

# The Role of Platelet Membrane Phospholipids in the Platelet Release Reaction

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**ABSTRACT** The structure and function of the platelet surface was probed by phospholipase C (*Clostridium perfringens*) which hydrolyzes membrane phospholipids, particularly phosphatidylcholine. Platelet phospholipids were susceptible to phospholipase C, and extent of hydrolysis was dependent on concentration of phospholipase C and  $\text{Ca}^{++}$ . Phospholipase C (0.15 U/ml) with  $\text{Ca}^{++}$  (0.55 mM) hydrolyzed 15.6% phospholipids during 5 min. Phospholipase C released platelet serotonin (5HT), ADP, and platelet factor 4. Hydrolysis of 5% phospholipids resulted in release of 70% 5HT. Platelet 5HT release was rapid, occurring within 2 min. Phospholipase C (0.2 U/ml) with  $\text{Ca}^{++}$  (0.55 mM) also released 10.35 nmol storage pool ADP/ $10^9$  platelets and 63% platelet factor 4 during 3 min. Phospholipase C did not cause leakage of cytoplasmic metabolic pool ADP, since only 6.6% [ $^3\text{H}$ ]ADP was released. Ultrastructural analysis of phospholipase C-modified platelets showed that platelets were intact. After 2% phospholipid hydrolysis, centralization of granules and contraction of microtubules were evident. After 18% phospholipid hydrolysis, there were morphological indications of degranulation. Phospholipase C-induced phospholipid hydrolysis caused the release of ADP and 5HT since: (a) Phospholipase C purified by heating was shown to be free of protease and neuraminidase activity and capable of inducing the platelet release reaction. (b) Antitoxin (*Cl. perfringens*) neutralized phospholipase C-induced 5HT release which rules out a contaminant. (c) Phosphorylcholine, the hydrolysis product, did not induce platelet 5HT release. This study demonstrates that minimal hydrolysis of platelet phospholipids triggers the release reaction. Our hypothesis is that phospholipids,

presumably phosphatidylcholine, are situated at or near the active site or "receptor" on the platelet surface and function as the modulator for the release reaction.

## INTRODUCTION

Insight into the interrelationships between the structure and function of the surface of the platelet is necessary for understanding the molecular basis of platelet physiological activities. The surface of the platelet is complex and is thought to be organized into a mucosubstance coat and a plasma membrane. The structural arrangement of the platelet surface enables the cell to respond rapidly to appropriate stimuli by integrating and mediating platelet reactions. There is little information about the precise structure of the platelet exterior and its role in platelet physiological reactions. One approach to probing the function of a membrane component is to observe the physiological consequences of its selective destruction. Platelet membrane phospholipids have been shown to be susceptible to hydrolysis by phospholipase A (1, 2). In this study we induced platelet membrane modification by the use of purified phospholipase C (phosphatidylcholine cholinephosphohydrolase) which is known to preferentially hydrolyze phosphatidylcholine (3), the most abundant platelet phospholipid. Phospholipids are known to be major constituents of the platelet plasma membrane (4). The effects of the resultant minimal membrane modification on the platelet release reaction are reported and interpreted in this paper.

## METHODS

### Materials

The following materials were used in the experimental procedures: phospholipase C from *Clostridium perfringens* (Worthington Biochemical Corp., Freehold, N. J., PLC 5640), neuraminidase from *Cl. perfringens* (Worthington Biochemical Corp., purified NEUP), phosphorylcholine chloride (Sigma Chemical Co., St. Louis, Mo.), [ $^3\text{H}$ ]-adenine (New England Nuclear, Boston, Mass., Net-063),

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[<sup>14</sup>C]serotonin (5HT)<sup>1</sup> (Amersham/Searle Corp., Arlington Heights, Ill., CFA 170), gas gangrene polyvalent antitoxin (Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.), Azocoll, hide powder azure, fibrin blue (Calbiochem, La Jolla, Calif.).

### Preparation of platelet suspensions

Platelet suspensions were prepared as previously described (5). Intact platelets were resuspended in a medium composed of isotonic saline with glucose (0.05 M) and buffered with Tris (15.4 mM) at pH 7.4. Cell counts performed by phase-contrast microscopy showed that the number of platelets varied from 300,000 to 400,000/mm<sup>3</sup> and that the suspensions were not significantly contaminated by erythrocytes and leukocytes.

