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Loss of phospholipid asymmetry in dilauroylphosphatidylcholine induced plasma membrane vesicles from human platelets

Edouard M. Bevers, Peter F.J. Verhallen, Willie M.A. Linskens, Paul Comfurius and Robert F.A. Zwaal

Department of Biochemistry, University of Limburg, Maastricht (The Netherlands)

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Incubation of human platelets with unilamellar vesicles composed of dilauroylphosphatidylcholine (DLPC) induces shedding of small vesicular structures from the platelet plasma membrane. No significant cell lysis is observed during the process of shedding. Isolated spicules contain the major membrane glycoproteins, Ib, IIb, and IIIa, which are used to define the sidedness of the spicule membrane. These glycoproteins are completely susceptible to chymotrypsin treatment, whereas cytoskeletal proteins are inaccessible towards this enzyme. This demonstrates that the spicule membranes have a right-side-out orientation in as far as membrane proteins are concerned. Isolated spicules were 30-fold more active than platelets in stimulating prothrombin conversion to thrombin by the prothrombinase complex (factors Xa, Va and Ca^{2+}). The increased prothrombinase activity reflects an increased amount of phosphatidylserine in the outer leaflet of the spicule membrane. Protein analysis of platelet spicules and native platelets reveals a number of differences, the most conspicuous of which is the virtual absence of myosin in the spicule preparations. It is proposed that a lack of myosin produces a different cytoskeletal organization in the spicules. This enables phosphatidylserine to become exposed at the outer surface of the spicule membrane.

Introduction

As reviewed by Op den Kamp [1], asymmetric distribution of phospholipids is a common feature of most, if not all, biological membranes. In the plasma membrane of platelets the negatively charged phospholipids, phosphatidylserine and phosphatidylinositol, are mainly confined to the cytoplasmic leaflet, whereas sphingomyelin is predominantly located in the outer half of the membrane. In contrast to the rather extreme distribution of these phospholipids, phosphatidylcholine and phosphatidylethanolamine are present on either side of the membrane, though not to the same extent [2–5].

Upon activation of platelets, in particular by collagen plus thrombin or by the non-physiological Ca^{2+}-ionophore A23187, the non-random distribution of phospholipids is partially lost, which results in an increased exposure of phosphatidylserine at the outer surface of the platelet [6,7]. The increased exposure of phosphatidylserine is reflected by an increased capability of platelets to enhance two sequential reactions of the coagulation cascade i.e. the activation of factor X by a complex of factors IXa and VIIIa and the activation of prothrombin by a complex of factors Xa

Correspondence: E.M. Bevers, Department of Biochemistry, Biomedical Centre, University of Limburg, P.O. Box 616, 6200 MD Maastricht, The Netherlands.

and Va [6–8]. The rate of both coagulation reactions is critically dependent on the presence of a negatively charged phospholipid surface. A close correlation was found between platelet procoagulant activity and exposure of phosphatidylserine at the outer surface [7].

Exposure of phosphatidylserine in the outer leaflet of the plasma membrane presumably results from an increased transbilayer movement of phospholipids (flip-flop) [6,7]. Recent data demonstrated that an increased exposure of phosphatidylserine strictly coincides with an increased breakdown of several cytoskeletal proteins by the action of calpain, an endogenous Ca^{2+}-dependent protease in platelets [9]. From this it was suggested that, in analogy to the erythrocyte membrane [10–16], interactions between membrane phospholipids and underlying cytoskeletal proteins play an important role in regulation of membrane phospholipid asymmetry.

Recently, Kobayashi and co-workers [17] found that spicules, derived from rabbit platelets upon incubation with dilauroylphosphatidylcholine (DLPC) vesicles, were essentially devoid of a 200 kDa protein, representing the heavy chain of myosin, one of the major cytoskeletal proteins in platelets. The aim of the present study was to investigate whether similar spicules could also be derived from human platelets and to see if and to what extent an alteration in composition of cytoskeletal proteins is accompanied by a change in phospholipid distribution in the membranes of these spicules. The ability of the spicules to enhance the activation of prothrombin by a complex of factors Xa and Va (prothrombinase activity) was used to monitor the exposure of phosphatidylserine in the outer leaflet of the spicule membrane.

