 10 ml methanol. A 4-ml sample was transferred to a scintillation vial, evaporated, and assayed for radioactivity. A second 4-ml sample was assayed for phospholipid phosphorus, which is indicative of either total PC or total PE, by the method of Harris and Popat (9).

Materials. [¹⁴C]Arachidonic, linoleic, and oleic acid (sp act 55 mCi/mM) as well as 10% Silar-10C on Gas-Chrom Q was purchased from Applied Science Labs, Inc., State College, Pa. [¹⁴C]8,11,14-Eicosatrienoic acid (sp act 57 mCi/mM) was purchased from New England Nuclear, Boston, Mass. and bovine serum albumin (Fraction V powder, fatty acid poor) from Pentex Biochemical, Kankakee, Ill. Docosanoic acid was purchased from Supelco, Inc., Bellefonte, Pa. All other materials are described elsewhere (7).

RESULTS

Studies of comparative fatty acid incorporation. Table I summarizes the results of experiments in which different radioactive fatty acids were incubated singly with human platelets under identical conditions. Arachidonic acid and 8,11,14-eicosatrienoic acid were readily incorporated into platelets whereas oleic and linoleic acids were poorly incorporated. Of the radioactivity incorporated into platelets, greater than 90% of that of arachidonic acid and 8,11,14-eicosatrienoic acid, and greater than 75% of that of the other fatty acids, was present in phospholipids. 8,11,14-Eicosatrienoic acid was incorporated into platelet triglycerides to a greater extent than the other fatty acids.

Thrombin-induced changes in ¹⁴C-fatty acid-labeled platelets. When platelets that had incorporated the ¹⁴C fatty acids exactly as described above were treated with thrombin, the changes in radioactivity of PC, summarized in Table II, were observed. Column 2 of Table II shows the relative incorporation of the ¹⁴C-fatty acids into PC for this set of experiments, while column 3 shows the percentage decrease in radioactivity of PC induced by thrombin. Only the thrombin-induced decrease in [¹⁴C]arachidonic acid-labeled PC was statistically significant.

TABLE I
Distribution of Radioactivity Incorporated into Platelet Lipids after Incubation with Radioactive Fatty Acids

Radioactive fatty acid added	Platelet lipids					Total incorporated
	FFA*	TG	PS + PI	PC	PE	
	% of ¹⁴ C-fatty acid added					
Arachidonic	0.5 (±0.0)‡	1.8 (±0.3)	14.9 (±2.0)	22.5 (±2.5)	6.2 (±1.1)	45.9
8,11,14-Eicosatrienoic	0.9 (±0.2)	4.6 (±0.1)	14.7 (±1.3)	37.9 (±0.8)	3.6 (±0.6)	61.7
Linoleic	0.4 (±0.0)	0.9 (±0.1)	0.8 (±0.1)	4.1 (±0.1)	0.6 (±0.1)	6.8
Oleic	0.4 (±0.2)	0.9 (±0.0)	0.6 (±0.1)	3.0 (±0.5)	0.4 (±0.3)	5.3

Human platelets were resuspended in a medium containing 1.0% albumin and incubated with an individual ¹⁴C-fatty acid. After incubation, the lipids were extracted, isolated and assayed for radioactivity (for details see Methods). Values are given as a percentage of the added radioactivity and are the means ± SE of at least four determinations.

* FFA, free fatty acids; TG, triglycerides.

‡ Values in parentheses represent SEM.

In contrast to the selective hydrolysis of [¹⁴C]arachidonyl-PC, a significant decrease in the radioactivity of PS + PI was noted for all of the fatty acids studied (Table III). The percentage decrease in radioactivity of PS + PI labeled with different fatty acids varied between 14.3 and 24.3% (Table III, column 3). However, the actual decrease in the radioactivity of these phospholipids was very small when platelets were labeled with the 18 carbon unsaturated fatty acids which were poorly incorporated, and great when they were labeled with the 20 carbon prostaglandin precursors which were readily incorporated. If the amount of radioactive arachidonic acid incorporated into this phospholipid fraction is considered to be 100%, then the relative decreases seen with the 18 carbon unsaturated fatty acids were 0.5 and 1.0% whereas those seen with arachidonic acid and 8,11,14-eicosatrienoic acid were 24.3 and 16.9% respectively (Table III, column 4).

No decrease in radioactivity of platelet PE was seen when platelets labeled with any of the fatty acids studied were treated with thrombin.