### Incubation conditions

Platelet suspensions were incubated at 37°C with phospholipase C, phosphorylcholine, neuraminidase, and thrombin. Immediately after the addition of these agents, Ca<sup>++</sup> (1/10 vol) was added to achieve the desired final concentration of the cation, and unless otherwise stated incubation mixtures in the experimental procedure contained 0.55 mM Ca<sup>++</sup>. Incubation was terminated by placing the mixtures on ice. In several experiments platelets were frozen and thawed five times in order to cause platelet damage. Platelets were examined for residual lipid phosphorus and prepared for electron microscopy. Filtrates of the incubation mixtures were prepared as previously described (6) and analyzed for the release of 5HT, [<sup>3</sup>H]ADP, nonradioactive ADP, and platelet factor 4 (PF4). Platelets were examined by phase-contrast microscopy during the incubations for clumping and morphological changes.

### Assay and determination of phospholipid hydrolysis

Platelet phospholipids were extracted and analyzed for lipid phosphorus as previously described (6). The percentage phospholipid hydrolysis was calculated from the ratio of residual lipid phosphorus in an aliquot of platelet suspension that had been incubated with phospholipase C to the lipid phosphorus present in control platelets. Separation and identification of phospholipids were done by thin-layer chromatography as previously described except that butylated hydroxytoluene was not used (6).

### Platelet 5HT release

The release of radiotracer 5HT was determined, and percent release was calculated as previously described (5) except that platelets were preloaded with [<sup>14</sup>C]5HT after they had been resuspended in incubation medium.

### Loss of metabolic pool [<sup>3</sup>H]ADP

Cytoplasmic ADP was labeled by incubating platelet suspension with [<sup>3</sup>H]adenine (0.9 nmol/ml platelet suspension) for 40 min. Leakage of radiotracer ADP was measured, and percent loss was calculated as previously described (5).

<sup>1</sup> Abbreviations used in this paper: 5HT, serotonin; NANA, N-acetyl neuraminic acid; PF4, platelet factor 4.

### Release of storage pool ADP

ADP present in filtrates of incubation mixtures was extracted with ethanol-EDTA and analyzed by the firefly luciferase assay (7).

### PF4 release

PF4 present in filtrates of incubation mixtures was estimated by the method of Donati, Palestro-Chlebowczyk, de Gaetano, and Vermeylen (8). Platelet suspensions were concentrated 2.5-fold, and 0.1 ml of filtrates of the incubation mixtures was combined with the 0.15 ml donor plasma and then tested for PF4. The results were expressed as percent of PF4 released from platelets that had been frozen and thawed.

### Electron microscopy

Platelets were prepared for electron microscopy as described by White (9). Incubation was terminated by the addition to platelet suspensions of an equal volume 0.1% glutaraldehyde that had been warmed at 37°C. After centrifugation and removal of the supernate, the platelet pellet was fixed with 3% glutaraldehyde and 1% buffered osmium tetroxide and then embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 300 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.).

### Evaluation of whether phospholipase C-induced platelet phospholipid hydrolysis caused the release of platelet constituents

#### PROTEASE CONTAMINATION

Phospholipase C preparation was tested against standard sensitive proteolysis substrates, substrate-dye complexes, at several pH's in order to detect trace contamination. Azocoll (10) and hide powder azure (11) were suspended, 5 mg per ml, in phosphate buffer (0.1 M) at pH 5.6, 7.0, and 7.8. Fibrin blue (12) was suspended, 4 mg/ml, in 0.018 M HCl at pH 2. Phospholipase C (100 times the concentration necessary to induce the release reaction) and Ca<sup>++</sup> (0.55 mM) were added to the suspensions which were then incubated at 37°C for 15 h. After incubation supernates were prepared by centrifugation. The Azocoll supernate was examined spectrophotometrically at 520 nm for released dye indicative of proteolysis; hide powder azure supernate was read at 595 nm, and the fibrin blue supernate at 620 nm.