Materials and Methods

Dilauroylglycerophosphocholine, α-chymotrypsin, fatty acid-free human serum albumin, peroxidase-conjugated concanavalin A and peroxidase-conjugated wheat germ agglutinin were purchased from Sigma Chemical Co. (St. Louis). The prostacyclin analogue ZK 36374 was a kind gift of Schering (Berlin). H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride (S2238), a chromogenic substrate specific for thrombin was from AB Kabi Diagnostica (Sweden). All other reagents used were of the highest grade commercially available.

Isolation of platelets. Washed human platelets were obtained from freshly drawn blood by differential centrifugation as described before [7]. Platelets were finally resuspended in a Hepes buffer composed of 136 mM NaCl, 2.7 mM KCl, 2.0 mM $MgCl_2$, 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 100 nM [ZK] 36374, 5 mM glucose (pH 7.5).

Preparation of DLPC vesicles. DLPC was dried from a solution in chloroform/methanol (1 : 2, v/v) by a stream of nitrogen. Hepes buffer (pH 7.5) was added and after sonication for 15 min, large particles were removed by centrifugation at $50\,000 \times g$ for 30 min. The phospholipid concentration was determined according to Böttger et al. [18].

Incubation of platelets with phospholipid vesicles. One volume of DLPC vesicles was added to nine volumes of a suspension containing $5 \cdot 10^8$ platelets/ml. The final concentration DLPC was 100 μM. Incubation was carried out at 22° C without stirring. To determine the amount of platelet lysis, lactate dehydrogenase activity present in a $30\,000 \times g$ supernatant was measured according to Wroblewski and La Due [19].

Isolation and characterization of platelet spicules. Platelet spicules were isolated by centrifugation of the total platelet/vesicle incubation at $800 \times g$ for 10 min. The resuspended $800 \times g$ pellet is further referred to as remnant platelets. The supernatant, which contains spicules, DLPC vesicles and some residual platelets, was centrifuged at $30\,000 \times g$ for 30 min to separate spicules from DLPC vesicles. The pellet containing the spicules was resuspended in 1 ml Hepes buffer and layered on top of 15 ml 30% Percoll prepared in the same buffer. After another 30 min centrifugation at $30\,000 \times g$ a single band of pure platelet spicules was isolated. Percoll was removed afterwards by three additional washings in Hepes buffer. Prothrombinase activity was measured as described below. Phospholipid analysis was carried out after extraction according to Bligh and Dyer [20] and two-dimensional thin-layer chromatography as described previously [21].

Fatty acid composition of total platelet phospholipids was analyzed by gas-liquid chromatography. From the ratio lauric acid over total fatty acid, the amount of exogenously incorporated DLPC could be calculated. Polyacrylamide gel electrophoreses was performed in the Laemmli system [22] using 7.5% polyacrylamide gels with a 4% stacking gel. Samples for electrophoresis were dissolved in 2% SDS (w/v), 5% β-mercaptoethanol (v/v), 50 mM Tris-HCl and 1 mM EDTA (pH 8). Protein determinations in the electrophoresis samples were done after 100-fold dilution using the method of Lowry et al. [23]. Gels were stained with Coomassie blue for total protein. Glycoproteins were visualised on nitrocellulose blots according to Clegg [24] using peroxidase-conjugated lectins (wheat germ agglutinin or concanavalin A).

Assay of prothrombinase activity. Purified coagulation factors Xa, Va and prothrombin were prepared as described elsewhere [8,25]. Prothrombinase activity of either the total incubation mixture of platelets and phospholipid vesicles or the isolated platelet spicules was measured as follows: 312 μl platelet or spicule suspension was incubated for 3 min in the presence of 13 μl $CaCl_2$ (final concn. 3 mM). Subsequently 50 μl of a mixture of factors Xa and Va (30 and 60 nM, respectively) was added, two minutes later followed by addition of 125 μl prothrombin (16 μM in a buffer containing 50 mM Tris, 175 mM NaCl, 14 mM $CaCl_2$ and 0.5 mg/ml human serum albumin (pH 7.9)). After one min prothrombin activation, a 25 μl sample is transferred into a cuvette containing 1 ml 50 mM Tris, 20 mM EDTA, 175 mM NaCl (pH 7.9) to stop the reaction. The amount of thrombin formed was calculated from the change in absorbance at 405 nm after addition of chromogenic substrate S2238 to a final concentration of 150μM, using a calibration curve made with active site titrated thrombin.

