Effect of phospholipase C (*Bacillus cereus*) on freshly isolated and 4-day-stored human platelets

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Phospholipase C (from *Bacillus cereus*) was used to study fresh and stored human platelets. Provided that the enzyme was inactivated before lipid extraction, no significant degradation of phospholipid in fresh cells was noted, even when platelets were activated or induced to change shape by ADP, collagen or thrombin. With platelets isolated from concentrates stored for transfusion for 4 days at 22°C, membrane phospholipids were degraded by the enzyme to an extent depending on the pH in the platelet concentrate at day 4 of storage. The extent of phospholipid hydrolysis in platelets correlated well with the extent of release of lactate dehydrogenase during storage, with both being minimal for platelets from concentrates of final pH 6.5–6.9. Under non-lytic conditions, phosphatidylcholine was the phospholipid most degraded (40%), with no significant degradation of phosphatidylserine being detected. Storage does not seem to alter the distribution of phospholipids at the external leaflet of the plasma membrane.

Blood platelets are a normal blood-bank product used in the treatment of haematologically depressed patients suffering from, for example, various forms of cancer and bone-marrow disorder (Schiffer *et al.*, 1978; Weiden *et al.*, 1978; Kelton & Blajchman, 1979). However, unlike erythrocytes, which have a storage life of several weeks, platelets fairly rapidly lose their viability and function *in vivo* during a few days of storage (Slichter & Harker, 1976; Filip & Aster, 1978; Murphy *et al.*, 1982). It has been shown that, whereas the fresh human erythrocyte is extremely resistant to attack by the membrane-degrading enzyme phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Bacillus cereus*, stored erythrocytes become progressively more susceptible to attack and lysis by this enzyme (Shukla *et al.*, 1978; Little & Rumsby, 1980). The effect of phospholipase C (*B. cereus*) on freshly isolated human platelets has been studied by Otnaess & Holm (1976) and by Chap *et al.* (1977). The former workers reported extensive degradation of phospholipid (up to 40%) in intact human platelets by this enzyme and used it to study the disposition of individual phospholipids in the platelet plasma

membrane. In marked contrast, Chap *et al.* (1977) found phospholipase C (*B. cereus*) totally unable to cause phospholipid hydrolysis in intact human platelets. In the present work we confirm the findings of Chap *et al.* (1977), and have also studied the effects of phospholipase C on platelets stored for transfusion.

Materials and methods

Crystalline phospholipase C was isolated from *B. cereus* as described by Myrnes & Little (1980) and assayed as described by Little (1981). Potato apyrase (EC 3.6.1.5) was isolated and assayed as described by Traverso-Cori *et al.* (1965). ADP, collagen and thrombin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation of platelets

Freshly donated human blood was taken into standard blood-bank citrate/phosphate/dextrose/adenine anticoagulant in Fenwall triple packs types R1616 and R9030 (450 ml of blood with 63 ml of anticoagulant mixture). The pack was centrifuged (1000g for 10 min) and the platelet-rich plasma supernatant squeezed out into a satellite pack, which was then centrifuged (3000g for 20 min) to obtain a platelet button. The platelet button was left undisturbed for 1.5 h and then

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resuspended in 50 ml of plasma by gentle manual squeezing. All handling procedures were carried out at room temperature (22–24°C), and the platelet concentrate was stored at room temperature in a circular rotator operating at 6 rev./min.

When platelets were required for study, the concentrate was centrifuged (150g for 10 min) and the sediment was rejected. The pH of the supernatant was recorded, and if above 6.8 was adjusted with citric acid to pH 6.6–6.8 before centrifugation (1000g for 10 min). The pellet was resuspended in Ca^{2+} -free Tyrode buffer (pH 6.8) containing 2 mM MgCl_2 , 1 g of glucose/l, 3.6 mg of bovine serum albumin/ml, 1 μg of prostaglandin E_1 (Sigma, St. Louis, MO, U.S.A.)/ml and 5 units of potato apyrase/ml. The final platelet count was adjusted to 2×10^6 cells/ mm^3 , except when enzyme attack on individual phospholipids was studied. In this case the final cell count was $4 \times 10^6/\text{mm}^3$.

Incubation procedure and extraction and determination of phospholipids

Platelets suspended in the above buffer plus supplements were incubated at 37°C and 30 min with phospholipase C (50 μg of enzyme/ml, unless otherwise stated). The enzyme was inactivated after incubation by the addition of *o*-phenanthroline and EDTA (final concns. 4 mM and 15 mM respectively).