Protease contamination was also tested by determining whether the phospholipase C preparation would inactivate enzymes. Phospholipase C (100 times the concentration required to induce the release reaction) was incubated for 10 min with either lactic dehydrogenase (Sigma Chemical Co.) or yeast alcohol dehydrogenase (Sigma Chemical Co.) under conditions identical to that in which phospholipase C released platelet ADP and 5HT. The incubation medium contained Ca<sup>++</sup> (0.55 mM) and was buffered at pH 7.4 by Tris (15.4 mM). After the incubation of the phospholipase C preparation with either of the enzymes, the activity of lactic dehydrogenase (13) or alcohol dehydrogenase (14) was assayed and compared to control experiments to determine whether the dehydrogenases had been inactivated.

(a) Phospholipase C preparation was analyzed for neuraminidase activity by the incubation of platelet suspensions ( $10^9$  platelets/0.5 ml) with phospholipase C and then assaying filtrates for release of *N*-acetyl neuraminic acid (NANA) by the thiobarbituric acid method (15). The results were expressed as percent of total platelet NANA. Total platelet NANA was determined by treating platelets ( $10^9$  cells/0.5 ml) with 0.1 N  $H_2SO_4$  at  $80^\circ C$  for 1 h and then measuring NANA in the filtrate.

(b) The  $Ca^{++}$  requirement of neuraminidase (*Cl. perfringens*) and phospholipase C in their respective activities, release of NANA and phospholipid hydrolysis, was investigated. The testing of the ability of the phospholipase C to induce the hydrolysis of platelet phospholipids and release 5HT when  $Ca^{++}$  had been omitted from the incubation medium provided a basis for excluding neuraminidase contamination.

#### HEAT STABILITY

Since phospholipase C is one of the few enzymes that is extremely heat stable, phospholipase C as well as neuraminidase were heated at  $100^\circ C$  at pH 7.4 for 10 min (16). Heated phospholipase C was then tested for its ability to hydrolyze platelet phospholipids and release ADP and 5HT. Heated neuraminidase was tested for its ability to release platelet NANA.

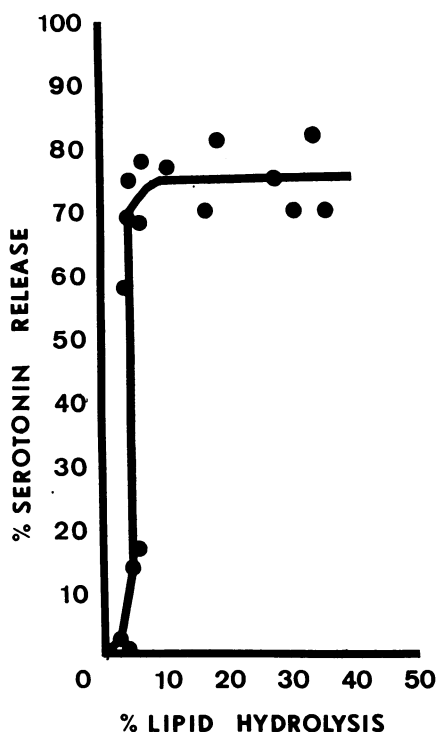


FIGURE 1 The release of 5HT in relation to extent of phospholipase C-induced phospholipid hydrolysis. Platelet suspensions were incubated with phospholipase C at  $37^\circ C$  for 5 min. The resuspension medium contained  $Ca^{++}$  (0.55 mM) and was buffered with Tris (15.4 mM) at pH 7.4.

#### NEUTRALIZATION BY ANTITOXIN.

Antitoxin derived from *Cl. perfringens* was combined with phospholipase C 5 min before the addition of this mixture to platelet suspensions. The ability of the phospholipase C-antitoxin combination to hydrolyze platelet phospholipids and to release ADP and 5HT was determined.

#### EFFECT OF HYDROLYSIS PRODUCT

Phosphorylcholine (0.075, 0.15, and 0.30  $\mu g$  per ml platelet suspension), the hydrolysis product of phospholipase C-treated phospholipids, was incubated with platelets. These concentrations corresponded with the degradation of 12.5, 25, and 50% platelet phospholipids by phospholipase C.