Thrombin-induced changes in total free fatty acid. When [¹⁴C]arachidonic acid-labeled platelets, pre-incubated with 5,8,11,14-eicosatetraenoic acid, were treated with thrombin there was release of radioactivity from PC and PI as previously reported (7). In this case, intact [¹⁴C]arachidonic acid accumulated since its oxygenation was blocked by 5,8,11,14-eicosatetraenoic acid. The thrombin-induced changes of total platelet free fatty acids are summarized in Table IV. Large amounts of free arachidonic acid accumulated in response to thrombin, but there was no significant increase in any of the other fatty acids.

Estimation of [¹⁴C]arachidonic acid-labeled PC pool size. Table V summarizes our results for this group of experiments. In these experiments, thrombin induced a 25.6% loss of radioactivity from PC but no

loss of radioactivity from PE, which is consistent with our previous report. (7). There was no net loss of radioactivity from platelet suspensions treated with either saline or thrombin; recovery after extraction was >90% in both cases. Phospholipid phosphorus determinations (Table V) demonstrated that thrombin induced a 7.6% loss of PC phosphorus but no loss of PE phosphorus. We interpret the loss of PC

TABLE II
Relative Incorporation into and Thrombin-Induced decreases in Platelet PC of Different 1-¹⁴C-Fatty Acids

Radioactive fatty acid	Relative incorporation into PC	Decrease in radioactivity of PC		Relative decrease of PC
	%	%	n‡	
Arachidonic	100.0	34.2‡	20	34.2
8,11,14-Eicosatrienoic	130.7 (±2.8)*	1.9 [¶]	7	—
Linoleic	14.2 (±0.4)	1.0 [¶]	5	—
Oleic	9.7 (±2.2)	0.9 [¶]	8	—

Suspensions of human platelets, prelabeled with an individual ¹⁴C-fatty acid, were treated with either saline or thrombin. Platelet PC was isolated and assayed for radioactivity (for details see Methods). For the purpose of comparison, the amount of [¹⁴C]arachidonic acid incorporated into PC has been designated as 100% (column 2). Incorporation of the other fatty acids is expressed relative to arachidonic acid incorporation. Column 3 gives the actual percentage decrease in radioactive PC induced by thrombin. The figures in column 4 were obtained by multiplying the numbers in columns 2 and 3. These figures reflect the actual amount of radioactive fatty acid released from PC.

* Values in parentheses represent standard error of the mean.

‡ P < 0.005.

¶ Number of determinations.

^{||} Values did not differ significantly from zero.

TABLE III
Relative Incorporation into and Thrombin-Induced
Decreases in Platelet PI + PS of Different
1-¹⁴C-Fatty Acids

Radioactive fatty acid	Relative incorporation into PS + PI	Decrease in radioactivity of PS + PI		Relative decrease of PS + PI
	%	%	n§	
Arachidonic	100.0	24.3†	20	24.3
8,11,14-Eicosatrienoic	79.9 (±7.1)*	21.2†	8	16.9
Linoleic	4.1 (±0.3)	21.8†	5	1.0
Oleic	3.6 (±0.5)	14.3†	8	0.5

Suspensions of human platelets, prelabeled with an individual ¹⁴C-fatty acid, were treated with either saline or thrombin. Platelet PS + PI was then isolated and assayed for radioactivity (for details see Methods). For the purpose of comparison the amount of [¹⁴C]arachidonic acid incorporated into PS + PI has been designated as 100% (column 2); incorporation of the other fatty acids is expressed as relative to arachidonic acid incorporation. Column 3 represents the actual percentage decrease in radioactive PS + PI induced by thrombin. The figures in column 4 were obtained by multiplying the numbers in columns 2 and 3. These figures reflect the actual amount of radioactive fatty acid released from PS + PI.

* Values in parentheses represent SEM.

† P < 0.01.

§ Number of determinations.

phosphorus to mean that lyso-PC and/or glycerylphosphorylcholine are formed, since these polar substances would not chromatograph with PC. The fact that there was no significant change in PE phosphorus supports our contention that this phosphatide is not a substrate for the stimulated phospholipase.