Lipids were extracted by the procedure recommended by Roelofsen *et al.* (1974) for phospholipase C-treated membranes. Individual phospholipids were determined after separation in two-dimensional t.l.c. on silica-gel H (Roelofsen *et al.*, 1974). Each lipid-extract sample was analysed in duplicate. Phosphorus was determined by the method of Ames (1966), and each determination was made in duplicate.

Lactate dehydrogenase activity was determined in a Clinicon Corona automatic analyser (from LKB, Bromma, Sweden) (Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology, 1974). The activity was determined in plasma from stored platelet concentrates and in the incubation system after enzyme treatment. In each case the cells were removed by centrifugation (1000g for 10 min). For total platelet activity measurements, the suspended cells were lysed with Triton X-100 (1%, v/v).

Platelet counts were measured with a Coulter counter model D. The settings were attenuation = 0.5, indicated aperture current = 0.033, volume measured 0.05 ml and an orifice tube of 70 μm .

Statistical analyses were performed with a 'Minitab II' program system obtained from the Department of Statistics, Pennsylvania State University, University Park, PA 16802, U.S.A.

Results

Effect of phospholipase C on freshly isolated platelets

Fresh platelets were incubated with 10–50 μg of phospholipase C/ml as described in the Materials and methods section. Provided that the enzyme was inactivated by the addition of chelating agents before lipid extraction, little or no significant hydrolysis of the platelet phospholipid could be detected (<5%). Omission of the enzyme-inactivation step resulted in marked phospholipid hydrolysis, the extent of which was independent of the incubation time before lipid extraction. Indeed, the same extent of phospholipid hydrolysis was noted after a 30 min incubation of platelets with enzyme as was observed if the lipid-extraction solvent was added immediately after the addition of enzyme. More than 90% of phosphatidylcholine and phosphatidylethanolamine were degraded when platelets containing 50 μg of phospholipase C/ml were subjected to lipid extraction without prior inactivation of enzyme.

The effect of phospholipase C on fresh platelets was also examined with platelet-activating agents present in the incubation system (prostaglandin E_1 was omitted). Agents such as thrombin (3 i.u./ml), collagen (1 $\mu\text{g}/\text{ml}$) and ADP (10 μM) were used, and only very limited extents of phospholipid degradation (<10%) were noted. In one study apyrase was present in the incubation mixture, and ADP portions were added every 5–10 min sufficient in each case to give a final concentration of 10 μM . It was clear that under these conditions the platelets were going repeatedly to and fro between discoid and spherical forms (the 'swirling' characteristics of discoid platelets are visible to the naked eye). Even under these conditions, phospholipid hydrolysis by phospholipase C was less than 5%.

Studies on 4-day-stored platelets

With a batch of platelets obtained from a platelet concentrate of plasma, pH 7.1, at day 4, the relation between total phospholipid hydrolysis and enzyme concentration was studied. The extent of hydrolysis reached a plateau value of 35% at an enzyme concentration of 20 $\mu\text{g}/\text{ml}$ in the incubation system (Fig. 1). With other samples of platelets, the extent of hydrolysis reached a plateau value at similar enzyme concentrations, and so, for assessing the amount of hydrolysable phospholipid, the standard enzyme concentration selected was 50 $\mu\text{g}/\text{ml}$.

With different batches of stored platelets, very variable extents of enzyme attack were noted. However, when the extent of attack by phospholipase C on platelets from different concentrates was measured and related to the plasma pH of the platelets at day 4, these two parameters correlated

Fig. 1. Digestion of total phospholipid in 4-day-stored platelets by phospholipase C

Platelets resuspended in Ca^{2+} -free Tyrode buffer (pH 6.8) were incubated for 30 min at 37°C with different concentrations of phospholipase C (*B. cereus*). Phospholipid was then extracted and quantified. Further details are given in the Materials and methods section.