#### RESULTS

The incubation of platelets with phospholipase C resulted in the hydrolysis of platelet phospholipids. The magnitude of phospholipid hydrolysis was dependent on the concentration of phospholipase C and  $Ca^{++}$ . In the absence of  $Ca^{++}$  phospholipase C did not induce hydrolysis of phospholipids in five experiments. Optimal phospholipid hydrolysis occurred when the final concentration of  $Ca^{++}$  was 0.55 mM. In five experiments under these conditions phospholipase C (0.15 U/ml platelet suspension) caused the hydrolysis of  $15.6\% \pm 6.4$  SD platelet phospholipids during 5 min. In order to achieve the hydrolysis of from 0 to 36% platelet phospholipids, phospholipase C in concentrations from 0.05 to 0.3 U/ml platelet suspension and  $Ca^{++}$  (0.55 mM) were used. Higher concentrations of  $Ca^{++}$  and phospholipase C caused platelet phospholipid destruction as high as 68% phospholipids.

Several experiments were designed to identify which phospholipids were attacked by phospholipase C in intact platelets. Four experiments showed that when 10.4% total platelet phospholipid had been hydrolyzed during 5-min incubations,  $24.7\% \pm 5.6$  SD platelet phosphatidylcholine and  $8.83\% \pm 3.2$  SD sphingomyelin had been hydrolyzed while other platelet phosphatides were spared. With more extensive phospholipid hydrolysis platelet phosphatidylethanolamine and eventually phosphatidylserine were also degraded.

This study also demonstrated that phospholipase C can induce the release of platelet 5HT. Fig. 1 demonstrates the relationship between the degree of 5HT released and the extent of phospholipid hydrolysis. Only minimal amounts of 5HT were released when less than 5% lipid hydrolysis had occurred. After 5% platelet phospholipids had been hydrolyzed, 70% 5HT was released. Further destruction of platelet phospholipid released only small amounts of 5HT. Fig. 2 compares the rate of phospholipase C-induced 5HT release to the rate of phospholipid hydrolysis. After an initial 2-min lag period, 60% platelet 5HT was released within 2 min, but very little additional 5HT was released during subsequent 6 min of incubation. Phospholipid hydrolysis pro-

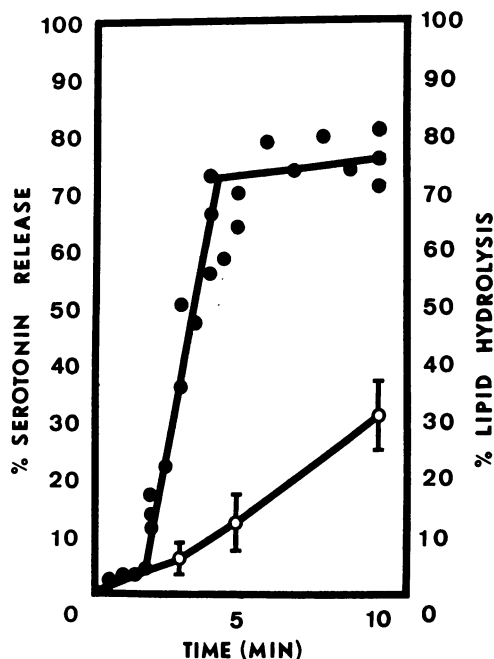


FIGURE 2 The relationship of the rate of 5HT release to the rate of phospholipase C-induced phospholipid hydrolysis. 5HT (●); phospholipid hydrolysis (○). Platelet suspensions were incubated with phospholipase C (0.2 U/ml) at 37°C. The resuspension medium contained  $\text{Ca}^{++}$  (0.55 mM) and was buffered with Tris (15.4 mM) at pH 7.4.

ceeded linearly, and 31% hydrolysis occurred during 10 min of incubation. Fig. 1 also shows that 5% phospholipid hydrolysis coincided with the onset of the rapid phase of 5HT release.

Phospholipase C also induced the release of other platelet constituents. Table I shows that phospholipase C (0.2 U/ml) released 10.35 nmol ADP/ $10^9$  platelets during 3-min incubations. In the same experiments thrombin (10 U/ml) released 10.1 nmol ADP, whereas in controls 0.59 nmol ADP was released per  $10^9$  platelets. Phospholipase C (0.2 U/ml) released 63% of PF4 which was present in filtrates of platelets that had been frozen and thawed five times. The effects of phospholipase C on the cytoplasmic metabolic pool ADP which had been labeled by the incubation of platelet suspensions with [ $^3\text{H}$ ]adenine is shown in Table I. Phospholipase C released 6.6% [ $^3\text{H}$ ]ADP whereas 70% [ $^3\text{H}$ ]ADP had been released from platelets that had been damaged by freezing and thawing.

