Location of Phosphatidylethanolamine and Phosphatidylserine in the Human Platelet Plasma Membrane

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ABSTRACT The location of phospholipids in the human platelet plasma membrane was probed with 2,4,6-trinitrobenzenesulfonate (TNBS). TNBS does not penetrate intact cells and can label phosphatidylethanolamine (PE) and phosphatidylserine (PS). In intact platelets, PE is not accessible to TNBS during the initial 15 min. However, 6.9% PE reacts with TNBS after 30 min and 17.9% PE is labeled after 90 min. In intact platelets, PS is not labeled even after 2 h. In contrast, in phospholipids extracted from platelets 71% PE and 26.5% PS react with TNBS within 5 min. This indicates that PS is inaccessible and PE is relatively inaccessible to TNBS in intact platelets. After incubation of platelets with thrombin, there is increased labeling of PE but no labeling of PS. The incubation of platelets with thrombin (0.05 U/ml) for 5 min results in 16.2% increase of PE labeling during subsequent 30-min incubation with TNBS. PS does not appear to be a component of the functional surface of platelets. However, exposure of PE may have a critical role in platelet hemostatic function. The implication of the study is that there is asymmetry of phospholipids in the platelet plasma membrane which has considerable physiological significance.

INTRODUCTION

This study was undertaken to determine the location of phospholipids in the platelet plasma membrane. There is evidence that platelet phospholipids are important for the cell's physiological activities. Altered phospholipid synthesis occurs in activated platelets (1–3) and pure phospholipids can mimic platelet procoagulant activity (4, 5). Phospholipids are major membrane components, and their physiological role can only be assessed by considering their structural interrelationships with proteins and other membrane components. Presently, very little is known about the location of phospholipids on the platelet surface. The structure of the erythrocyte surface has been investigated by using a membrane probe, 2,4,6-trinitrobenzenesulfonate (TNBS).[1] It is one of a group of agents which is thought not to penetrate the intact cell and only labels molecules on the cell surface. It forms stable covalent derivatives with amine groups and therefore has been used to identify aminophospholipids, phosphatidylethanolamine (PE), and phosphatidylserine (PS) (6, 7). In the present study, TNBS was used to probe the exterior of the platelet. The resultant information about the location of aminophospholipids in the lipid bilayer of the platelet plasma membrane is presented. The effect of thrombin on the availability of PE and PS to TNBS on the platelet surface is also considered.

METHODS

Materials. The following materials were used in the experimental procedure: TNBS (Aldrich Chemical Co., Inc., Milwaukee, Wis.; 10,408-6), [^{14}C]serotonin (5HT) (Amersham/Searle Corp., Arlington Heights, Ill.; CFA 170), [^{3}H]adenine (New England Nuclear, Boston, Mass.; Net-063), luciferase-luciferin (E. I. du Pont de Nemours & Co., Wilmington, Del.; 1504A), pronase CB (Calbiochem, San Diego, Calif.; 537011), bovine thrombin (Sigma Chemical Co., St. Louis, Mo.; T6646), bovine thrombin (Parke, Davis & Co., Detroit, Mich.) which had been purified by the method described by Lundblad (8).

Preparation of platelet suspensions. Platelets were prepared as previously described (9). Intact platelets were washed once with and resuspended in medium composed of isotonic saline with glucose (50 mM) and buffered with Tris (20 mM) at pH 7.4. For incubation with TNBS, platelets were resuspended in medium buffered with Tris (20 mM) at pH 8.6. Cell counts performed by phase-con-

This data was presented at the FASEB meeting, 14 April 1975. Part of this work appeared in abstract form in 1975. *Fed. Proc.* **34**: 126.

Received for publication 12 August 1975 and in revised form 29 December 1975.

[1] *Abbreviations used in this paper:* 5HT, serotonin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TNBS, 2,4,6-trinitrobenzenesulfonate.

trast microscopy showed that platelet counts ranged from 300,000 to 400,000/mm^3 and that the suspensions were free of significant erythrocyte and leukocyte contamination.

