The Effect of Phospholipase C on Human Blood Platelets

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ABSTRACT The effect of phospholipase C (EC 3.1.4.3) on human blood platelets has been studied. Phospholipase C from Bacillus cereus was purified to homogeneity as judged by analytical and sodium dodecyl sulphate disc gel electrophoresis and by immunoelectrophoresis. Human platelets isolated from platelet-rich plasma by gel filtration or by centrifugation and washing were incubated with phospholipase C.

A loss of 20–45% of the total platelet phospholipid was observed, whereas 88% was hydrolyzed when platelet homogenates were submitted to identical enzyme treatment. Intact platelets lost 50–75% phosphatidylethanolamine, 20–50% phosphatidylcholine, and 20–25% phosphatidylserine. Sphingomyelin was not a substrate for the enzyme under the conditions used. The platelets contained no detectable endogenous phospholipase C activity.

The loss of phospholipid was not accompanied by aggregation of the platelets, nor did the platelets lose their ability to aggregate with ADP or thrombin. Total platelet factor 3 releasable by freezing and thawing was reduced. Measurements of releasable platelet factor 4 and the efflux of serotonin showed that no release reaction was triggered even when up to 45% of the total phospholipid in the platelets was hydrolyzed. When sphingomyelinase was added together with, before, or after phospholipase C, aggregation occurred. Sphingomyelinase alone gave no aggregation. The gel-filtered platelets also aggregated upon addition of purified phospholipase C from Clostridium perfringens. The distribution of phospholipids in the platelet membrane is discussed.

INTRODUCTION

Recently, Schick and Yu (1) reported that a commercially available preparation of phospholipase C (EC 3.1.4.3) from Clostridium perfringens caused the release reaction of platelets, together with the hydrolysis of platelet phospholipids, which were situated at or near the active site or "receptor" on the platelet surface and functioned as the modulator for the release reaction. Chap and Douste-Blazy (2) found essentially the same in similar experiments using the C. perfringens phospholipase C from another commercial source. However, neither of these enzyme preparations was pure; hence it was of interest to examine the effects of a highly purified, electrophoretically homogeneous PLC from B. cereus. The latter enzyme differs from the C. perfringens enzyme in that it does not attack sphingomyelin under the conditions used here.

METHODS

Materials. ADP, sodium salt, albumin, and apyrase (Grade A) were obtained from Sigma Chemical Co., St. Louis, Mo. Bovine thrombin was obtained from Hoffman-La Roche, Basel, Switzerland. Human fibrinogen (90% clottable) from Kabi, Stockholm, Sweden, was dissolved in 0.15 M sodium chloride. Casein was obtained from Merck AG, Inc., Darmstadt, Germany. Sepharose 2B was obtained from Pharmacia AB, Uppsala, Sweden. PLC (C. perfringens) was purified and characterized as described by Möllby and Wadström (3). Sphingomyelin phosphodiesterase (SMase) (Staphylococcus aureus) (EC 3.1.4.12) was purified and characterized as described by Wadström and Möllby (4, 5) and Möllby and Wadström (6). Both enzymes were kindly donated by Dr. Roland Möllby, Karolinska Institutet, Stockholm, Sweden.

Preparation of platelet suspension. Human platelets were prepared from blood drawn from fasting donors into plastic equipment containing 0.1 vol of acid citrate-dextrose anticoagulant (7). Platelet-rich plasma was obtained by centrifugation at 120 g for 15 min at 22°C in a Sorvall

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GLC-1 centrifuge (Dupont Instruments, Sorvall Operations, Newtown, Conn.).

The platelets were separated from the plasma proteins as described (8) by the gel filtration method of Tangen and Berman (9). The platelets were eluted with a solution containing 1 part 0.14 M NaCl and 9 parts 0.145 M Tris-HCl, pH 8.0, containing 50 mM CaCl2, 0.98 mM MgCl2, 5.4 mM KCl, 1.5 g/100 ml of albumin, and 5 mg/100 ml of apyrase. The albumin preparation contained less than 0.3% phospholipid. Platelets were also prepared by washing and centrifugation as described by Mustard et al. (10). Platelet-poor plasma was obtained by centrifugation at 12,000 g for 10 min at 4°C. Platelets were counted in a Thrombocounter (Coulter Electronics Ltd., Harpenden, England).