The ultrastructural integrity of phospholipase C-modified platelets observed at 25,000 magnification is shown in Fig. 3. Phospholipase C had induced the hydrolysis of 2% phospholipids during 5 min in platelets shown in Fig. 3a. The platelets and their membranes appeared to be intact. It should be pointed out that distinct cen-

tralization of granules and contraction of microtubules were evident. In Fig. 3b, 18% lipid hydrolysis had occurred. There was no apparent disruption of the platelet plasma membrane and the cells appeared to be discoid. However, there was evidence of degranulation. Also, large dilated granules which contained an amorphous substance could be seen.

The following are the results of experiments designed to establish whether phospholipase C-induced platelet phospholipid hydrolysis caused the observed release of platelet constituents.

(a) Phospholipase C that had been purified by heating retained its ability to release 5HT and ADP as shown in Table II and in addition caused the same ultrastructural changes as those induced by unheated phospholipase C that are demonstrated in Fig. 3. This suggested that heat-labile enzymes such as proteases and neuraminidase did not trigger the observed release reaction. Higher concentrations of heated phospholipase C were used because it is known that when phospholipase C is heated as described in Methods 55% of the enzyme's activity is lost (16).

(b) Heated phospholipase C was shown to be free of proteolytic contamination. Heated phospholipase C (100 times the concentration necessary to cause the release reaction) did not hydrolyze in three experiments Azocol (sensitive for neutral proteases) or hide powder azure (basic proteases) at pH 5.6, 7.0, and 7.8 and in addition did not hydrolyze in three experiments fibrin blue (sensitive for acid proteases) at pH 2.0 during 15-h incubations at 37°C. Unheated phospholipase C was shown

TABLE I  
Release of Other Platelet Constituents

Agents* (No. of experiments)	ADP	PF4	[ $^3\text{H}$ ]ADP
	nmol/ $10^9$ platelets	%	%
Control (4)	0.59 $\pm$ 0.05†	0	0
Phospholipase C (0.2 U/ml§) (4)	10.35 $\pm$ 0.62	63 $\pm$ 4	6.6 $\pm$ 5.2
Thrombin (10 U/ml) (3)	10.10 $\pm$ 0.71	—	—
Frozen-thawed platelet (five times) (3)	—	100	70.0 $\pm$ 6.5

\* Platelet suspensions were incubated with phospholipase C and thrombin for 3 min. The resuspension medium contained  $\text{Ca}^{++}$  (0.55 mM) and was buffered with Tris (15.4 mM) at pH 7.4.

† All values are means $\pm$ SD.

§ ml = milliliter platelet suspension.

|| Percentage of PF4 released by freezing and thawing platelets.