Preparation of aqueous suspensions of extracted platelet phospholipids, isolated platelet plasma membranes, and aged platelets. Lipids were extracted from platelets by the method of Bligh and Dyer (10). Phospholipids in chloroform were evaporated to dryness under nitrogen and resuspended in incubation medium (pH 8.6) by sonication. Sonication of phospholipids (1 μg lipid phosphorus per ml) was carried out at setting 8 on a Branson sonifier (model S125, Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) for three 1-min periods with minute intervals at 0°C. Platelet plasma membranes isolated by hypotonic lysis of glycerol-loaded platelets (11) and by the method of Marcus et al. (12) were used within 24 h after being prepared. Aged platelets were prepared by incubating intact platelets in medium (pH 7.4) at 23°C for 6 or 23 h.

Preparation of TNBS. A solution of TNBS (9.75 mM) buffered with sodium bicarbonate (780 mM) at pH 8.6 was prepared immediately before addition to platelet suspensions and other incubations.

Labeling conditions. TNBS buffered with sodium bicarbonate was added to intact platelets, platelet plasma membranes, and extracted phospholipids to a final concentration of 1.5 mM. Incubation was carried out at 23°C in medium buffered with Tris (20 mM) at pH 8.6. Tris is known to react weakly with TNBS (13). However, the presence of sodium bicarbonate in the labeling medium maintained the pH at 8.6. After incubation, intact platelets were recovered by centrifugation at 1,900 *g* for 10 min and plasma membranes at 44,000 *g* for 45 min. Platelets and plasma membranes were washed once with medium (pH 7.4) and then subjected to lipid extraction. Pure phospholipid suspensions that had been incubated with TNBS were not centrifuged before lipid extraction.

Incubation of platelets with thrombin and pronase. Intact platelets were incubated with thrombin or pronase for 5 min at 37°C. The medium was buffered at pH 7.4 and contained EDTA (2 mM) to avoid clumping of platelets during incubation with the enzymes. After incubation, enzyme-treated platelets were centrifuged at 480 *g* for 10 min and then resuspended in labeling medium (pH 8.6) for reaction with TNBS. Several experiments were designed to exclude the possibility that centrifugation of thrombin-treated platelets contributed to the extent of TNBS-labeling of aminophospholipids. Instead of centrifuging platelets after incubation with thrombin, these platelets were directly reacted with TNBS. The pH of the labeling medium was maintained at 8.6 by sodium bicarbonate buffer.

Analysis of phospholipids and quantitation of lipid labeling. Phospholipids were extracted from platelets and plasma membranes by the method of Bligh and Dyer (10) and separated into components by thin-layer chromatography as previously described (14) to $\frac{3}{4}$ of the height of the chromatoplate. The plate was then developed in chloroform to its full length to further separate neutral lipids from the TNBS-PE derivative. The lipid and derivative bands were visualized by charring with sulfuric acid, scraped, and analyzed for lipid phosphorus content (15). Percentage PE or PS labeled was calculated from the ratio of lipid phosphorus remaining in the PE or PS bands to the total lipid phosphorus on the chromatoplate. The results were confirmed by determining the percentage of total lipid phosphorus present in the TNBS-PE(PS) derivative bands which should correspond to the percentage PE or PS that had been labeled by TNBS.

Release of platelet constituents. Release of platelet 5HT was measured by using platelets labeled with [^{14}C]5HT, and the release of storage pool ADP and ATP was determined by using the firefly luciferase assay. The leakage of metabolic pool adenine nucleotides and their metabolites was measured by using platelets in which cytoplasmic adenine nucleotides had been labeled by incubation with [^{3}H]adenine. The methodology used in these experiments has been described previously (9, 16). The radioactive substances released from platelets in which cytoplasmic adenine nucleotides had been labeled were analyzed by high-voltage electrophoresis as described by Holmsen and Weiss (17).

Detection of TNBS-5HT derivative. After incubation of [^{14}C]5HT with TNBS at pH 8.6, absolute ethanol was added to prepare a 70% ethanolic mixture. The mixture, along with cold 5HT in 70% ethanol, was separated by thin-layer chromatography by a previously described method (9). Chromatoplates were developed with ethyl acetate, isopropyl alcohol, and concentrated NH_4OH, 9/7/4 (vol/vol/vol). The reaction of TNBS with 5HT was detected by the presence of radioactivity in the TNBS-5HT derivative band which was found near the solvent front and clearly separated from the 5HT band.