Fig. 2. Relation between plasma pH after storage and extent of total phospholipid degradation by phospholipase C (*B. cereus*) in 4-day-stored platelets

Platelets were isolated from 4-day-stored concentrates and the plasma pH was recorded. After resuspension in Ca^{2+} -free Tyrode buffer, pH 6.8, the platelets were incubated for 30 min at 37°C with $50 \mu\text{g}$ of phospholipase C/ml. Phospholipid was then extracted and quantified. Further details are given in the Materials and methods section. Symbols: ●, individual values; ○, average value over 0.2 pH unit; —, regression line; ----, 95% confidence limits on regression line.

well and a V-shaped curve was observed (Fig. 2). Except for platelets from rather alkaline concentrates (pH > 7.2), the extent of phospholipid hydrolysis was less than 40%, with a minimum value of 10–15% occurring with platelets isolated from concentrates of pH 6.6–6.8.

Fig. 3. Release of lactate dehydrogenase after incubation of 4-day-stored platelets with phospholipase C (*B. cereus*)

Platelets (stored 4 days) were isolated from concentrates in plasma and the plasma pH was recorded. After resuspension in Ca^{2+} -free Tyrode buffer, pH 6.8, and subsequent incubation for 30 min at 37°C with phospholipase C ($50 \mu\text{g}/\text{ml}$), the cells were removed by centrifugation and the lactate dehydrogenase activity in the supernatant was measured and expressed as a percentage of the activity released by detergent-induced cell lysis. Further details are given in the Materials and methods section. Symbols: ●, individual values; ○, average value over 0.2 pH unit.

To check for cell lysis, the release of lactate dehydrogenase from the platelets after phospholipase C treatment was followed. For platelets isolated from plasma of pH 6.1–7.2, less than 5% of the platelet lactate dehydrogenase was released, and hence only minimal cell lysis had occurred (Fig. 3). Marked cell lysis seemed to occur only with platelets from very acid plasma (pH 5.8–6.0).

Lactate dehydrogenase activities were also measured in the storage plasma at day 4. Minimal release seemed to occur with platelet concentrates of final pH 6.6–6.8, and the relationship between plasma pH and lactate dehydrogenase activity (both at day 4) showed a profile almost identical with that observed for plasma pH/phospholipase C attack. The plasma pH at day 4 was itself related to

Fig. 4. Hydrolysis of individual phospholipid classes by phospholipase C (*B. cereus*) in resuspended 4-day-stored platelets. Platelets resuspended in Ca^{2+} -free Tyrode buffer, pH 6.8, were incubated for 30 min at 37°C with various amounts of phospholipase C. The reaction was stopped by the addition of chelating agents, and the phospholipids were extracted, separated and quantified. Further details are given in the Materials and methods section. Platelets were isolated from concentrates of plasma pH of 5.96 (a) and 7.19 (b) on day 4. Symbols: □, phosphatidylserine; ▲, phosphatidylethanolamine; △, sphingomyelin; ○, phosphatidylcholine; ●, total phospholipid.

the platelet concentration during storage. High platelet counts ($>0.9 \times 10^6$ cells/mm³) led to an acid pH in the plasma, whereas below this concentration the plasma pH became more basic (results not shown).

The attack by phospholipase C on individual phospholipid classes was also investigated. Samples of stored platelets from two concentrates, of pH 5.96 and 7.19, were incubated with different amounts of enzyme. The enzyme was then inactivated and the phospholipids were extracted, separated by t.l.c. and quantified. The pattern of results obtained with the two sets of platelets was rather similar, and was also confirmed with two other batches of stored platelets. In all cases, phosphatidylcholine was the most markedly degraded phospholipid, with little or no attack occurring on phosphatidylserine (Fig. 4).

For non-enzyme-treated platelets, the phospholipid composition (average of six different batches) was 24% phosphatidylethanolamine, 12% phosphatidylserine, 21% sphingomyelin and 43% phosphatidylcholine. These values seemed independent of the pH in the sera at day 4, and are similar to those observed by other workers (see Hamid *et al.*, 1980).

Discussion

In agreement with the results of Chap *et al.* (1977), we find that fresh human platelets are extremely resistant to attack by phospholipase C (*B. cereus*), even at very high enzyme concentrations (50 μg/ml). Under these conditions it was necessary to inactivate the enzyme after incubation, otherwise extensive phospholipid degra-

dation occurred during extraction. It is therefore strongly advisable to inactivate phospholipase C (*B. cereus*) after incubation, as suggested by Roelofsens (1982). For this purpose, a mixture of *o*-phenanthroline and EDTA is recommended, EDTA alone being relatively slow at inactivating this enzyme (Little & Otnæss, 1975). The report of a successful attack by phospholipase C (*B. cereus*) on fresh human platelets (Otnæss & Holm, 1976) is almost certainly due to a failure to inactivate the enzyme before lipid extraction. Furthermore, Otnæss & Holm (1976) reported extensive degradation of phosphatidylethanolamine by this enzyme. We find that this phospholipid is the one most heavily degraded during lipid extraction in the presence of active enzyme. Activated and aggregated fresh platelets have a drastically altered morphology and possess long spikes [Hovig (1970), and also confirmed in a simultaneous electron-microscopic study (C. S. Solberg & C. Little, unpublished work)], but were still very resistant to attack by this enzyme. These results suggest an intact membrane with tightly packed phospholipids (Demel *et al.*, 1975).