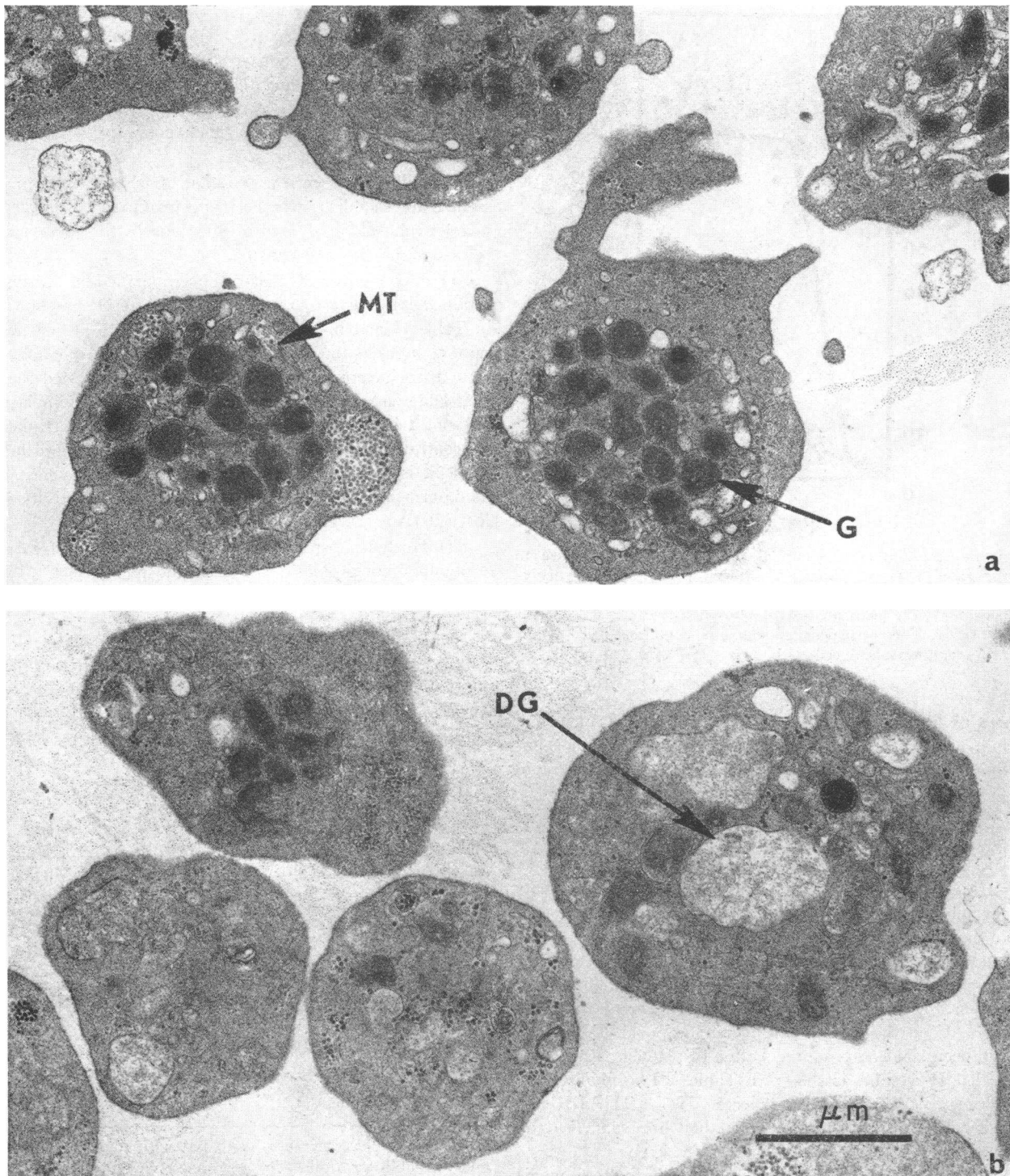


FIGURE 3 The ultrastructural integrity of phospholipase C-modified platelets magnified 25,000 $\times$ . (a) Phospholipase C-induced 2% platelet phospholipid hydrolysis. (b) Phospholipase C-induced 18% platelet phospholipid hydrolysis. *G*, granules; *MT*, microtubules; *DG*, dilated granules. Platelet suspensions were incubated with phospholipase C at 37°C for 5 min. The resuspension medium contained  $\text{Ca}^{++}$  (0.55 mM) and was buffered with Tris (15.4 mM) at pH 7.4.

to hydrolyze both Azocoll and hide powder azure at pH 7.0 and 7.8 while fibrin blue was not attacked.

The incubation of heated or unheated phospholipase C (100 times the concentration that caused the release reaction) with either lactic dehydrogenase (four experiments) or yeast alcohol dehydrogenase (four experiments) at pH 7.4 for 10 min did not reduce the activity of either of the dehydrogenases, further indicating that our observations were not due to a protease contaminant in the phospholipase C preparation.

(c) Neuraminidase (0.01 U/ml platelet suspension) released 59%, and 0.1 U/ml released 71% total platelet NANA during 5 min. In the absence of  $\text{Ca}^{++}$  neuraminidase (0.1 U/ml) was still capable of releasing 70% platelet NANA. Heated neuraminidase (0.1 U/ml) did not release platelet NANA. Therefore, neuraminidase differs from phospholipase C which required  $\text{Ca}^{++}$  for activation and is extremely heat-stable. Since phospholipase C did not induce the release of platelet constituents in the absence of  $\text{Ca}^{++}$ , neuraminidase as a contaminant in the phospholipase C preparation was not responsible for our observations.

