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CHANGES IN MEMBRANE PHOSPHOLIPID DISTRIBUTION DURING PLATELET ACTIVATION

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(1) Exposure of phospholipids at the outer surface of activated and control platelets was studied by incubation with a mixture of phospholipase A_2 from *Naja naja* and bee venom, solely or in combination with sphingomyelinase from *Staphylococcus aureus*, using conditions under which cell lysis remained below 10%. (2) Incubation with phospholipase A_2 alone revealed a markedly increased susceptibility of the phospholipids in platelets activated by a mixture of collagen plus thrombin, by the SH-oxydizing compound diamide, or by calcium ionophore A23187, as compared to control platelets or platelets activated separately by collagen or thrombin. (3) Collagen plus thrombin, diamide, and ionophore treated platelets revealed an increased exposure of phosphatidylserine at the outer surface accompanied by a decreased exposure of sphingomyelin, as could be concluded from incubations with a combination of phospholipase A_2 and sphingomyelinase. These alterations were much less apparent in platelets activated either by thrombin or by collagen alone. (4) The increased exposure of phosphatidylserine in activated platelets is accompanied by an increased ability of the platelets to enhance the conversion of prothrombin to thrombin by coagulation factor Xa, in the presence of factor Va and calcium. (5) It is concluded that the altered orientation of the phospholipids in the plasma membrane of platelets activated by collagen plus thrombin, by diamide, or by calcium ionophore, is the result of a transbilayer movement. Moreover, the increased exposure of phosphatidylserine in platelets stimulated by the combined action of collagen and thrombin might be of considerable importance for the hemostatic process.

Introduction

Phospholipids in the plasma membrane of human platelets are not homogeneously distributed between both halves of the membrane bilayer [1–3]. In a resting, non-activated platelet, the outer surface of the membrane is characterized by the presence of neutral phospholipids, particularly phosphatidylcholine and sphingomyelin. The negatively-charged platelet phospholipids, phosphatidylserine and phosphatidylinositol, are almost exclusively present in the inner leaflet of the bilayer. We have previously shown that activation of platelets by simultaneous action of thrombin and collagen changes the distribution of the lipids in the plasma membrane in such a way that a substantial amount of the negatively-charged phosphatidylserine becomes exposed at the membrane outer surface [4]. This property of activated platelets is of significant importance for their role in the hemostatic process, since negatively-charged phospholipids markedly enhance the conversion of factor X to factor Xa by a complex of factor IXa, factor VIIIa and Ca^{2+} as well as the conversion of prothrombin to thrombin by a complex of factor Xa, factor Va and Ca^{2+} [5–12]. Non-activated

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

platelets have a low capacity to stimulate the formation of factor Xa or thrombin, because of the nearly absolute absence of negatively-charged phospholipids at the outer surface of the membrane. Simultaneous activation of platelets by collagen and thrombin results in a large enhancement of the rate of factor Xa or thrombin formation [4,13]. This increased activity has been ascribed to the increased exposure of negatively-charged phosphatidylserine, which makes the platelet surface more suitable for the formation of prothrombinase and factor X activating complexes.

In our previous experiments, exposure of phosphatidylserine at the outer surface of activated platelets was demonstrated using phospholipase A_2 from *Naja naja* or bee venom [4]. However, only the phospholipids of platelets activated by the combination of collagen and thrombin can be hydrolyzed by these lipolytic enzymes; the phospholipids of unactivated platelets or platelets activated either by collagen or by thrombin alone are hardly susceptible towards phospholipase A_2. Thus, from incubations with phospholipase A_2 alone it cannot always be concluded whether activated platelets have an increased exposure of negatively-charged phospholipids. However, phospholipids of unactivated platelets become available for hydrolysis by phospholipase A_2 when sphingomyelinase (*Staphylococcus aureus*) is also present [1]. Therefore, the accessibility of phospholipids of activated platelets to a combination of phospholipase A_2 and sphingomyelinase was investigated to establish whether a correlation exists between exposure of phosphatidylserine and platelet prothrombin converting activity after activation of platelets by collagen, thrombin or a combination of these agents. Since platelets treated with calcium ionophore A23187 also show a dramatic increase in prothrombin- and factor X-activating capacity [14], it was of interest to investigate whether these platelets also show alterations in the phospholipid distribution across the membrane. Moreover, Haest et al. [15,16] have found that treatment of erythrocytes with the SH-oxidizing agent diamide results in an increased accessibility of the aminophospholipids. Therefore, it was investigated whether this agent is also able to alter the phospholipid arrangement in the platelet membrane and if this accompanied by an increase of the prothrombin converting activity of platelets.