DISCUSSION

Incorporation of unsaturated fatty acids. We have previously compared the rates of incorporation of [¹⁴C]arachidonic acid into the various platelet phospholipids with the amounts of arachidonic acid reported to be present in the respective phospholipids of platelets (7). This comparison showed that the rates of incorporation could not fully explain the normal distribution of arachidonic acid in platelet phospholipids. The PE (including PE plasmalogen) and PS of human platelets normally contain large amounts of arachidonic acid (10) but we observed relatively little incorporation of radioactive arachidonic acid into PE and PS. Arachidonic acid found in these phospholipids therefore may be derived via other anabolic pathways. On the other hand, radioactive arachidonic acid was incorporated into platelet PC and PI from

plasma at rates that could account for the levels of this fatty acid normally esterified to PC and PI (10). Thus, the major source of the arachidonic acid normally esterified to platelet PC and PI may be free arachidonic acid which is known to circulate in plasma (11). Since PC and PI are the source of the arachidonic acid that is converted to prostaglandins and thromboxane A₂ during thrombin-induced aggregation, both the levels of free arachidonic acid circulating in plasma and the capacity of platelets to esterify this fatty acid to PC and PI would be expected to influence platelet function.

The present studies show that radioactive arachidonic acid is incorporated into the phospholipids of washed human platelets at a much greater rate than radioactive linoleic or oleic acids. Since this occurs in the presence of a pool of free linoleic and oleic acids (Table IV) it is possible that the rates of incorporation of all three fatty acids are similar, the specific activity of the arachidonic acid being much higher than that of oleic or linoleic acids.

It has been suggested that the linoleic acid in plasma is normally taken up by platelets, converted into arachidonic acid and then esterified to phospholipids (12). However, this hypothesis is based on studies of lysates of human platelets and, although such a pathway may exist, our results suggest that it is of little importance for the production of the prostaglandins and thromboxane A₂. Although [¹⁴C]linoleic acid is incorporated into the PC of platelets, it is not released from this phospholipid in response to thrombin. If the [¹⁴C]linoleic acid was converted to [¹⁴C]arachidonic

TABLE IV
Thrombin-Induced Changes in the Free Fatty Acid Content of Human Platelet Suspensions

Free fatty acid	Saline control	Thrombin
	nmol FFA/10 ⁹ platelets	
Palmitic	172.2 (±35.8)*	165.9 (±4.1)
Stearic	73.6 (±13.4)	81.9 (±4.5)
Oleic	125.3 (±33.1)	127.2 (±12.7)
Linoleic	36.4 (±8.9)	32.3 (±7.0)
Arachidonic	ND†	20.2 (±2.2)

Suspensions of human platelets, prelabeled with [¹⁴C]arachidonic acid, were incubated with either saline or thrombin. The lipids of these suspensions were extracted and isolated chromatographically. The free fatty acids were obtained as described in methods. A portion was assayed for radioactivity and the remainder was quantitatively analyzed by gas-liquid chromatography (for details see Methods). Values are given as nanomoles of free fatty acid/10⁹ platelets and are the means ± SE of six determinations. This procedure had a detection limit of 1 nmol fatty acid/10⁹ platelets.

* Values in parentheses represent SEM.

† ND means not detected.

acid before incorporation then a decrease in the amount of radioactivity in platelet PC after thrombin stimulation would have been detected. It was not. Furthermore, ¹⁴C-labeled products of platelet lipoxygenase and cyclooxygenase would also have been detected and they were not. This supports our contention that the arachidonic acid released from the phospholipids and utilized by the platelet oxygenation pathways is derived largely from the plasma pool of free arachidonic acid.

8,11,14-Eicosatrienoic acid is normally present at very low levels in both platelet phospholipids (10) and plasma (11). However, this prostaglandin precursor was readily incorporated into platelet phospholipids. It has been reported that rats and rabbits fed a diet enriched with 8,11,14-eicosatrienoic acid have elevated levels of this fatty acid in their plasmas as well as esterified to platelet phospholipids and triglycerides (13, 14). Our results suggest that similarly elevated levels would be obtained in platelets if humans were fed a diet enriched with 8,11,14-eicosatrienoic acid. Since this fatty acid is the precursor of prostaglandins known to inhibit platelet aggregation (15, 16), such a diet may provide a rationale for the prophylaxis of thrombosis.

Platelet phospholipase activities. The thrombin-induced decrease in phospholipid radioactivity in platelets labeled with [¹⁴C]arachidonic acid is due to a stimulated phospholipase activity (7). Based on the present evidence, we suggest that human platelets contain two phospholipase A₂ activities which are stimulated by thrombin. One activity utilizes platelet PC and releases only arachidonic acid. The other activity utilizes platelet PI and/or PS, two phosphatides that are highly enriched with arachidonic acid. It is not yet known whether these activities are due to one or more enzymes.