PLC. The enzyme (PLC) was purified from the culture filtrate of B. cereus ATCC 10987 AB-1 (11) by affinity chromatography as described earlier (12). The enzyme preparations were homogeneous in analytical disc electrophoresis and sodium dodecyl sulfate electrophoresis. When injected into goats and rabbits, they gave rise to antiserum which were monospecific in immunoprecipitation against purified PLC as well as against a concentrate of crude bacterial culture supernate.* The enzyme had no proteolytic activity on a casein substrate and less than 2% hemolytic activity when 10 μg PLC was incubated for 1 h at 37°C with a 2.5% suspension of washed human erythrocytes. The concentration of the stock solution of enzyme in tris buffered saline (13) containing 1 mM ZnCl2 was 0.3 μg/ml. Under these conditions the enzyme is stable for at least 12 mo at −20°C.

Incubation conditions. Gel-filtered platelets (1.8-3.2 × 10^10 platelets/ml) were incubated in nylon centrifuge tubes for 20 min at 37°C with PLC or buffer. The final concentration of PLC was 2.5-22.7 μg PLC/10^9 platelets (5.9-37.5 pg PLC/PL). Before and at the end of the incubation period, samples were withdrawn for (a) aggregation measurement, (b) testing of platelet factor 3 (PF-3) activity, and (c) measurement of platelet factor 4 (PF-4). The remaining incubation mixture was centrifuged (40,000 g for 30 min at 4°C), and residual lipid phosphorus in the platelets was examined.

Extractions of lipids. The centrifuged platelets were suspended in 1 ml 0.15 M saline, and lipids were extracted with methanol and chloroform (2:1) according to the method of Vikrot (14), with 5 ml of saturated NaCl in the procedure instead of CaCl2 (15). In three experiments this extraction method was compared with that of Folch et al. (16), as modified by Marcus et al. (17), except that NaCl was used instead of CaCl2. No significant differences were found.

Phospholipid fractionation. The phospholipid distribution was determined by thin-layer chromatography with 0.5 mm silica gel H (Merck) on glass plates measuring 20 × 6 cm or 20 × 20 cm. The plates were activated at 120°C for 80 min before use, and the solvent system was chloroform: methanol:glacial acetic acid:water, 65:25:8:4 hr vol (18). The chromatography was done in nomenclature chambers since this in our hands gave a better separation. The plates were sprayed with dichlorofluorescein (Merck), and the spots were scraped off into sintered glass tubes. The phospholipids were eluted with chloroform: methanol: glutaric acid: water, 50:39:1:10, and dichlorofluorescein was extracted by using 4 M NH4OH followed by 50% methanol in water, as described by Arvidson (19). Phospholipid-phosphorus was determined by the method of Chen et al. (20) after digestion with sulfuric acid. The recovery of phospholipids from the plates was regularly 80-95%.

Platelet aggregation. Platelet aggregation was monitored at 37°C with a Payton single-channel aggregometer (Payton Associates Ltd.,Scarborough, Ont.). The platelet response to ADP, thrombin, and collagen was examined before and after treatment with PLC. Final concentrations were 2 mM CaCl2, 0.05 NIH U of thrombin/ml, or 10 μM ADP and 0.5 mg/ml fibrinogen instead of thrombin. The collagen suspension (2 mg/ml) was prepared as described by Holmsen et al. (21).

PF-3. For the purpose of these experiments total PF-3 was operationally defined as the ability of isolated platelets, frozen and thawed three times, to shorten the coagulation time of platelet-poor plasma in the absence of added cephalin. PF-3 was tested in triplicate with the one-stage method of Husom (22) with 0.1 ml activated human plasma plus 0.1 ml test material. Platelet-poor human plasma was activated with 30 mg Celite/ml (Johns-Manville, New York) (22). A standard curve was prepared with dilutions of a suspension of three times frozen and thawed platelets (3.7 × 10^9 platelets/ml) as source of PF-3.

PF-4. Aliquots of the incubation mixture were centrifuged (12,000 g/10 min/22°C), and the supernates were frozen before testing by the electroimmunoassay method of Gjesdal (23). Standards of purified PF-4 were included for comparison. Total PF-4 was determined after treatment of 2.5% Triton X-100 and freezing and thawing the platelets once, as well as after freezing and thawing three times. Activity is expressed as units of heparin neutralized.