Materials and Methods

Fatty-acid-free human serum albumin, nicotinamide-adenine dinucleotide, reduced form (NADH), indomethacin and diamide were obtained from Sigma Chemicals Co. (St. Louis). Dithiothreitol and *o*-phenanthroline were from Baker. Collagen (Type I) was from Hormon Chemie (Munich) and calcium ionophore A23187 from Calbiochem. *Naja naja* venom and bee venom (*Apis mellifica*) were purchased from Koch Light. Chromogenic substrate H-D-phenylalanyl-L-pipecolylarginine-*p*-nitroanilide (S2238) was from Kabi Diagnostica (Stockholm, Sweden). Methylphosphatidic acid was prepared according to the method of Comfurius and Zwaal [17]. Coagulation factors Xa, Va, prothrombin and thrombin were isolated and purified as described elsewhere [4]. Phospholipase A_2 (phosphatide-2-acylhydrolase; EC 3.1.1.4) from *N. naja* and bee venom and sphingomyelinase (sphingomyelin-cholinephosphohydrolase; EC 3.1.4.12) from *Staphylococcus aureus* were purified according to Zwaal et al. [18]. One international unit (IU) is defined as the amount of enzyme which hydrolyzes one μmol of phospholipid per min under optimal conditions.

Isolation of platelets

Because relatively large amounts of platelets were required, a method was developed to isolate platelets from buffy coats obtained from the local hospital. The buffy coats were prepared from blood of healthy donors which was collected into the anticoagulant acid citrate dextrose: 0.052 M citric acid, 0.08 M trisodium citrate, 0.18 M glucose. One volume of buffy coat was diluted with five volumes of Hepes buffer pH 6.6, containing 137 mM NaCl, 2.68 mM KCl, 10 mM Hepes, 1.7 mM $MgCl_2$, 25 mM glucose and 0.05% fatty acid free human serum albumin. After centrifugation for 15 min at $200 \times g$, the platelet-rich upper phase was recentrifuged for 10 min at $1400 \times g$ to obtain a platelet pellet. This pellet was resuspended in Hepes-buffer pH 6.6, and one volume of acid citrate dextrose was added to fourteen volumes of the platelet suspension. The platelets were washed

twice and finally resuspended in the same Hepes buffer pH 7.5, without addition of acid citrate dextrose. Platelet concentration was determined with a Coulter Counter. The washed platelet preparations contained less than one percent leukocytes or erythrocytes. All platelet handling was carried out at room temperature.

Phospholipase treatment of platelets

Stirred platelet suspensions (10 ml), at a concentration of $3 \cdot 10^8$/ml, were activated with various stimulators in the presence of 3 mM $CaCl_2$ at 37°C. After 15 min, an additional amount of $CaCl_2$ (final concentration 10 mM) as well as *o*-phenanthroline (final concentration 2 mM) and indomethacin (final concentration 1 μg/ml) were added, followed by a mixture of phospholipase A_2 from *N. naja* (36 IU) and bee venom (26 IU). In some experiments sphingomyelinase (3.5 IU) was added 5 min after the addition of phospholipase A_2. At various time intervals, phospholipase activity was inhibited by addition of 1 ml 0.2 M EDTA. Prior to the addition of EDTA, a sample of 1 ml was taken to measure the amount of platelet lysis. It should be noted that for every time period of phospholipase treatment, a separate incubation was required, since platelet aggregation as a result of the activation procedure makes homogeneous subsampling unreliable.

Platelet lipids were extracted according to the procedure of Bligh and Dyer [19]. Methylphosphatidic acid (250 μg) was added as an external standard prior to the extraction procedure. Phospholipid analysis was carried out by two dimensional thin layer chromatography followed by phosphorous determination of the isolated spots as described earlier [20]. To evaluate phospholipid degradation in intact platelets only, corrections were made for phospholipid breakdown in the population of lysed cells assuming that the phospholipids of the lysed cells are completely degraded. Only those experiments where lysis did not exceed 10% were taken into consideration.