Electron microscopy. Platelet suspensions were fixed with glutaraldehyde (2.5% in phosphate buffer 0.1 M at pH 7.3). The samples were washed and postfixed in osmium tetroxide (1% in the same phosphate buffer). Dehydration was carried out in a graded series of ethanol after which the preparations were embedded in Epon 812 [26]. Ultrathin sections with stained with a saturated solution of uranylacetate and lead citrate (0.25% w/v) adjusted to pH 12 using NaOH. Micrographs were taken with a Philips CM12 microscope. Isolated spicules were negatively stained with 2% uranylacetate on carbon-coated formvar-films.

Results

The prothrombinase activity of a platelet suspension, rapidly increases upon addition of DLPC vesicles and reaches a maximal value after approximately 45 min incubation (Fig. 1). The increased prothrombinase activity is not the result of exposure of the cytoplasmic side of the membrane due to cell rupture, since less than 3% of the cells was lysed after 60 min incubation. Addition of 100 nM ZK36374, a stable carbacyclin derivative of prostacyclin, which is a powerful inhibitor of platelet aggregation and release [27–29], did not prevent expression of procoagulant activity. Significant morphological changes accompany the process in which procoagulant activity develops (Fig. 2). Although it cannot be excluded that the process of spicule formation is not immediately arrested by addition of glutaraldehyde, the appearance of small vesicular structures, pinched off from the platelets, can already be observed one minute after addition of DLPC. Apart from the

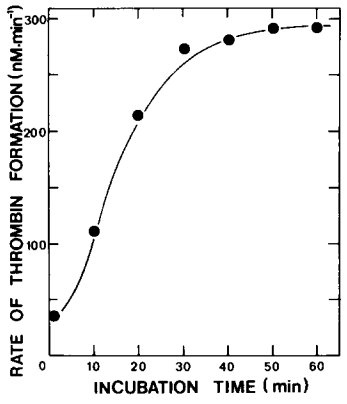

Fig. 1. Time-dependent prothrombinase activity upon incubation of washed human platelets with DLPC vesicles. Samples were taken from the incubation mixture at different time intervals and diluted with Hepes buffer (pH 7.5) for measuring prothrombinase activity. Platelet concentration in the prothrombinase assay was $1.2 \cdot 10^7$ ml^{-1}. Prothrombinase activity is expressed as the rate of thrombin formation (nM per min).

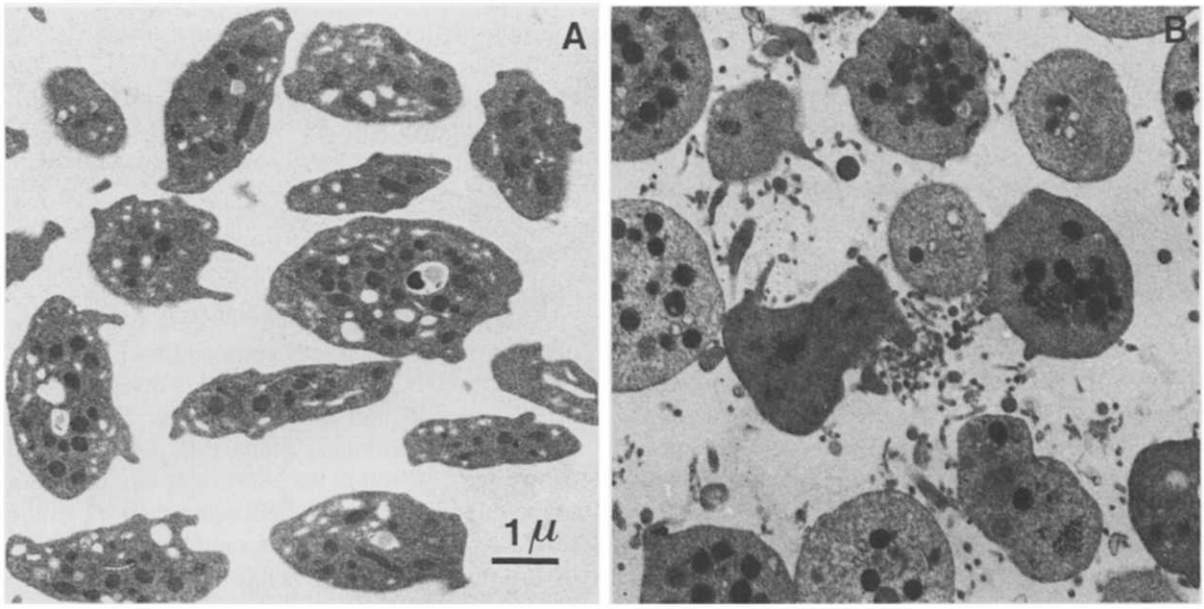

Fig. 2. Electron micrographs of control platelets (A) and platelets incubated with DLPC vesicles for 1 min (B).