Serotonin (5HT) efflux. The effect of PLC on SHT efflux was investigated as described by Lingjerde (24) in separate experiments. PRP with 0.1 vol 5% (wt/vol) diNa-EDTA as anticoagulant was incubated with [3H]SHT (Radiochemical Centre, Amersham, U. K., final conc. 0.67 nmo/l/ml, with sp act 10 μCi/ml) in 0.5 vol isotonic sodium phosphate buffer, pH 6.4, for 6 min at 37°C, to a total uptake of about 80 pmol [3H]SHT/10^9 platelets. The platelets were then isolated by centrifugation (1,000 g for 12 min at 4°C) and resuspended in 2 vol of the buffer used for gel filtration. 2-ml aliquots were withdrawn, and 50 μl PLC (15 μg) or 50 μl buffer was added. Unlabeled SHT (final conc. 10 μM) was added to block competitively the uptake of released [3H]SHT. The samples were incubated at 37°C for 10-40 min and then chilled. Control samples were kept at 4°C throughout. No efflux took place at this low temperature for at least 40 min. All samples were centrifuged again (2,500 g for 15 min at 4°C). The supernates were discarded, the test tubes were washed, and the remaining medium was carefully wiped off. Finally, the platelets were lysed in 1 ml distilled water at 37°C for 20 min. After a brief centrifugation, 0.6 ml of the supernate was mixed with 6 ml Diluene (Packard Instrument International, Zurich, Switzerland) and counted in a Packard TriCarb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). The control platelets, from which no release had taken place, contained [3H]SHT corresponding to about 5,000 cpm in the sample withdrawn for counting. The efflux was expressed as percent loss of platelet-bound [3H]SHT during the incubation at 37°C, compared to the control platelets kept at 4°C.

Other methods. Analytical disc and sodium dodecyl sulfate polyacrylamide gel electrophoresis were carried out as

*Otness, A.-B. Unpublished observations.

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described by Davis (25) and by Laemmli (26). Immuno-electrophoresis was carried out as described by Scheidegger (27) except that 1% agarose was used. Protein was determined according to Lowry et al. (28). Lactic acid dehydrogenase was determined with the Baker kit (Baker Chemicals, Deventer, Holland) according to Wroblewski and LaDue (29).

RESULTS

Aggregation measurements. The incubation of PLC with gel-filtered platelets did not cause aggregation either in the absence or in the presence of 1 mM ZnCl₂ (final concentration) and did not significantly alter the response of the platelets to thrombin or collagen. In 16 out of 18 experiments the response to ADP was either unchanged or slightly reduced (5–15%) (Fig. 1). Very similar results were obtained with collagen. No correlation was found between the loss of sensitivity of the platelets to ADP and either the amount of PLC used or the amount of phospholipid hydrolyzed. When SMase (15 µg/ml) was added after incubation of PLC, aggregation occurred within 3–6 min at 37°C. The same result was obtained if the platelets were incubated with SMase before PLC, or if the two enzymes were added together. However, SMase alone gave no aggregation. PLC from C. perfringens (final concn. 15 µg/ml) caused aggregation within 1 min at 37°C.

PF-3. Total PF-3 decreased markedly after PLC treatment of the platelets. In two experiments the mean decrease was 93%. The corresponding mean increase in coagulation time was from 43.5 s to 73.5 s.

PF-4. The amounts of PF-4 in the supernates from unincubated, PLC-treated, and control incubated platelets were compared (Table I). The total releasable PF-4 is taken as 100%. Incubation with PLC resulted in little or no increase of PF-4 above the amount seen in the supernates from the unincubated and incubated controls. No correlation between amount of PLC added and increase of PF-4 was observed. The experiments showed that PLC did not induce any significant release of PF-4.

Phospholipids. In good accordance with data of others (30, 31), the platelets were found to contain 10.0–13.9 µg lipid phosphorus/10⁸ platelets. The composition of the phospholipid fraction is given in Table II. Our data agree reasonably well with data in the literature (31–39). A small amount of phosphatidylinositol was detectable but was not quantified. Control extractions of phospholipids from platelets isolated by centrifugation and washing showed no significant differences from the data in Table II. The phospholipid fraction contained 43.5% phosphatidylcholine (PC) and 15.8% SM. The phospholipid content of the platelets was reduced after treatment with PLC, the reduction depending on the amount of PLC added when small amounts of enzyme were used (Fig. 2). The release of lipid phos-

![Figure 1](image)

**Figure 1** Effect of PLC on ADP-induced aggregation of human platelets. 1-ml platelet suspension, 2 mM CaCl₂, 0.5 mg/ml fibrinogen, and 10 µM ADP (all final concentrations) were used. (A) After incubation with 26 µl PLC/ml (4.3 µg PLC/10⁸ platelets) for 20 min at 37°C. (B) After incubation with 26 µl/ml Veronal-buffered saline containing 1 mM ZnCl₂ for 20 min at 37°C.