RESULTS

TNBS reacts with and forms stable covalent derivatives with platelet PE and PS. The derivatives, along with platelet phospholipids, can be extracted from platelets and separated into components by thin-layer chromatography. In Fig. 1, the control chromatoplate demon-

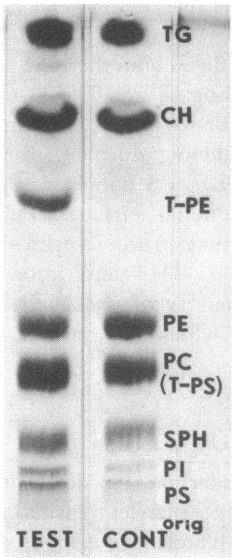

FIGURE 1 Thin-layer chromatography of platelet phospholipids and aminophospholipid-TNBS derivatives. The control (CONT) chromatoplate demonstrates the separation of the major platelet phospholipids: PS, phosphatidylinositol (PI), sphingomyelin (SPH), PC, and PE. Also shown are cholesterol (CH) and triglyceride (TG). The TEST represents an experiment in which 48% platelet PE had been labeled by TNBS. The TNBS-PE derivative (T-PE) is clearly separated from the other bands. TNBS-PS derivative (T-PS) when formed is present in the PC band.

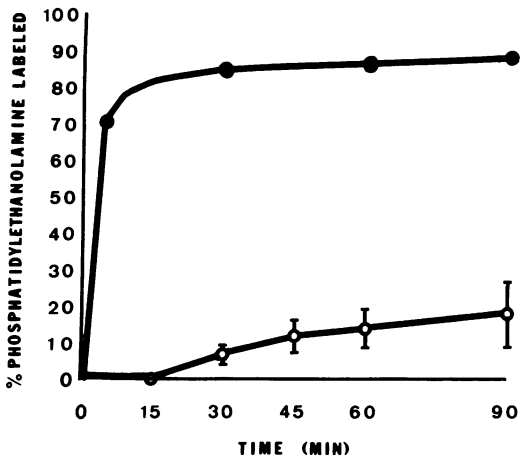

FIGURE 2 Comparison of PE labeled by TNBS in intact platelets to that in lipids extracted from platelets. Intact platelets (○); extracted platelet phospholipids (●). Labeling with TNBS (1.5 mM) was carried out at 23°C. Labeling medium was composed of isotonic saline and glucose (50 mM) and buffered with Tris (20 mM) at pH 8.6.

strated the separation of the major platelet phospholipid species. The test represents an experiment in which 48% platelet PE had reacted with TNBS. The resultant TNBS-PE derivative is clearly separated from the other phospholipid bands and the PE band is visibly diminished. TNBS-PS derivative, when formed, is present in the phosphatidylcholine (PC) band. Preliminary experiments indicated that TNBS reacts with PE and PS more completely if the pH of the labeling medium is above 8.2. A pH of 8.6 was used during the labeling experiments.

This study demonstrates the extent of accessibility of PE and PS to TNBS in intact platelets. In Fig. 2, PE labeling by TNBS is shown and compared to that in phospholipids which had been extracted from platelets. In intact platelets, PE is not available to TNBS during the initial 15 min. However, 6.9% total platelet PE reacts with TNBS after 30 min and 17.9% PE is labeled after 90 min. The percentage of total PE labeled underestimates the percentage of plasma membrane PE labeled by TNBS. Since TNBS does not penetrate the platelet, only PE in the plasma membrane can potentially react with TNBS. The composition of phospholipids in the platelet plasma membrane is similar to that of the granule membrane (18). Therefore, if plasma membranes were to represent 50% if the membrane systems in platelets, 6.9% total platelet PE labeled would actually represent 13.8% plasma membrane PE labeled. In contrast, 71% PE in extracted phospholipid suspensions reacts with TNBS within 5 min. Several experiments show that 26.5% PS in extracted platelet phospholipids is labeled by TNBS after 5 min.