Measurement of platelet lysis

Lactate dehydrogenase activity in the supernatant was used as a parameter for platelet lysis and was measured according to the method of Wrobleski and La Due [21]. In the samples containing diamide, the measurements were carried out in the presence of excess dithiothreitol (twice the concentration of diamide). The amount of platelet lysis was determined from a calibration curve made with different dilutions of a platelet preparation which was lysed completely by sonication for 3 min.

Measurement of platelet prothrombin converting activity

Prothrombin-converting activity was measured as described before [4]. Final concentrations in the reaction mixture were: $5 \cdot 10^6$ platelets/ml, 15 nM factor Xa, 30 nM factor Va, 4 μM prothrombin and 6 mM $CaCl_2$. Samples (25 μl) for measuring the amount of thrombin formed were taken 30 s and 60 s after addition of prothrombin and assayed in a cuvette containing 1 ml buffer (50 mM Tris, 120 mM NaCl, 2 mM EDTA, pH 7.5) and 0.25 mM S2238. From the rate of change in absorbance at 405 nm, the amount of thrombin formed was calculated using a calibration curve with known amounts of active site-titrated thrombin.

Results

Exposure of phospholipids at the platelet outer surface was studied using a mixture of two phospholipases A_2 (the enzymes from *N. naja* and bee venom) solely or in combination with sphingomyelinase from *S. aureus*. An essential requirement for such a study is that all phospholipids are suitable substrates for the enzymes used and that the amount of enzyme and time of incubation are sufficient to guarantee complete degradation in lysed cells. Therefore, lysed platelets obtained by sonication for 3 min were incubated with a mixture of phospholipase A_2 (*N. naja* and bee venom) and sphingomyelinase, and phospholipid hydrolysis was measured. As shown in Fig. 1, all phospholipid classes with the exception of phosphatidylinositol are almost completely degraded within 5 min under the given experimental conditions. Since hydrolysis of phosphatidylinositol is incomplete, this phospholipid was not considered in further experiments. The results in Fig. 1 can be obtained by incubation of lysed platelets with phospholipase A_2 and sphingomye-

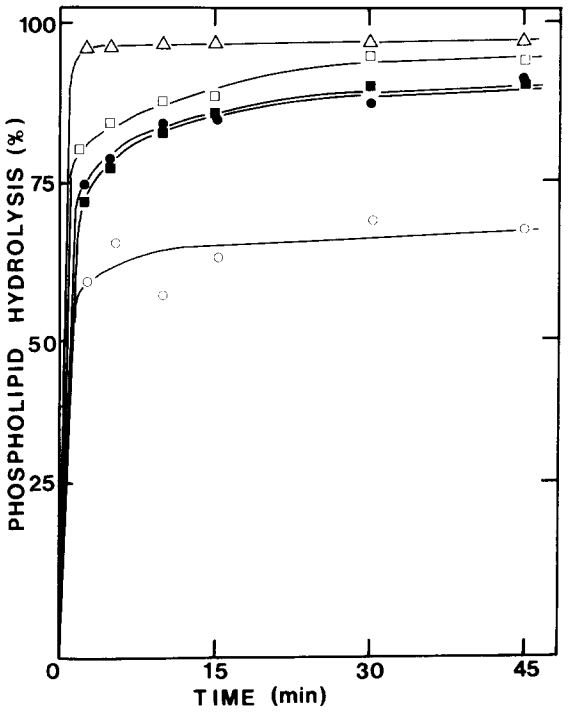

Fig. 1. Phospholipid hydrolysis in platelet lysate by phospholipase A_2 and sphingomyelinase. Platelets ($3 \cdot 10^8$/ml) were intermittantly sonicated at 15 sec intervals for 6 min at 0°C using a Branson Sonifier. At the end of this period, platelets were completely lysed. Phospholipase A_2 and sphingomyelinase were added and at different time intervals the incubation was stopped with EDTA and analyzed for phospholipid hydrolysis. Phosphatidylcholine, ■; phosphatidylethanolamine, △; phosphatidylserine, ●; phosphatidylinositol, ○; sphingomyelin, □.

TABLE I

NON-LYTIC DEGRADATION OF PHOSPHOLIPIDS IN ACTIVATED PLATELETS BY EXOGENOUS PHOSPHOLIPASE A_2 (*N. NAJA* PLUS BEE VENOM)

Platelets ($3 \cdot 10^8$/ml) were activated with various agents for 15 min. At that time phospholipase A_2 was added, and the incubation was continued for another 45 min. Values have been corrected for platelet lysis, which was lower than 8%. Data are the average (mean ± S.D.) of six experiments. For experimental details see Materials and Methods.