(d) Table II also demonstrates that antitoxin isolated from *Cl. perfringens* neutralized and inhibited both the phospholipase C (*Cl. perfringens*)-induced lipid hydrolysis as well as the release of ADP and 5HT. However, antitoxin did not inhibit thrombin-induced 5HT release.

(e) The incubation of platelets with phosphorylcholine, the hydrolysis product of phospholipids, treated with phospholipase C, in concentrations equivalent to that produced after 12.5, 25, and 50% phospholipase C-induced platelet phospholipid hydrolysis did not release platelet 5HT as shown in Table II.

## DISCUSSION

The probing of platelet membranes with phospholipase C has better defined the structure of the platelet surface. In this study phospholipase C (*Cl. perfringens*) has been shown to hydrolyze primarily platelet phosphatidylcholine and to a lesser extent sphingomyelin. Platelet phospholipids have been shown to be major components of platelet plasma and organelle membranes, and probably platelet granules (4). Several considerations indicate that phospholipase C only hydrolyzed phospholipids present in the platelet plasma membrane. The platelet was extremely susceptible to the actions of phospholipase C in that lipid hydrolysis occurred within minutes. Also, most of the significant phospholipase C-induced physiological reactions occurred when less than 5% platelet phospholipid had been hydrolyzed. Phospholipase C which has a molecular weight of 100,000 is thought to be too large to penetrate through the surface of intact cells (17, 18). Therefore, it seems

TABLE II  
Relationship between Phospholipase C-Induced Phospholipid Hydrolysis and the Release Reaction

Agents* (No. of experiments)	5HT release	ADP	Phospholipid hydrolysis
	%	nmol/10 <sup>9</sup> platelets	%
Control (4)	0	0.61 ± 0.04	0
Heated phospholipase C (0.6 U/ml)† (4)	73 ± 4§	10.6 ± 0.30	32.3 ± 3.2
Phospholipase C (0.2 U/ml) + antitoxin (0.4 U/ml) (4)	0	0	0
Thrombin (5 U/ml) + antitoxin (0.4 U/ml) (3)	75 ± 6	—	—
Phosphorylcholine (0.075, 0.150, 0.30 µg/ml) (4)	0	—	—

\* Platelet suspensions were incubated with agents listed below at 37°C for 5 min. The resuspension medium contained  $\text{Ca}^{++}$  (0.55 mM) and was buffered Tris (15.4 mM) at pH 7.4.

† ml = milliliter platelet suspension.

§ All values are means ± SD.

reasonable to conclude that phospholipase C only modified the platelet plasma membrane. The structure of the platelet's exterior is complex. In addition to the plasma membrane, which is rich with phospholipids, it consists of a mucosubstance coat and adsorbed plasma proteins (4). However, the precise arrangement of phospholipids in the platelet surface is not known. This study suggests that plasma membrane phospholipids are available for hydrolysis and are located at or near the outermost surface of the platelet.

In order to evaluate the physiological significance of the observed phospholipase C-induced release of platelet constituents, it is necessary to compare the characteristics of this release to that induced by other agents. Certain agents, such as reserpine, release only platelet 5HT (5) while thrombin is an example of agents which rapidly release both 5HT and ADP and trigger the physiologically important platelet release reaction. The rate of phospholipase C-induced release of 5HT is comparable to that caused by thrombin (19), which can release 75% 5HT within minutes, but considerably more rapid than that induced by methylene blue or reserpine, which can release 48% within 15 min and 30% 5HT within 60 min, respectively (5). Phospholipase C also released ADP and PF4 and therefore further differs from agents, such as reserpine, which only release 5HT. The release of ADP after phospholipase C-induced phospholipid hydrolysis is substantial and equivalent to that released by thrombin (19). Nonspecific platelet permeability as seen in damaged platelets could not account for the observed release of platelet constituents, since phospholipase C did not cause a corresponding leakage of cytoplasmic metabolic pool ADP. The ultra-