PE and PS labeling by TNBS under conditions which potentially can alter the platelet plasma membrane is demonstrated in this study. When 23-h aged intact platelets or isolated platelet plasma membranes were reacted with TNBS for 1 h, 49.1±4.9% PE and 81.4±2.6% PE were labeled, respectively. Under the same conditions 14.3±5.7% PE in freshly prepared platelets and 17.6 ±3.0% PE in 6-h aged platelets react with TNBS. Aged platelets are defined as platelets incubated at 23°C in medium buffered with Tris (20 mM) at pH 7.4 for 6 or 23 h. PS in freshly prepared intact platelets or 6-h aged platelets is not labeled after 1 h incubation with TNBS. Under the same conditions 13.5±4.6% PS in 23-h aged platelets and 30.8±7% PS in isolated plasma membranes react with TNBS. The values for experiments with intact platelets, 6-h aged platelets, and plasma membranes are mean±SD of four experiments. Values for experiments with 23-h aged platelets are the means±SD of three experiments. In additional experiments designed to test the effects of extended labeling it was shown that after a 2-h incubation of intact platelets with TNBS, PS is still not labeled. In experiments in which intact platelets were labeled with TNBS for 7 h, substantial amounts of both PE- and PS-TNBS derivatives are formed.

The incubation of intact platelets with thrombin or pronase resulted in increased labeling of PE by TNBS. Fig. 3 demonstrates the extent of PE labeling in platelets treated with thrombin (0.1 U/ml) for 5 min and

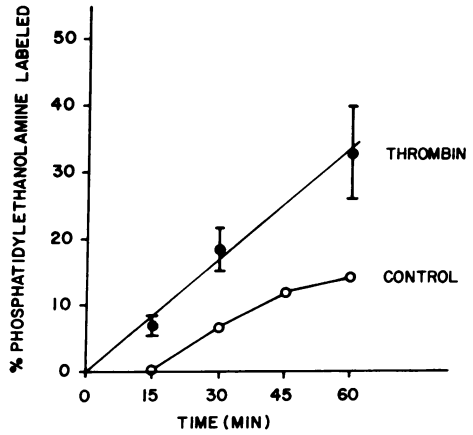

FIGURE 3 Comparison of PE labeled by TNBS in thrombin-treated platelets to that in control platelets. Intact platelets were incubated with thrombin (0.1 U/ml) or 5 min at 37°C. The incubation medium was composed of isotonic saline and glucose (50 mM) and buffered with Tris (20 mM) at pH 7.4 and contained EDTA (2 mM) to avoid clumping of platelets. After incubation, thrombin-treated platelets were centrifuged and resuspended in labeling medium buffered with Tris (20 mM) at pH 8.6 for reaction with TNBS (1.5 mM).

compares it to that in control intact platelets. At 15 min of incubation with TNBS, 7% PE is labeled in thrombin-treated platelets and none is labeled by TNBS in control intact platelets. After each subsequent time point, more PE reacts with TNBS in thrombin-treated platelets than in controls. Fig. 4 demonstrates the relationship between percentage of PE labeled and the concentration of thrombin. After the incubation of platelets with thrombin for 5 min, labeling with TNBS was carried out for 30 min. Thrombin concentrations less than 0.05 U/ml did not cause increased labeling of PE. Beginning with thrombin (0.05 U/ml), 16.2% more PE is available to TNBS than in control intact platelets. However, it should be emphasized that PS was not labeled by TNBS in thrombin-treated platelets in these experiments. Experiments designed to exclude the effects of pelleting show that comparable amounts of PE labeling occur, 14.6±4.6% SD (four experiments), in thrombin-treated platelets that were not centrifuged before reaction with TNBS. Thrombin and not a contaminant caused the increased labeling of platelet PE by TNBS. In four experiments, thrombin purified by the method of Lundblad (8) caused the same increase in PE labeling as shown in Fig. 3, which was obtained by using a purified thrombin purchased from Sigma.