formation of more spicules, no further morphological changes are observed upon prolonged incubation up to 60 min. Isolation of these platelet spicules on Percoll, followed by negative staining, showed under the electron microscope spherical structure with diameters ranging from 100 to 150 nm. Prothrombinase activities of isolated platelet spicules, control platelets and remnant cells are presented in Table I. The prothrombinase activity of a cell or vesicle preparation depends on the concentration as well as the composition of the phospholipid. To eliminate the effect of different phospholipid concentrations, prothrombinase activities were expressed per μmol phospholipid. Isolated spicules exhibit a prothrombinase activity of 218 nmol thrombin/min per μmol phospholipid, which is a 30-fold increase with respect to the activity of intact native platelets. Also remnant platelets have an increased prothrombinase activity (52 nmol thrombin/min per μmol phospholipid), though considerably less than observed for the platelet spicules. Maximal prothrombinase activities of platelets and platelet spicules can be obtained after complete cell disruption by sonication. As shown in Table I the prothrombinase values for the various sonicated preparations do not differ significantly. This is consistent with the observation that the phospholipid composition of platelets, platelet spicules and remnant cells are similar (Table II). Although the amount of phosphatidylcholine is increased in the spicules and remnant platelets due to the incorporation of exogenous DLPC (17.3 and 14.9% in the spicules and remnant platelets, respectively), nos significant changes in the composition of the other phospholipids is seen. It is therefore concluded that the elevated prothrombinase activity of the platelet spicules is not the result of a non-random shedding of membrane phospholipids upon spicule

TABLE I

PROTHROMBINASE ACTIVITIES OF CONTROL PLATELETS, PLATELET SPICULES AND REMNANT PLATELETS AFTER INCUBATION WITH DLPC VESICLES

Activities are expressed per μmol phospholipid. Values in parenthesis are obtained after complete lysis by extensive sonication of all preparations.

	Prothrombinase activity (nM thrombin/min per μmol phospholipid)
Control platelets	7.5 (1250)
Platelet spicules	218.4 (1442)
Remnant platelets	52.0 (1012)

TABLE II

PHOSPHOLIPID COMPOSITION OF CONTROL PLATELETS, PLATELET SPICULES AND REMNANT PLATELETS AFTER TREATMENT WITH DLPC VESICLES

Data are expressed as mol% of total phospholipid fraction (mean values ± S.D. from three experiments). The numbers in parenthesis represent mol% of total phospholipids corrected for incorporated DLPC, which amounts 17.3 and 14.9% in the spicules and remnant platelets, respectively.

Phospholipid class	Control platelets	Platelet spicules	Remnant platelets
Phosphatidyl-serine	10.8 ± 0.5	8.5 ± 0.5 (10.3)	9.2 ± 1.1 (10.9)
Phosphatidyl-inositol	3.1 ± 1.3	1.7 ± 0.4 (2.1)	3.1 ± 1.8 (3.7)
Phosphatidyl-ethanolamine	26.8 ± 1.8	19.7 ± 0.9 (23.8)	21.3 ± 1.0 (25.1)
Phosphatidyl-choline	39.8 ± 1.6	52.1 ± 0.9 (42.1)	48.2 ± 2.0 (39.3)
Sphingo-myelin	19.3 ± 0.5	18.0 ± 0.8 (21.8)	17.8 ± 0.6 (21.0)

formation. Phospholipases, either alone or in combination with transfer protein, have been used successfully to localize phospholipids in the membrane of various cells [1,10]. An attempt to apply this technique on isolated spicules resulted in virtually complete hydrolysis of all phospholipids and extensive lysis of the spicules as judged from the liberation of lactate dehydrogenase (data not shown). Presumably, the small size of the platelet spicules does not allow the use of these techniques, as was also found for spicules derived from sickle cells [10]. Therefore, it remains uncertain whether or not other phospholipids also undergo a change in distribution over the membrane.