Platelet activator	Total phospholipid hydrolysis (% of total lipid phosphorous)
None	2.2 ± 1.4
Thrombin (2 nM)	5.8 + 2.1
Collagen (10 μg/ml)	4.9 ± 3.0
Thrombin + collagen	20.4 ± 2.1
Diamide (5 mM)	19.2 ± 2.7
A23187 (1 μM)	46.8 ± 3.1

linase separately or in combination. In contrast, very little phospholipid hydrolysis occurs when intact, non-activated platelets are treated with phospholipase A_2 alone (Table I). This is also the case when platelets are activated either by thrombin or by collagen; less than 6% of the total phospholipids is hydrolyzed by phospholipase A_2. However, when platelets are activated by a mixture of collagen and thrombin, by the SH-oxydizing compound diamide, or by the calcium ionophore A23187, increased amounts of phospholipid become susceptible towards exogenously added phospholipase A_2 without significant lysis of the cells. The increased accessibility of platelet phospholipids towards phospholipase A_2 indicates that changes in the membrane structure have occurred as a result of these activation procedures. Activation of platelets for 15 min with the agents listed in Table I, does not cause detectable changes in over-all phospholipid composition of the platelets, and platelet release and aggregation occur to a similar extent with all activators except for diamide with which neither release nor aggregation is observed (data not shown). It should be noted that sphingomyelinase treatment of non-activated as well as activated platelets results in all cases in significant degradation of sphingomyelin, without cell lysis. The amount of sphingomyelin degradation is the same whether sphingomyelinase is added alone or in combination with phospholipase A_2 (see below).

In order to obtain more detailed information on the exposure of phospholipids at the outer surface of platelets and to compare the effect of various activating agents on this exposure, a mixture of phospholipase A_2 and sphingomyelinase was used. However, the use of this combination of enzymes increases the risk of platelet lysis, particularly if the platelets are activated prior to addition of the phospholipases. Therefore, careful examination of platelet lysis during treatment with the combination of phospholipase A_2 and sphingomyelinase was essential. With non-activated platelets and platelets activated either by thrombin, collagen or diamide, breakdown of phospholipid levels off at a moment where platelet lysis is still less than 10%, and neither phospholipid breakdown nor cell-lysis increase upon a further 30 min incubation. A different picture was obtained using platelets tri-

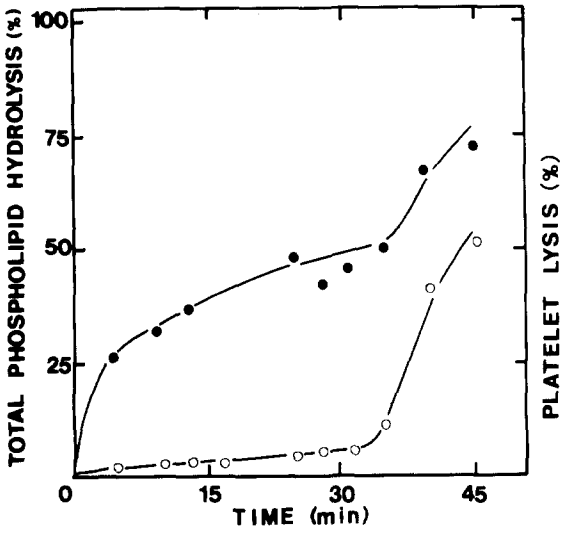

Fig. 2. Total phospholipid hydrolysis and cell lysis during phospholipase A_2 and sphingomyelinase treatment of platelets activated by collagen and thrombin. Platelets (10 ml at a concentration of $3 \cdot 10^8$/ml) were activated for 15 min with collagen (10 μg/ml) and thrombin (2 nM). After the activation period, phospholipase A_2 and sphingomyelinase were added. The incubation was stopped by addition of EDTA. Total phospholipid degradation (●) and platelet lysis (○) were measured. Further details are given in Materials and Methods.

ggered with collagen plus thrombin, or with A23187. Fig. 2 shows the hydrolysis of total phospholipid as a function of time together with the lysis pattern during phospholipase treatment of platelets that have been activated with collagen plus thrombin. Phospholipid hydrolysis does not reach a plateau in this incubation. During the first 30 min of phospholipase treatment, there is a small increase in cell lysis. At this time, phospholipid hydrolysis approximates 50%. Prolonged incubation gives rise to a sudden increase in cell lysis as well as phospholipid degradation, suggesting that extensive destruction of cell structure occurs. A similar course of hydrolysis and lysis was found for phospholipase treatment of platelets activated by A23187.