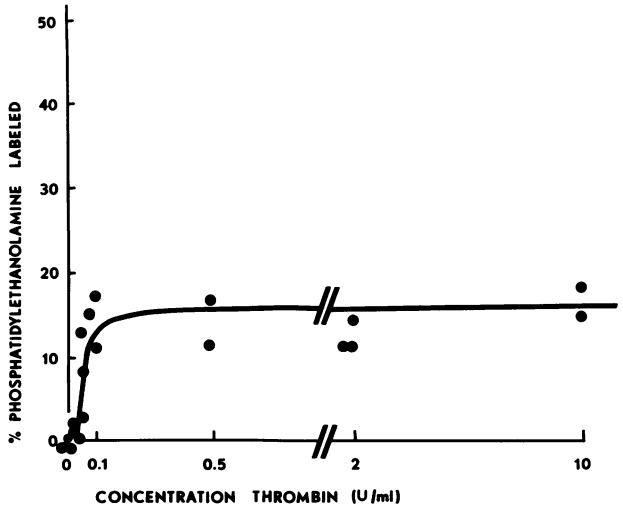

FIGURE 4 Percentage PE labeled by TNBS in relation to the concentration of thrombin. Intact platelets were incubated with thrombin for 5 min at 37°C. The incubation medium was buffered with Tris (20 mM) at pH 7.4 and contained EDTA (2 mM) to avoid clumping of platelets. After incubation, enzyme-treated platelets were centrifuged and then resuspended in labeling medium buffered with Tris (20 mM) at pH 8.6 for reaction with TNBS (1.5 mM). Labeling was carried out for 30 min. Values express increase of PE labeled due to enzyme (percentage of PE labeled in the enzyme-treated platelets minus percentage of PE labeled by TNBS in controls).

When platelets had been incubated with pronase (0.2, 0.5, and 2.0 mg/ml) for 5 min in seven experiments, subsequent incubation with TNBS for 30 min resulted in the labeling of 20.9±5.5% PE over controls. However, PS did not become accessible to TNBS in pronase-treated platelets in these experiments.

The results of experiments to determine the effects of TNBS on platelets showed that incubation with TNBS did not cause platelet clumping or a reduction in the platelet count. The effect of TNBS on the release of platelet constituents is shown in Table I. TNBS did not cause platelet lysis since significant leakage of cytoplasmic pool adenine nucleotides did not occur even after 45 min. Storage pool adenine nucleotides were not released after 15 min, but 26.9% ADP was released after 45 min. Hypoxanthine was also released from platelets after 45 min incubation with TNBS.

The incubation of platelets with TNBS is associated with a loss of platelet 5HT. Table I shows that 10.2% 5HT is released after 5 min and substantial amounts of 5HT are released after 15 min. Our experiments also show that TNBS forms a derivative with 5HT. In four experiments 80% 5HT reacted with TNBS and was converted to TNBS-5HT derivative during 5-min incubations.

DISCUSSION

The probing of platelets with TNBS, an agent considered to label only the cell surface, has provided new information about the structural arrangement of phospholipids in the platelet plasma membrane. TNBS forms derivatives with membrane components containing primary amine groups (6, 7, 19) and thus can potentially react with only two of the major platelet phospholipids, PE and PS. This study demonstrates that in intact platelets PS is inaccessible and PE is relatively inaccessible to TNBS. PE is not labeled during the initial 15 min and only 12% of total platelet PE reacts with TNBS after 45 min. The unavailability of PS to TNBS is emphasized by the observation that pronase digestion of the platelet surface does not result in the labeling of this aminophospholipid. However, when phospholipids are extracted from platelets and incubated with TNBS, 71% PE and 26% PS are labeled within 5 min. In addition, both PE and PS in isolated platelet plasma membranes react with TNBS. This series of experiments indicates that in intact platelets PS and probably PE are located primarily in the inner lipid bilayer of the plasma membrane. However, there is also the possibility that platelet aminophospholipids are intimately associated with proteins and other membrane components and therefore unable to react with TNBS.

The implication of this study is that there is asymmetry of phospholipids in the platelet plasma membrane.

TABLE I

TNBS-Associated Loss of Platelet Constituents

Incubation time*	No. of experiments	[^{3}H]Adenine nucleotides	[^{3}H]Hypoxanthine	[^{14}C]5HT	ADP (storage)	ATP (storage)
min		%‡	%	%	%	%
5	4	0	0	10.2±2.1	—	—
15	4	0	0	43.0±3.6	0	4.4±0.7
45	4	3±0.4	13.2±0.8	62.2±4.1	26.9±9.4	13.4±4.0

* Intact platelets were labeled with TNBS (1.5 mM) at 23°C in medium composed of isotonic saline and glucose (50 mM) and buffered with Tris (20 mM) at pH 8.6.