Protein analysis of isolated spicules and control platelets reveals a number of differences as illustrated in Fig. 3. The most conspicuous difference is the virtual absence of a band at 200 kDa in the platelet spicules, corresponding to the heavy chain of myosin. Furthermore, two increased bands at 125 kDa and 98 kDa are observed in the spicules. These bands likely represent increased amounts of glycoproteins IIb and IIIa (see also Fig. 4). Finally, the spicules show a distinctly increased band at 76 kDa and lack of a band at 59 kDa. Both protein bands have not been identified. Similar protein patterns were obtained by

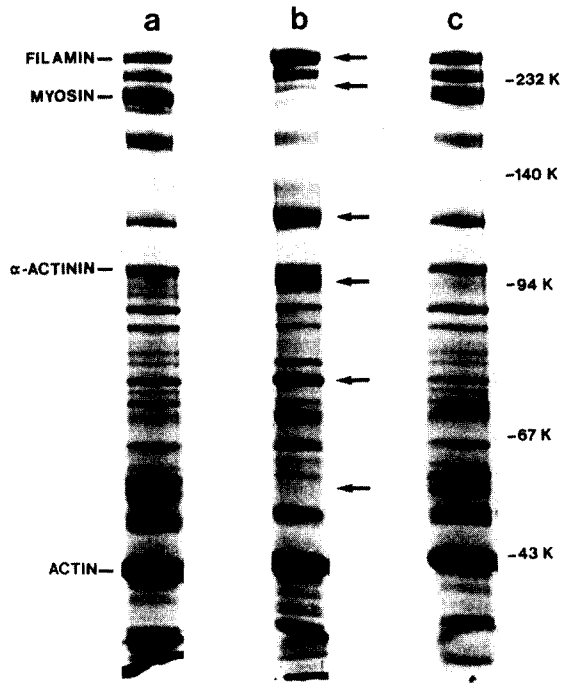

Fig. 3. SDS-polyacrylamide gel electrophoresis of control platelets (lane a), isolated DLPC-induced platelet spicules (lane b) and remnant platelets after removal of platelet spicules and DLPC-vesicles (lane c). Gels were stained with Coomassie brilliant blue. Cytoskeletal proteins are indicated on the left and molecular weight standards on the right. The arrows indicate major differences in protein patterns. Spicules were isolated from a 45 min incubation of platelets ($5 \cdot 10^8$ platelets/ml) with DLPC vesicles (100 μM) as described in Materials and Methods.

Kobayashi et al. [17] for spicules isolated from rabbit platelets upon treatment with DLPC.

Advantage was taken of the presence of glycoproteins in the spicules to define the sidedness of the spicule membranes. Glycoproteins were detected on nitrocellulose blots stained with peroxidase-conjugated concanavalin A or wheat germ agglutinin before and after treatment of the spicules with α-chymotrypsin. As a control, intact platelets were treated and analysed in the same manner. Fig. 4A shows the concanavalin A-stained blots of platelets and spicules before and after treatment with chymotrypsin. Complete loss of the concanavalin A-binding sites of glycoproteins IIb and IIIa was found after chymotrypsin treatment of both platelets and platelet spicules. A similar finding was obtained upon wheat germ