Besides total phospholipid hydrolysis, we also determined the hydrolysis of each phospholipid class upon platelet incubation with a combination of sphingomyelinase and phospholipase A_2. The data are summarized in Table II. For control platelets and platelets activated by collagen, thrombin, or diamide, the hydrolysis of each phospholipid class leveled off after 30 min; values at $t = 45$ min are presented in this table. The data for platelets activated by collagen plus thrombin or by A23187 were taken at a time of phospholipase treatment just before the sudden rise in platelet lysis (e.g. in Fig. 2 at 35 min). The percentages of lipid degradation are corrected for cell lysis assuming complete hydrolysis of the phospholipids in the lysed cell population. Treatment of control cells with a combination of phospholipase A_2 and

TABLE II

NON-LYTIC DEGRADATION OF PHOSPHOLIPIDS BY PHOSPHOLIPASE A2 (*N. NAJA* AND BEE VENOM) AND SPHINGOMYELINASE (*S. AUREUS*) IN ACTIVATED HUMAN PLATELETS

The values are expressed as percentage of total lipid phosphorus (mean ± S.D.). The numbers in parenthesis refer to the percent degradation of the corresponding phospholipid class. n = number of experiments. PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; total PL, total phospholipid.

	Phospholipid composition ($n = 12$)	Hydrolysis					
		Control ($n = 7$)	Thrombin ($n = 9$)	Collagen ($n = 9$)	Thrombin + collagen ($n = 12$)	Diamide ($n = 7$)	A23187 ($n = 7$)
PS	10.9 ± 0.7	0.5 ± 0.5 (4)	1.6 ± 1.0(15)	2.1 ± 2.0(19)	5.0 ± 0.3(46)	5.3 ± 0.4(49)	8.2 ± 0.7(75)
PC	38.3 ± 1.4	6.5 ± 1.5(17)	12.9 ± 4.6(34)	14.4 ± 4.3(37)	15.1 ± 1.6(39)	15.5 ± 0.5(41)	19.2 ± 1.5(50)
PE	26.8 ± 0.8	2.0 ± 0.9 (7)	5.1 ± 3.1(19)	5.9 ± 3.9(22)	16.0 ± 0.9(60)	10.3 ± 0.1(38)	23.3 ± 0.8(87)
SM	19.0 ± 1.4	12.0 ± 1.5(63)	13.2 ± 3.8(69)	12.4 ± 0.6(65)	8.3 ± 1.5(43)	8.6 ± 0.2(45)	14.3 ± 0.6(75)
Total PL	95.0 [a]	21.0 ± 2.4	32.8 ± 6.8	34.8 ± 6.2	44.5 ± 2.3	39.7 ± 0.7	65.0 ± 1.9

[a] Phosphatidylinositol (PI) is omitted for reasons explained in Results.

sphingomyelinase leads to hydrolysis of 21% of total phospholipid which is in agreement with the findings of Perret et al. [3]. The hydrolyzed phospholipid fraction is mainly composed of the choline containing phospholipids, sphingomyelin and phosphatidylcholine. Activation of platelets by collagen or by thrombin prior to addition of phospholipases results in an increased phospholipid hydrolysis. The amount of sphingomyelin hydrolyzed in these platelets is not different from that in control platelets, but an increased degradation of the glycerophospholipids is observed. However, the small quantities of phosphatidylserine and phosphatidylethanolamine that are hydrolyzed together with the large standard deviations make it difficult to discern whether there is an increased susceptibility of these phospholipids towards phospholipase A_2. Remarkably deviating patterns of phospholipid hydrolysis are found for platelets activated by collagen plus thrombin, diamide, or A23187. Apart from a further increase in the extent of total phospholipid hydrolysis (even to a maximum of 65% for platelets activated by A23187) the most striking feature is the significant increase in the breakdown of phosphatidylserine and phosphatidylethanolamine in these platelets.