**Table I**

<table>
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<tr>
<th>Conc. of platelets</th>
<th>Unincubated platelets</th>
<th>Platelets incubated with:</th>
<th>Total in platelets</th>
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<tr>
<td></td>
<td></td>
<td>Buffer</td>
<td>PLC</td>
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<td></td>
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<td>X10⁸/ml</td>
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<td>3.2</td>
<td>30 (0%)</td>
<td>50 (3%)</td>
<td>83 (7%)</td>
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<td>1.5</td>
<td>80 (0%)</td>
<td>72 (0%)</td>
<td>90 (2%)</td>
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<td>2.7</td>
<td>30 (0%)</td>
<td>36 (1%)</td>
<td>42 (2%)</td>
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PF-4 was measured in the supernate after removal of platelets by centrifugation (12,000 g/10 min per 20°C). FFA activity in supernates from unincubated platelets was subtracted as background.

The Effect of Phospholipase C on Human Blood Platelets
Table II

<table>
<thead>
<tr>
<th>Phospholipid Composition of Gel-Filtered Platelets*</th>
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<td>Percent of total</td>
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<td>PE</td>
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Phospholipids were separated by thin-layer chromatography, the spots scraped off, eluted with chloroform/methanol/glacial acetic acid/water and digested, and phosphate was determined (see Methods).
* Mean and range of six experiments.

that of sonicated control platelets, indicating that no endogenous phospholipase activity appeared in the platelets after sonication. In the intact platelets, only about 40% of the total phospholipid was hydrolyzed, with the different phospholipids being hydrolyzed to differing extents (Fig. 3). Phosphatidylethanolamine (PE) was the most susceptible to hydrolysis (50–75%), followed by PC (20–50%) and phosphatidylserine (PS) (20%), while SM was not attacked. The amount of SM recovered after PLC treatment appeared to increase 5–10%. The hydrolyses of the individual phospholipids reached a plateau value that was not further increased by increasing amounts of enzyme (Fig. 3). With SMase only 20–25% of the total platelet SM was hydrolyzed.

**Efflux of serotonin.** Monitoring the efflux of [3H]-5HT from preloaded platelets showed that incubation with PLC did not trigger any release of 5HT. The efflux curve (Fig. 4) showed the same slow efflux as seen with the preloaded control platelets. The only difference was that with the PLC-treated platelets the efflux was reproducibly about 3% higher at all time points.

**Lactic acid dehydrogenase.** 3.7 × 10^6 platelets were incubated for 20 min at 37°C with 19 μg PLC. No release of lactic acid dehydrogenase (EC 1.1.1.27) from the platelets to the supernate was observed.

**DISCUSSION**

The present study demonstrates that gel-filtered human platelets may lose up to at least 45% of their total phospholipid without induction of aggregation, or release or significant impairment of their response to ADP, thrombin, or collagen. The platelets fully retained their sensitivity and response to thrombin. The response to ADP was slightly reduced in most experiments, but the decrease did not correlate with the amount of phospholipid hydrolyzed and was therefore probably not related to the enzymatic activity. The response to collagen showed variations similar to those of the ADP response. These findings are in apparent contrast to the results of Schick and Yu (1), who found that crude PLC (C. perfringens) induced a release reaction when only a small amount (5–10%) of the phospholipid had been hydrolyzed. The corresponding loss of PC was 25% and of SM 9%. Chap and Douste-Blazy (2) reported essentially similar experiments with a similar preparation of PLC (C. perfringens) and obtained basically the same results.