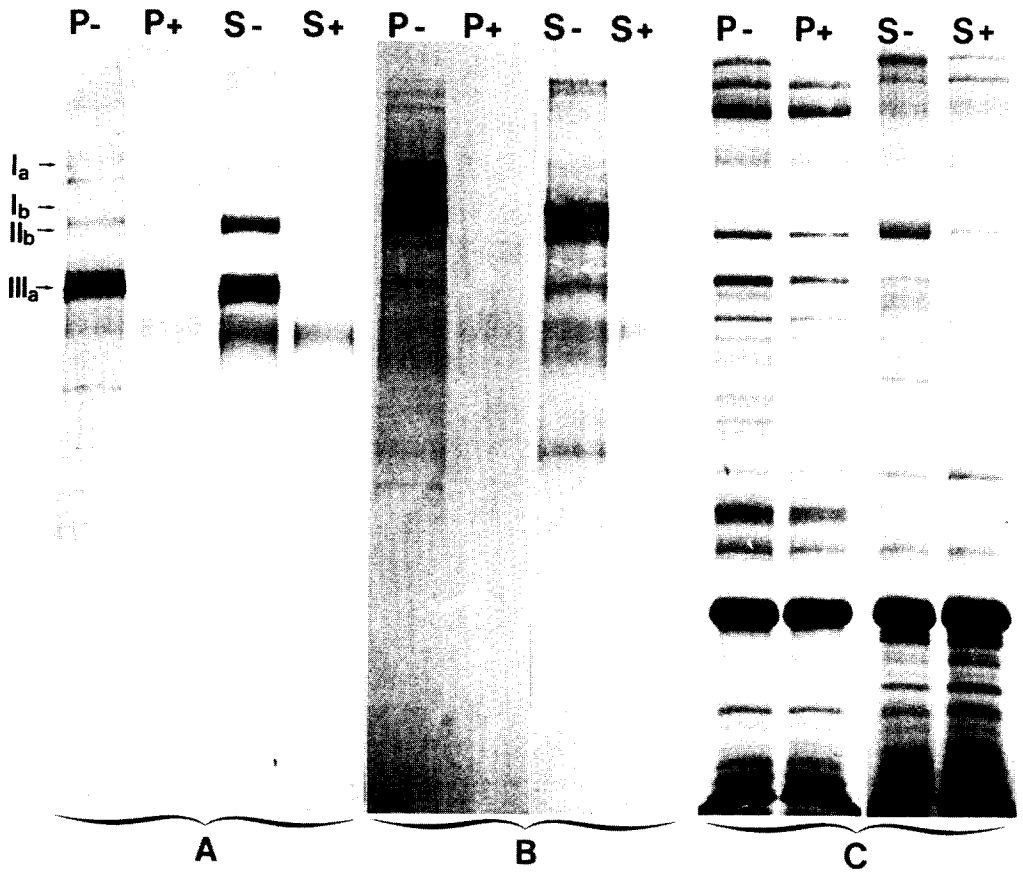

Fig. 4. SDS-polyacrylamide gel electrophoresis of control platelets (P) and isolated DLPC-induced platelet spicules (S) before (−) and after (+) treatment with chymotrypsin. (A) Nitrocellulose blot stained with peroxidase-conjugated concanavalin A. (B) Nitrocellulose blot stained with peroxidase-conjugated wheat germ agglutinin. (C) Gel stained with Coomassie brilliant blue. Major glycoproteins (GP) are indicated in the figure: GPIa at 150 kDa, GPIb at 132 kDa, GPIIb at 125 kDa and GPIIIa at 98 kDa.

agglutinin-staining (Fig. 4B), showing complete removal of the wheat germ agglutinin-binding sites of glycoproteins Ib and Ia. Moreover, more than 97% of the sialic acid residues in the spicule preparation are removed upon chymotrypsin treatment. These findings demonstrate that, in comparison with the plasma membrane of control platelets, the spicule membranes are right-side out with respect to the orientation of the major glycoproteins. In addition, Coomassie-stained gels of untreated and chymotrypsin-treated platelets and spicules (Fig. 4C) reveal no detectable hydrolysis of the cytoskeletal proteins talin (235 kDa), myosin (200 kDa), α-actinin (105 kDa) and actin (43 kDa), indicating that these proteins are indeed localized in the interior of the spicules. Moreover, this finding indicates that the spicule membrane and platelet membrane maintain their integrity upon chymotrypsin treatment as also indicated by the fact that no lactate dehydrogenase was liberated from the spicules during the incubation. The small, but apparent degradation of filamin (250 kDa) in both intact platelets and spicules upon chymotrypsin treatment is rather unexpected and difficult to reconcile with the notion that filamin is an actin-binding protein of the cytoskeletal network. It is possible, however, that an unknown ectoprotein migrates at the same position in the gel as filamin.

Additional information on the protein composition of the spicules comes from comparison of the concanavalin A- and wheat germ agglutinin-

stained protein patterns of the untreated samples in Figs. 4A and 4B. Platelet spicules seem to be enriched in glycoproteins IIb and IIIa, although this could partially be a reflection of the increased ratio of membrane proteins over total protein, due to the small size of the spicules. Furthermore, from the patterns stained with wheat germ agglutinin it appears that the spicules lack a glycoprotein at approximately 160 kDa, which is possibly identical to glycoprotein Ia. Preliminary studies using two-dimensional gel electrophoresis have confirmed the lack of glycoprotein Ia and in addition showed that also glycoprotein IIa is virtually absent in the spicules (Clemetson, K.J., personal communication).