In our experiments with different ¹⁴C-fatty acids, more radioactivity was incorporated into platelet PC than into any other lipid component (Table I). However, only the PC labeled with [¹⁴C]arachidonic acid showed a decrease in radioactivity in response to thrombin (Table II). Since arachidonic acid has been reported to be esterified almost exclusively at the 2 position of platelet phospholipids (17) this decrease is probably the result of a stimulated phospholipase A₂ activity.

By contrast, the activity that utilized PI and PS showed no such specificity. When platelets, labeled with different ¹⁴C-fatty acids were treated with thrombin, decreases in the radioactivity of these two phospholipids were always seen (Table III). The actual amount of ¹⁴C-fatty acid released was quite small for oleic and linoleic acids because only small amounts of these radioactive fatty acids were present. Further, since both of these platelet phosphatides

TABLE V
Comparison of Changes in Radioactivity and Phosphorus Content of Platelet PC and PE Induced by Thrombin

Phospholipid	Saline control	Thrombin	Loss
Radioactivity, (% of saline control)			
PC	100.0	74.5 (±4.0)*	25.5‡
PE	100.0	103.8 (±3.2)	NS§
Phosphorus, (% of saline control)			
PC	100.0	92.4 (±3.1)	7.6‡
PE	100.0	97.5 (±2.1)	NS§

Suspensions of human platelets, prelabeled with [¹⁴C]arachidonic acid, were incubated with either saline or thrombin. The PC and PE of these platelets were isolated and assayed for both radioactivity and phospholipid phosphorus (for details see Methods). For the purpose of comparison, the mean values of both the radioactivity and the phosphorus content of PC and PE in saline controls has been expressed arbitrarily as 100%.

* Values in parentheses represent SEM.

‡ *P* < 0.005.

§ NS means not significant.

are highly enriched with arachidonic acid, the actual amounts of endogenous oleic and linoleic acids released would also be small.

The major unsaturated fatty acid released from PI and/or PS in response to thrombin would be arachidonic acid.

The studies discussed above demonstrate that radioactive oleic and linoleic acids are not hydrolyzed from platelet PC and PE although trace amounts are released from PI and/or PS. It is possible, however, that pools of platelet phosphatides that were not labeled do release large amounts of these unsaturated fatty acids. We therefore measured the amounts of non-radioactive fatty acids to ascertain whether thrombin induced the release of oleic and linoleic acids from unlabeled, endogenous phospholipid pools. These experiments confirm the tracer studies. Only arachidonic acid was seen to accumulate in response to thrombin. Thus none of the other commonly occurring fatty acids were released from platelet phospholipids in response to thrombin.

All of the above findings suggest that human platelets contain a thrombin-stimulated phospholipase A₂ activity that utilizes only 1-acyl-2-arachidonoyl-PC and changes in total platelet PC should reflect this fact. For example, if 100% of platelet PC contained arachidonic acid at the 2 position, then a thrombin-induced decrease in [¹⁴C]arachidonoyl-PC of 25% should be matched by a 25% decrease in total PC (assuming that no reesterification of lyso-PC occurs). However, only about 24–30% of human platelet PC contains arachidonic acid (6, 10). Therefore, a 25% decrease in

[¹⁴C]arachidonyl-PC should only result in a 25% decrease in this 24–30% pool if arachidonyl-PC is the only substrate. Thus total PC should only decrease by 6–7.5%. The observed 7.6% decrease in total PC (Table V) therefore supports the above hypothesis.

It has been demonstrated that unsaturated fatty acids such as linoleic and oleic acid will both competitively and nonreversibly inhibit prostaglandin cyclooxygenase (18, 19). If the phospholipase activities stimulated by thrombin were to release these fatty acids, the production of prostaglandins G₂ and H₂, and thromboxane A₂ would be inhibited. The observed selectivity results in the release of large amounts of arachidonic acid, the substrate for platelet cyclooxygenase, and only trace amounts of those fatty acids which could inhibit the cyclooxygenase. Thus, the production of the endoperoxides and thromboxane A₂ is facilitated.

The selective mechanism involved in the release of arachidonic acid by platelets can be considered to be the initial step in platelet prostaglandin and thromboxane synthesis and may be of importance in hemostasis and thrombosis.

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