We confirm their results with regard to the ability of the C. perfringens enzyme to aggregate platelets. Both PC and SM are good substrates for this enzyme (40), whereas the Bacillus enzyme did not hydrolyze SM under the present reaction conditions. An extensive hydrolysis by the Bacillus enzyme of PE (up to 60–75%), of PC (about 50%), and of PS (20%) was not enough to induce platelet aggregation or release. Aggregation was observed only when gel-filtered platelets were treated with the Clostridium enzyme or with SMase and the Bacillus enzyme in combination or in sequence. This clearly demonstrates that even when extensive hydrolysis of the other phospholipids occurs, some SM (about 20–25%) must be hydrolyzed for aggregation and release to take place. When a certain degradation of the other phospholipids has taken place, this small degree of hydrolysis of SM (3.5–4.5% of the total platelet phospholipid) apparently determines
whether the platelets remain intact or aggregate. Hydrolysis of SM alone is, however, not sufficient to induce aggregation. These findings are comparable to those of Colley et al. (41), obtained with erythrocytes, except that the erythrocyte phospholipids were not accessible to PLC unless the erythrocytes were exposed to SMase before PLC.

No evidence for induction of a release reaction by incubation with PLC was found. Less than 4% of total releasable PF-4 was released during the PLC treatment if the release from the incubated control platelets was subtracted as background. It has been suggested (42) that about 10% of PF-4 is bound to the plasma membrane. The 2-4% released by PLC may derive from this fraction.

The efflux of [14C]5HT from preloaded platelets also showed only a very slight increase (3%) in the presence of PLC, in contrast to the situation when a release reaction takes place and about 70-85% of total platelet 5HT appears in the supernate in less than 40 s (43). The parallel course of the efflux curves (Fig. 4) suggests that there was probably no difference in the efflux from the main intracellular pool of 5HT in the PLC-treated and in the control platelets. The small constant 3% difference remains unexplained, but may possibly be due to loss of a small plasma membrane-bound fraction of 5HT. In conclusion, both the PF-4 and the [14C]5HT determinations indicate that no release reaction is induced by the Bacillus enzyme.

The loss of phospholipids reached a plateau of 30-45% when intact platelets were exposed to PLC and this loss was not increased further by increasing the amount of enzyme. A similar figure was found when platelets isolated by centrifugation and washing were used. The hydrolysis of the various phospholipids also reached different plateau levels (Fig. 3), whereas in platelet sonicates the phospholipids were completely hydrolyzed by the same amounts of enzyme. Taken together with the absence of release of PF-4 and 5HT and the lack of lactic acid dehydrogenase leakage from the platelets, these findings strongly suggest that only the phospholipids of the outer layers of the platelet membrane (except SM) are accessible to the enzyme and that the PLC attack was limited to the outside of the membrane. This is in accordance with observations in other systems (41, 44). If PLC hydrolyzes only the outer layer phospholipids, then the distribution of phospholipids in the platelet membrane may be calculated (Fig. 5), provided that the following assumptions are made: (a) the accessible membranes (plasma membrane and main surface-connecting channels) account for about two-thirds of the total phospholipids of platelets; (b) the phospholipid compositions of accessible and internal membranes are not grossly different (32); and (c) the inner and outer layers of the plasma membranes have about the same protein-to-phospholipid ratio. (This latter assumption may not be strictly correct, since it has been suggested that the inner layer contains more protein than the outer (44).

Since 60-70% of PE is hydrolyzable, the remaining 30-40% is either part of the inner layer of the plasma membrane or of the intracellular membranes. SMase
hydrolyzed 20–25% of SM. From the same reasoning, this fraction must be located in the external membrane to be available to the enzyme. The estimate of phospholipid distribution (Fig. 5) based on these assumptions and considerations is obviously not accurate in detail, but demonstrates the asymmetry in the platelet plasma membrane. The main difference from results obtained with other membranes (41, 44, 45) is that more PE seems to be located in the outer plasma membrane layer in the platelets. This feature may be related to the special origin of platelets and correlates nicely with the well-known procoagulant activity of PE and PS + PE, thought to be responsible for the PF-3 activity (46).

PF-3 as defined here is generally assumed to be partly localized in the plasma membrane (available PF-3) and partly in the interior of the platelet, being made available during the release reaction. The decrease in total PF-3 activity after PLC treatment must mean that the main part of the structures carrying the PF-3 activity are accessible to PLC, i.e., if our interpretation of the present data is correct, they are localized in the plasma membrane. The exact nature of these structures is currently unknown, although Vecchione and Zucker (47) recently have produced evidence that phospholipids alone can substitute completely for platelets in a plasma recalcification system.

ACKNOWLEDGMENTS
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