Discussion

In the present paper we demonstrate that the prothrombinase activity of a suspension of human platelets rapidly increases upon incubation with DLPC vesicles. The increased prothrombinase activity predominantly resides on the surface of small vesicular structures which can be separated from remnant platelets and DLPC vesicles on a percoll gradient. Since the ability of a membrane surface to enhance factor Xa and Va catalyzed activation of prothrombin is determined by the presence of negatively charged phospholipids, in particular phosphatidylserine [7,8], it is concluded that spicule formation is accompanied by an increased exposure of phosphatidylserine to the external medium containing the coagulation factors. The increased exposure of phosphatidylserine on the spicule surface is not due to an inversion process of the platelet plasma membranes, which would lead to exposure of phosphatidylserine present in the cytoplasmic leaflet of the plasma membrane. This is based on the following observations: (i) no appreciable lysis accompanies the process of spicule formation and (ii) spicule membranes are right-side out, as judged from the orientation of the membrane (sialo)-glycoproteins and the localization of cytoskeletal proteins within the spicules. Furthermore, the increased exposure of phosphatidylserine is not the result of a selective enrichement of this phospholipid, since, apart from the amount of incorporated DLPC, no significant change in composition of the other lipids could be observed in the isolated spicules. The small increase in phosphatidylserine content of the isolated spicules in comparison to control platelets, as reported by Kobayashi et al. [17], might be due to a different isolation procedure of the spicules or merely reflects a species difference (rabbit vs. human platelets). Finally, the increased exposure of phosphatidylserine is not caused by a platelet release reaction, because this is effectively inhibited by the presence of a stable prostacyclin analogue. It is therefore concluded that the shedding process, induced by DLPC, causes a spontaneous loss of the asymmetric distribution of phosphatidylserine. It is presently unknown whether the increased exposure of phosphatidylserine occurs during the actual shedding process or is the result of a redistribution of this lipid in the spicule membranes, after their detachment from the main cell body. It might be speculated, however, that the exfoliation of the plasma membrane as such, which preceeds the formation of membrane vesicles, is sufficient to cause at least some loss of phosphatidylserine asymmetry, since the remnant cells also exhibit an increased prothrombinase activity.

The rate at which the prothrombinase activity of platelets increases upon incubation with DLPC, suggests a fast redistribution of phosphatidylserine in the platelet or spicule membrane. A rapid loss of phosphatidylserine asymmetry is likely to require both enhancement of transbilayer movement and a loss of stabilizing forces, as for instance provided by cytoskeletal interactions. An increased transbilayer movement could be facilitated by the relatively large amount of incorporated DLPC (approx. 20%) in the membranes. The presence of these short fatty acyl chain phospholipids might be responsible for disturbances of the bilayer structure, with the formation of phospholipid reorientation sites.

Extensive studies on erythrocytes have led to the concept that the asymmetric distribution of phosphatidylserine in the red cell membrane is maintained by two independent mechanisms, i.e. (i) its ATP dependent translocation towards the inner monolayer [30–32] and (ii) its interaction with the membrane skeleton, in particular with spectrin [10–12,15]. Recently, the existence of both mechanisms has been demonstrated in a single

study by Middelkoop and co-workers (unpublished data).

The absence of myosin in the platelet spicules suggests an alteration of the molecular architecture of the cytoskeleton. In analogy to the model which explains the asymmetric distribution of phosphatidylserine in the red cell membrane, we propose that the different cytoskeletal organization in the platelet spicules is responsible for a loss of interaction between phosphatidylserine and cytoskeleton. As a result, phosphatidylserine (partially) randomizes over both membrane leaflets. The involvement of a membrane-cytoskeleton interaction in regulating phospholipid asymmetry in the platelet membrane is supported by a previous study in which it was demonstrated that upon stimulation with various agonists, the extent of phosphatidylserine exposure at the outer surface of the platelet, strictely correlates with the extent of cytoskeletal protein degradation (including myosin) by the endogenous Ca^{2+}-dependent protease [9]. In this respect it is of interest to note that two different myosin isoenzymes have been demonstrated in platelets [33]: a soluble form which represents cytosolic myosin and a slightly different form, which comprises about 25% of the total myosin and appears to be strongly bound to the membrane since it could not be removed by repeated washings.

A asymmetric distribution of phospholipids over the membrane bilayer is likely to involve an energy requiring process. It may be speculated that he myosin-ATPase activity in platelets could play an active role, either via a direct interaction with amino phospholipids or through maintenance of a particular cytoskeletal conformation, which would be required for an interaction with specific phospholipids.