A clear picture of the effects of the different platelet stimulators on the exposure of phospholipids at the membrane outer surface is obtained when the compositions of the hydrolyzed phospholipid fractions are compared. These compositions, which are calculated from the data in Table II, are presented in Table III. Compared to control platelets, there is an increase in phosphatidylserine and phosphatidylethanolamine accompanied by a decrease in sphingomyelin in the hydrolyzed phospholipid fraction, when platelets are activated by a mixture of collagen plus thrombin, by diamide or by A23187. It is not clear why the standard deviations for the phospholipid hydrolysis of thrombin or collagen treated platelets are relatively high. Therefore, it remains uncertain whether gross alterations in phospholipid orientation also occur in these platelets.

The effect of the various platelet activators on the ability of platelets to enhance the conversion

TABLE IV

COMPARISON BETWEEN PROTHROMBIN CONVERTING ACTIVITY OF ACTIVATED HUMAN PLATELETS AND EXPOSURE OF PHOSPHATIDYLSERINE

Rate of thrombin formation was measured after a 15 min activation period in a system containing $5.8 \cdot 10^6$ platelets/ml, factor Xa (15 nM), factor Va (30 nM) and prothrombin (4 μM) in the presence of 6 mM $CaCl_2$.

	Rate of thrombin formation (nM IIa/min)	Percentage of PS exposed [a]
No stimulator	34	4
Thrombin (2–20 nM)	41	15 ($p = 0.05$)
Collagen (10 μg/ml)	157 [b]	19 ($p = 0.10$)
Thrombin + collagen	352	46 ($p < 0.001$)
Diamide (5 mM)	356	49 ($p < 0.001$)
A23187 (1 μM)	793	75 ($p < 0.001$)
Lysed platelets	1170	96 ($p < 0.001$)

[a] Differences with respect to non-stimulated platelets were tested by two-sided Student's t-test. p values are given in parenthesis. Consult Table II for corresponding standard deviations.

[b] The increased rate of thrombin formation of collagen-activated platelets could be the result of a combined action of the added collagen and the thrombin that is formed during the assay (cf. Ref. 4).

TABLE III

COMPOSITION OF PHOSPHOLIPID FRACTIONS HYDROLYZED BY PHOSPHOLIPASE A_2 AND SPHINGOMYELINASE TREATMENT OF ACTIVATED HUMAN PLATELETS

Values are expressed as percentage of the hydrolyzed fraction of total phospholipid (mean ± S.D.). Data are calculated from Table II by setting the hydrolyzed total phospholipid fraction at 100%. PI is not taken into account for reasons explained in the results section.

	Control	Thrombin	Collagen	Thrombin + collagen	Diamide	A23187
PS	2.4 ± 2.4	4.9 ± 3.2	6.0 ± 5.8	11.2 ± 0.9	13.4 ± 1.0	12.6 ± 1.1
PC	31.0 ± 8.0	39.3 ± 16.2	41.4 ± 14.4	33.9 ± 3.4	39.0 ± 1.4	29.5 ± 2.5
PE	9.5 ± 4.4	15.5 ± 9.9	17.0 ± 11.6	36.0 ± 2.0	25.8 ± 0.5	35.8 ± 1.6
SM	57.1 ± 9.7	40.2 ± 14.3	35.6 ± 6.6	18.7 ± 3.4	21.7 ± 0.6	22.0 ± 1.1

of prothrombin into thrombin by factor Xa was investigated using a chromogenic substrate assay to measure the rate of thrombin formation. The results are presented in Table IV. It has previously been demonstrated that the rate of thrombin formation is strongly enhanced in the presence of a negatively-charged phospholipid surface, in particular, one containing phosphatidylserine [5–9]. Therefore, the corresponding values for phosphatidylserine hydrolysis by exogenous phospholipases are also presented in this table, since this may represent a measure of phosphatidylserine exposure at the outer surface. It is obvious from this comparison that an increased exposure of phosphatidylserine corresponds to an increased rate of thrombin formation.