structural evaluation of phospholipase C-modified platelets indicated that the cells were intact. However, significant centralization of granules and contraction of microtubules were evident when minimal phospholipid hydrolysis had occurred. These morphological features can be seen in platelets during early phases of the platelet release reaction (20). After more extensive phospholipase C-induced phospholipid hydrolysis and the release of ADP and 5HT, there was morphological evidence of platelet degranulation. Similar ultrastructural changes have been seen in thrombin-treated platelets. It is apparent that the biochemical and morphological characteristics of the phospholipase C-induced release of platelet constituents are virtually identical to that caused by thrombin, especially in respect to the selectivity and rapidity of the release of 5HT and ADP.

Since phospholipase C preparation from *Cl. perfringens* may contain other enzymes and contaminants, it was important to establish that phospholipase C-induced phospholipid hydrolysis caused the release reaction. This was achieved by several approaches:

(a) An extremely purified phospholipase C preparation, 133 U/mg, was obtained from Worthington Biochemical Corp. It had been back-extracted from a high ammonium sulfate-precipitated fraction of culture filtrates from *Cl. perfringens*. Subsequently the extract was purified by passage over a Sephadex G-100 and a DEAE Sephadex A-50 column.<sup>2</sup>

(b) One property that sets phospholipase C apart from most other enzymes is that it is extremely heat stable (16). The demonstration that phospholipase C that had been further purified by heating retained its ability to hydrolyze platelet phospholipids as well as to release ADP and 5HT and to produce ultrastructural evidence of degranulation speaks against the possibility that heat-labile enzyme such as neuraminidase caused the observed release reaction.

(c) Phospholipase C purified by heating was clearly free of proteolytic activity. Protease activity was not detected in massive concentrations of heated phospholipase C by three sensitive standard proteolysis tests and by the demonstration that phospholipase C did not decrease the activity of other enzymes,

(d) The observation that phospholipase C (*Cl. perfringens*) was neutralized by antitoxin to *Cl. perfringens* enzymes ruled out the possibility that a contaminant was responsible for the platelet release reaction.

(e) Several considerations indicate that neuraminidase contamination was not responsible for the release of platelet constituents. The phospholipase C preparation which had been heated in a manner than would readily inactivate neuraminidase still induced the release reaction, demonstrating that neuraminidase was not re-

sponsible for our observations. Also, the finding that phospholipase C preparation did not release platelet 5HT in the absence of  $Ca^{++}$  indicated that neuraminidase contamination did not cause the release reaction. This study showed that neuraminidase, unlike phospholipase C, did not require  $Ca^{++}$  for its activation.

(f) Platelet phosphatidylcholine was primarily attacked by phospholipase C. Therefore, the possibility that phosphorylcholine, the major hydrolysis product of phospholipase C-treated phosphatidylcholine, caused the release reaction was ruled out by the finding that exogenous phosphorylcholine did not release platelet 5HT. This phase of the research strongly indicates that phospholipase C-induced platelet phospholipid hydrolysis caused the platelet release reaction.

The platelet surface undoubtedly provides active sites for the reception of stimuli that initiate the release reaction. There is evidence that ADP binds to specific sites on the platelet surface (21) and that thrombin interacts with a membrane-labile protein (22). The concept of a lipid-ADP receptor has been advanced by the demonstration of rapid incorporation of  $^{32}P$  into platelet phospholipids in aggregating platelets (23). This enhanced phospholipid synthesis was thought to represent a change in the platelet membrane in response to ADP. Alteration in the synthesis of platelet phospholipids have also been shown to occur in epinephrine- (24) and thrombin-treated (25) platelets. However, changes in platelet lipid synthesis may be a nonspecific phenomenon, since similar alterations occur in platelets incubated with phenazine methosulfate and methylene blue, which do not induce platelet aggregation (6). A different approach to the problem of assessing the role of membrane lipids in platelet function was used in this study. Our results clearly demonstrate that minimal hydrolysis of platelet phospholipids triggers the platelet release reaction. Our hypothesis is that phospholipids, possibly phosphatidylcholine molecules, are situated on or near the active site or "receptor" on the platelet surface and function as the modulator for the platelet release reaction.

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