Experiments determining the effect of energy depletion on the localization of phosphatidylserine in the platelet membrane are now in progress in our laboratory.

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References

1 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71
2 Zwaal, R.F.A. (1978) Biochim. Biophys. Acta 515, 163–205
3 Chap, H., Zwaal, R.F.A. and Van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 467, 146–64
4 Perret, B., Chap, H. and Douste-Blazy, L. (1979) Biochim. Biophys. Acta 556, 333–344
5 Schick, P.K. (1979) Sem. Haematol. 16, 221–233
6 Bevers, E.M., Comfurius, P., Van Rijn, J.L.M.L., Hemker, H.C. and Zwaal, R.F.A. (1982) Eur. J. Biochem. 122, 429–436
7 Bevers, E.M., Comfurius, P., and Zwaal, R.F.A. (1983) Biochim. Biophys. Acta 736, 57–60
8 Rosing, J., Van Rijn, J.L.M.L, Bevers, E.M., Van Deijen, G., Comfurius, P. and Zwaal, R.F.A. (1985) Blood 65, 319–322
9 Comfurius, P., Bevers, E.M. and Zwaal, R.F.A. (1985) Biochim. Biophys. Acta 815, 143–148
10 Franck, P.F.H., Bevers, E.M., Lubin, B.H., Comfurius, P., Chiu, D.T.-Y., Op den Kamp, J.A.F., Zwaal, R.F.A., Van Deenen, L.L.M. and Roelofsen, B. (1985) J. Clin. Invest. 75, 183–190
11 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32
12 Haest, C.W.H. (1982) Biochim. Biophys. Acta 694, 331–352
13 Franck, P.F.H., Roelofsen, B. and Op den Kamp, J.A.F. (1982) Biochim. Biophys. Acta 687, 105–108
14 Franck, P.F.H., Chiu, D.T.-Y., Op den Kamp, J.A.F., Lubin, B., Van Deenen, L.L.M. and Roelofsen, B. (1983) J. Biol. Chem. 258, 8435–8442
15 Williamson, P., Bateman, J., Kozarsky, K., Mattocks, K., Hermanowicz, N., Choe, H.R. and Schlegel, R.A. (1982) Cell 30, 725–733
16 Mohandas, N., Rossi, M., Bernstein, S., Ballas, S., Ravindranath, Y., Wyatt, J. and Mentzer, W. (1985) J. Biol. Chem. 260, 14264–14268
17 Kobayashi, T., Okamoto, H., Yamada, J.-I., Setaka, M. and Kwan, T. (1984) Biochim. Biophys. Acta 778, 210–218
18 Böttger, C.J.B., Van Gent, C.M. and Pries, C. (1961) Anal. Chim. Acta 24, 203–208
19 Wroblewski, R. and La Due, J.S. (1955) Proc. Soc. Exp. Biol. Med. 90, 210–215
20 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917
21 Verkleij, A.J., Zwaal, R.F.A., Roelofsen, B., Comfurius, P., Kastelij, D. and Van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 233, 178–193
22 Laemmli, U.K. (1970) Nature 227, 680–685
23 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–268
24 Clegg, J.C.S. (1982) Anal. Biochem. 127, 389–394

25 Rosing, J., Tans, G., Govers-Riemslag, J.W.P., Zwaal, R.F.A. and Hemker, H.C. (1980) J. Biol. Chem. 255, 274
26 Luft, J. (1961) J. Biophys. Biochem. Cytol. 9, 409–412
27 Schrör, K., Darius, H., Matzky, R. and Ohlendorf, R. (1981) Arch. Pharmacol. 316, 252–256
28 Baruch, D., Hemker, H.C. and Lindhout, T. (1986) Eur. J. Biochem. 154, 213–218
29 Soons, H., Janssen-Claessen, T., Hemker, H.C. and Tans, G. (1986) Blood 68, 140–148
30 Zachowski, A., Favre, E., Cribier, S., Hervé, P. and Devaux, P.F. (1986) Biochem. 25, 2585–2590
31 Tilley, L., Cribier, S., Roelofsen, B., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1986) FEBS Lett. 194, 21–27
32 Daleke, D.L. and Huestis, W.H. (1985) Biochemistry 24, 5406–5416
33 Peleg, I., Kahane, I., Eldor, A., Groschel-Stewart, U., Mestan, J. and Muhlrad, A. (1983) J. Biol. Chem. 258, 9290–9295