Discussion

Platelet activation leads to morphological and metabolic changes. In particular, the enhanced turnover of phosphatidylinositol is one of the primary metabolic events that take place in the membrane during platelet activation [22–27]. However, little evidence is available indicating that the plasma membrane is subjected to structural alterations. Using fluorescent probes, Nathan et al. [28] have found indications for changes in microviscosity of phospholipids in the membrane of human platelets after activation by thrombin. Changes in the distribution of membrane phospholipids have been reported by Schick et al. [29], who found increased labeling of phosphatidylethanolamine by trinitrobenzenesulfonic acid (TNBS) after triggering of platelets with thrombin. We have previously shown [4], that activation of platelets simultaneously by collagen and thrombin results in an increased susceptibility of phospholipids towards exogenously added phospholipase A_2. This was not observed for platelets activated by either one of these activators separately. The increased susceptibility of phospholipids is also apparent following treatment of platelets with diamide or A23187. Differences in availability of phospholipids for phospholipase A_2 in platelets activated by various means may reflect differences in structural organization of the membrane. In this respect, the difference between platelets activated by a combination of collagen and thrombin compared to platelets activated by either of the two is striking, since no differences are observed in the extent of platelet aggregation and release.

The activity of phospholipase A_2 towards platelet membranes is significantly facilitated if sphingomyelin is degraded at the same time by the action of sphingomyelinase [1,3]. When platelet lysis is carefully controlled, this allows the study of the exposure of all individual phospholipid classes at the outer surface of activated platelets in a similar manner as was described for unstimulated platelets by Perret et al. [3]. The amount of phospholipids that can be degraded under non-lytic conditions can be directly influenced by the extent of platelet aggregation and release resulting from the activation procedure. Intact unstimulated platelets contain several types of intracellular membranes such as granule membranes, mitochondrial membranes and membranes of the dense tubular system. As was elegantly demonstrated by Perret et al. [3], 57% of the total platelet phospholipids are present in the plasma membranes of unactivated human platelets. Consequently, degradation of the complete outer leaflet of the plasma membrane would be reflected in 28% hydrolysis of the total phospholipid content of the cell. During the platelet release reaction as a result of platelet activation, granule membranes are thought to fuse with the plasma membrane. This would result in an increased amount of phospholipids that can be hydrolyzed. Assuming that as a result of the release reaction, 60% of the intracellular membranes have fused with the plasma membrane, approx. 80% of the platelet phospholipids would be present in the plasma membrane. Thus, complete hydrolysis of the outer leaflet of the plasma membrane of activated platelets would result in 40% hydrolysis of total phospholipids. On the other hand, platelet aggregation forms a complicating factor since it may prevent efficient action of phospholipases, restricting the total amount of phospholipid hydrolysis.

The following remarks can be made concerning the data for total phospholipid hydrolysis from Table II. Total phospholipid hydrolysis of intact unstimulated platelets amounts to 21% which is in close agreement to the 25% reported by Perret et al. [3]. In the absence of aggregation or release this

implies a very substantial hydrolysis of the outer leaflet phospholipids. Activation of platelets by thrombin or collagen induces release as well as aggregation. The observed hydrolysis of 32.8% and 34.8%, respectively, are higher than observed with control platelets as a result of the release reaction, but lower than the expected 40%, presumably due to platelet aggregation. Activation of platelets with collagen plus thrombin results in release and aggregation to a similar extent as is induced by the activators separately, but the phospholipid hydrolysis is higher (44.5%). In the case of diamide treatment of platelets, there is neither aggregation nor release which would predict a phospholipid hydrolysis of maximally 28%. The observation that almost 40% of the phospholipids are hydrolyzed in these cells under non-lytic conditions indicates that more than half of the plasma membrane phospholipids have been degraded. The greatest amount of hydrolysis is observed with platelets activated by the calcium ionophore A23187; 65% of the total phospholipids are hydrolyzed by phospholipase A_2 and sphingomyelinase under non-lytic conditions. Activation by A23187 leads to substantial release and aggregation, although the size of the aggregates produced by A23187 is much smaller than induced by collagen or thrombin. Since phospholipid degradation in ionophore-treated platelets exceeds the expected 40%, hydrolysis is apparently not restricted to half of the membrane phospholipids. Considering that the data have been corrected for phospholipid degradation in the population of lysed cells and that the plasma membrane is impermeable to phospholipases, the extent of phospholipid degradation observed with platelets that are activated by collagen plus thrombin, diamide or A23187, can be explained if one assumed a transbilayer movement of phospholipids (flip-flop). From the present data, it is difficult to ascertain whether the exposure of phospholipids is significantly changed upon platelet activation by either collagen or thrombin, due to the relatively large standard deviations. Transbilayer movement of phospholipids does apparently not occur in control platelets, which indicates that the action of phospholipases as such, does not necessarily induce flip-flop of phospholipids in these membranes. On the other hand it cannot be excluded that phospholipase treatment induces flip-flop in platelets that have been activated by collagen plus thrombin, by diamide or by ionophore. However, if exposure of phosphatidylserine at the platelet outer surface would be a result of the phospholipase treatment as such, one would expect a delay in the onset of hydrolysis of this phospholipid directly after addition of phospholipase. Since neither phosphatidylserine nor the other phospholipids show any such lag period in hydrolysis, phospholipase induced flip-flop seems unlikely. On the other hand it is very well possible that transbilayer movement of the phospholipids continues during incubation with phospholipase. Therefore it cannot be distinguished whether the hydrolyzed phospholipids are continuously or transiently exposed at the outer surface during the time course of the phospholipase incubation.