Platelets from 4-day-stored concentrates were attacked by phospholipase C to an extent which correlated with the pH of the concentrate at day 4. The pH after storage is determined by the platelet count of the concentrate (Murphy & Gardner, 1975; Slichter & Harker, 1976; Moroff *et al.*, 1982; also confirmed in the present study). Platelets from stored concentrates of final pH 6.5–6.9 suffered a 10–15% degradation of phospholipid. Progressively more phospholipid hydrolysis was found with platelets from concentrates with pH decreasing below 6.5 or increasing above 6.9 (Fig. 2). Except

Table 1. *Phospholipid distribution at the external leaflet of the platelet plasma membrane*
Values are expressed as percentages of the total amount of a phospholipid class found in the platelet.

Phospholipid	Amount at external leaflet (%)		
	Present work (stored cells)	Chap <i>et al.</i> (1977) (fresh cells)	Otnæss & Holm (1976) (fresh cells)
Sphingomyelin	> 30	72	25
Phosphatidylcholine	40	23	50
Phosphatidylserine	0	1	20
Phosphatidylethanolamine	15	12	75
Total phospholipid	approx. 30–40	25	45

with platelets that had become rather acidic during storage (concentrate pH below 6.0), only minimal release of lactate dehydrogenase (<5%) occurred during enzyme treatment, thereby suggesting non-lytic attack.

Comparison of Figs. 2 and 3 suggests that the maximum amount of non-lytic degradation of the phospholipids in 4-day-old platelets is about 30–40%. This value is somewhat higher than that suggested by Chap *et al.* (1977) for the percentage of total phospholipid found in the outer leaflet of the plasma membrane of fresh human platelets. With platelets isolated from the most alkaline concentrates studied (pH > 7.4), very extensive phospholipid degradation by phospholipase C occurs (50–60%), with only around 5–6% release of lactate dehydrogenase. These cells had already lost large amounts of lactate dehydrogenase during storage and may thus already have lysed.

The pattern of hydrolysis of individual phospholipid classes during apparently non-lytic attack on 4-day-stored platelets is shown in Table 1 together with the results of Chap *et al.* (1977) and Otnæss & Holm (1966). Our results are in general agreement with those of Chap *et al.* (1977), i.e. virtually no attack on phosphatidylserine and a small amount of degradation of phosphatidylethanolamine. The extent of phosphatidylcholine degradation was somewhat greater in the present work. As for sphingomyelin, phospholipase C (*B. cereus*), having a very low sphingomyelinase activity, is a poor tool for studying this lipid (see discussion in Gwarsha *et al.*, 1984). The 30% degradation of sphingomyelin by this enzyme must therefore be regarded as a minimal value. Our phospholipid-disposition data give no indication of phospholipid reorientation in platelets during storage. Furthermore, total phospholipid composition was also unchanged. Clearly, however, some changes must have occurred in the plasma membrane during storage to permit enzyme attack. Equivalent observations to these have been made on stored human erythrocytes (Shukla *et al.*, 1978; Little & Rumsby, 1980). In this case, a build-up of membrane diacyl-

glycerol may have been responsible (Allan & Michell, 1975; Dawson *et al.*, 1983). Possibly, therefore, an equivalent process occurs in the platelet membrane. That lipid changes do occur in the platelet membrane during storage is shown by the work of Hamid *et al.* (1980), who reported a 15% loss of cholesterol and a 7–11% loss of total phospholipid during 3 days of storage at 20°C.

The susceptibility of platelet phospholipids to phospholipase C attack and the release of lactate dehydrogenase during storage showed the same relationship to plasma pH at day 4 and were minimal at plasma pH 6.5–6.9. The platelet membrane would thus seem to be best preserved at these storage pH values and possibly also so would platelet viability.

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