‡ All percentages in the table represent the percentage of total platelet constituent (mean±SD).

A previous study has indicated that PC and sphingomyelin are situated at or near the active site or "receptor" on the platelet surface and the hydrolysis of PC and sphingomyelin can cause the release reaction (16). Conceivably, PC and sphingomyelin are present in the outer part and PS and PE are located in the inner part of the platelet plasma membrane. Phospholipids situated on the surface of the platelet are most likely directly involved in the regulation of platelet hemostatic activities.

Rearrangement and/or reorientation of surface lipids and proteins are thought to occur in aggregating platelets. Pure PS and to a lesser extent PE are more efficient than other platelet phospholipids in promoting clot formation. Therefore, it has been suggested that PS and PE are either present on the platelet surface, or become available when aggregation occurs and then mediate platelet physiological activities (20). The thrombin experiments in this study show that PS does not become available to TNBS on the surface of thrombin-activated platelets. It would appear that the functional surface of platelets consists of proteins and phospholipids other than PS whose physical properties, however, resemble those of PS.

PE does become available to TNBS on the surface of thrombin-activated platelets and may provide the surface for clot-promoting activity. The increased exposure of membrane PE occurs at threshold concentrations of thrombin that can induce the release reaction, suggesting physiological importance. Thrombin has been shown to cause proteolysis (21) and actual rearrangement of the platelet surface (22, 23). Thrombin has been shown to alter the labeling pattern of plasma membrane glycoproteins (24, 25). It also has been suggested that thrombin causes the fusion of granule with canalicular membranes and exposes granule membranes (23). Any of these events could be responsible for the observed increase of PE labeling by TNBS in thrombin-treated platelets. Whether the increased availability of PE on the surface of thrombin-treated platelets is due to modification of the architecture of the plasma membrane or to exposure of granule membranes, the newly exposed PE may play a critical role in the regulation of platelet hemostatic activities.

Singer (26) points out that there is no evidence at present for an asymmetrical distribution of phospholipids in cell membranes other than the erythrocyte. He emphasizes that if an asymmetrical distribution of phospholipids is a general phenomenon of cell membranes, then its implications for the structure and function of membranes are considerable. The observation that lipid asymmetry probably also occurs in the platelet whose surface mediates extremely complex biological activities provides a new approach for investigating this problem.

TNBS most likely does not penetrate the platelet for the following reasons: (*a*) The incubation was carried out at 23°C and phosphate buffer was avoided. TNBS has been shown not to penetrate the erythrocyte under these conditions (19). (*b*) TNBS does not label PS in intact platelets even after 2-h incubations. When platelets had been disrupted or damaged by aging for 23 h, platelet membrane PS was shown to be labeled. (*c*) TNBS did not cause platelet lysis after 45 min as shown by the absence of leakage of cytoplasmic pool adenine nucleotides.

Platelet 5HT release may be due to modification of the platelet surface by TNBS or to the formation of a TNBS-5HT derivative. The formation of the TNBS-5HT complex may interfere with 5HT reuptake and storage (27). The release of ADP does not occur at 15 min but is evident at 45 min. Most of our observations about the accessibility of PS and PE were made during earlier time periods. The release of ADP is an active process since there is a concomitant release of hypoxanthine. The modification of platelet membrane PE as well as the reaction of TNBS with surface protein amine and sulfhydryl groups may trigger the TNBS-associated release of ADP.

This study indicates that PS and probably PE are located primarily in the inner lipid bilayer of the plate-

let plasma membrane. PS is not a component of the lipids and proteins that provide the surface for platelet hemostatic activities. PS does not become exposed to TNBS in thrombin-activated platelets. PE becomes available on the surface of thrombin-activated platelets and may have a critical role in platelet function.

ACKNOWLEDGMENTS

The authors would like to thank Drs. G. A. Jamieson and R. L. Nachman for preparing plasma membranes and Dr. Erik Mürer for his analysis of released adenine nucleotides. The authors appreciate Dr. E. Kirby's donation of a purified thrombin preparation.

This work was supported by a Grant-in-Aid from the American Heart Association and in part by National Institutes of Health Grant HL 18249.

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