As we have shown previously [4], the increased hydrolysis with platelets activated by collagen and thrombin cannot be explained by the action of endogenous phospholipases. Also platelet treatment by A23187 or diamide does not produce significant phospholipid breakdown on a percentage basis.

Transbilayer movement of phospholipids due to the activation of platelets could explain the increased exposure of phosphatidylserine and phosphatidylethanolamine. To balance this process, sphingomyelin seems to move from the outer leaflet to the inner leaflet of the plasma membrane. There is no large change in the orientation of phosphatidylcholine. Particularly after treatment with diamide, the phospholipid composition of the hydrolyzed fraction is very similar to the total phospholipid composition, suggesting a total randomization of the phospholipids over both membrane halves. Transbilayer movement of phospholipids has been reported [15,16] to occur in erythrocytes treated with diamide or tetrathionate. Approx. 50% of the phosphatidylethanolamine and 30% of the phosphatidylserine became accessible to exogenous phospholipase A_2 without hemolysis. The effect was correlated with a significant cross-linking of spectrin which was proposed to play a role in maintaining the asymmetric phospholipid orientation in the erythrocyte membrane. Although treatment of platelets with diamide also results in a significant cross-linking

of membrane proteins as was observed by polyacrylamide gel electrophoresis (data not shown), it is not clear whether in all cases exposure of phosphatidylserine at the platelet outer surface is dependent on a similar mechanism, since cross-linking of membrane proteins was not observed following treatment with collagen plus thrombin or A23187.

The increased exposure of phosphatidylserine at the outer surface of activated platelets may be of considerable importance for the hemostatic process. Unstimulated platelets have little phosphatidylserine exposed at their outer surface. This lack of negatively-charged phospholipids is reflected in a relatively poor capacity of resting platelets to stimulate the formation of thrombin from prothrombin by factor Xa, factor Va and calcium. The rate of thrombin formation is critically dependent on the presence of a negatively-charged phospholipid surface to which factors Xa, Va and the substrate prothrombin can bind [4–13]. Those platelet activators that induce increased exposure of phosphatidylserine at the outer surface of the membrane as measured by exogenously added phospholipases, also induce the platelets to become more active in enhancing the rate of thrombin formation. Remarkable in this respect is the relationship between the amount of phosphatidylserine exposed and the rate of thrombin formation.

The mechanism by which transbilayer movement of phospholipids in the platelet membrane can take place is not well understood. Although phospholipid flip-flop was demonstrated to be an extremely slow process in vesicles of pure phosphatidylcholine [30], rapid flip-flop in artificial and natural membranes has been reported (for a review, see Ref. 31). One possible mechanism for transbilayer movement of phospholipids is the existence or introduction of intrabilayer inverted micelles as described by Cullis and De Kruijff [32,33]. These structures can be formed with those lipids having a conical shape such as diacylglycerols and phosphatidic acid that do not adopt bilayer structures. The formation of these lipids has been suggested to play a triggering role for various cellular responses to exogenous stimuli by altering membrane physical states [32,34]. In this respect, it is tempting to speculate that intermediates of the phosphatidylinositol-cycle, which is activated during platelet stimulation [22–27], produce local bilayer disturbances that enable transbilayer movements of phospholipids. Preliminary experiments have demonstrated that the introduction of diglycerides in the platelet membrane by the action of phospholipase C from *Clostridium welchii* causes an increased exposure of phosphatidylserine at the outer surface of the plasma membrane. Whether or not differences in the activation of the phosphatidylinositol-cycle exist as a result of different platelet activation procedures is under current investigation.

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