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Contribution of the Platelet Factor V Content to Platelet Factor 3 Activity

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SUMMARY. The procoagulant activity obtained from bovine thrombocytes has been compared to that of lipids isolated from platelets, with and without the addition of purified bovine factor V. A one-stage assay, which consisted of delipidated bovine plasma containing RVV-activated factor X, was used to assess the activity. At low lipid concentrations no difference in coagulant activity was found between sonicated vesicles of extracted platelet lipid and lysed platelets. At higher lipid concentrations, however, the extracted lipids were found to be less active than lysed platelets. Determination of factor V in suspensions of gel-filtered platelets demonstrated that suspensions containing 2×10^9 platelets per ml possessed about 1% of the factor V activity present in a normal bovine plasma pool. Platelet lysis by sonication produced a five-fold increase in factor V activity. Addition of factor V to sonicated vesicles of extracted platelet lipid, so as to produce an identical factor V activity per amount of lipid as found in lysed platelets, decreased the clotting time only in the higher lipid concentration range. A further three-fold increase in the amount of factor V added to the lipid vesicles made the coagulant properties of the lipid vesicles indistinguishable from those of lysed platelets over the whole range of phospholipid concentrations tested. When the conditions of the test were changed by diminishing the concentration of factor Xa in the substrate plasma, the difference between lysed platelets and extracted platelet lipid disappeared completely. It is concluded that the higher coagulant activity of lysed platelets, as compared to that of extracted platelet lipid, can be ascribed to platelet factor V activity. Therefore there is no compelling necessity to postulate the existence of a specific procoagulant factor in the platelet other than factor V or phospholipids.

Whether phospholipids and known clotting factors can account for the procoagulant activity of blood platelets or that this activity arises from other platelet proteins as well, is a long-standing matter of debate. In 1966, it was reported that phospholipids extracted both from human platelets and isolated platelet plasma membranes showed lower coagulant activities than the membranes themselves (Marcus *et al*, 1966). This finding led the authors to conclude that platelet phospholipid alone had less clot-promoting activity than the lipoprotein complexes in

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platelet membranes. It was also suggested that platelet factor 3, the coagulant activity which becomes available during the platelet activation process, represented the activity of platelet membrane lipoprotein rather than the clot-promoting activity of membrane phospholipids *per se* (Marcus, 1969, 1978). Also, phosphatidylserine was found to be more active than other phospholipids in providing intrinsic factor Xa-forming activity but this was not interpreted as evidence that this phospholipid plays a specific role (Walsh & Biggs, 1972; Walsh, 1978).

It has been known for several years that human platelets, separated from plasma by high-speed centrifugation (Hjort *et al,* 1955) or albumin density gradient centrifugation (Walsh, 1972), possess factor V activity, presumably representing plasma factor V bound to the platelet. Recent studies with gel-filtered platelets have presented evidence that factor V is localized within the intact normal human platelet rather than being adsorbed to its surface (Breederveld *et al,* 1975; Østerud *et al,* 1977). Furthermore, it has been proposed that during the platelet activation process activated (platelet) factor V becomes available at the platelet surface (Østerud *et al,* 1977). This factor V activity seems to be related to the factor Xa binding site which is exposed after platelet activation with small amounts of thrombin (Miletich *et al,* 1977, 1978a, b).

Platelet factor Va is evidently a first candidate among the platelet factors that could explain the excess procoagulant activity reported by Marcus *et al* (1966). The purpose of the present study was to investigate whether or not factors, other than factor V, need be postulated. In an earlier paper (Tans *et al,* 1979) we reported studies on the clotting activity of various synthetic mixtures of phosphatidylserine and phosphatidylcholine using a one-stage prothrombinase assay with different phospholipid concentrations. In the present study this assay has been used to compare the coagulant activities of lipids isolated from bovine platelets with that of lysed platelets. The results indicate that the higher coagulant activity of lysed platelets need not be ascribed to anything other than platelet factor V activity.

MATERIALS AND METHODS

Preparation of Platelet Suspensions

800 ml of bovine blood was collected in 176 ml of a solution which was 0·183 M in glucose, 0·079 M in trisodium citrate and 0·051 M in citric acid. The blood was then centrifuged at 250 ***g*** for 25 min at room temperature and the platelet rich plasma (PRP) was collected. It was respun at 250 ***g*** for 20 min in order to remove contaminating erythrocytes.

To separate the platelets from the plasma about 350 ml of PRP were applied to a column of 1500 ml of Sepharose 2B (Tangen & Berman, 1973). Elution of the column was carried out with a 0·05 M Tris buffer, pH 7·4, which contained 0·12 M NaCl. Fractions containing platelets as judged by their turbidity were combined and counted by phase contrast microscopy. This always showed 0·5–1·0% of contaminating erythrocytes. The gel-filtered platelets (GFP) were then pelleted by centrifugation at 1400 ***g*** for 30 min and resuspended in the eluant buffer. The resulting platelet suspensions (2×10^9 platelets/ml) were kept at room temperature and used immediately in coagulation assays. Platelet suspensions to be lysed later by sonication were stored at −70°C. Siliconized glassware or plastic was used throughout the platelet isolation procedure.

Lipid Extracts and Lipid Analyses

Quantitative lipid extracts from known numbers of bovine platelets were made by methanol–chloroform extraction (Reed *et al*, 1960). The extracts were taken to dryness under reduced pressure and the residues were weighed and dissolved in known volumes of carbon tetrachloride–methanol (66:34 v/v). The lipid solutions were analysed for cholesterol (Webster, 1962) and phospholipid phosphorus (Böttcher *et al*, 1961). The phospholipid composition was obtained from two-dimensional thin-layer chromatography (Broekhuyse, 1969) and lipid phosphorus determination (Böttcher *et al*, 1961). The lipid solutions were stored under nitrogen at −20°C until use. Synthetic phospholipids were prepared as described recently (Tans *et al*, 1979). Phosphatidylinositol from plant was obtained from Applied Science Laboratories Inc. Cholesterol was purchased from J. T. Baker.

Coagulation Assays

The procoagulant activities of bovine platelet suspensions and lipids extracted from the platelets were measured in a one-stage prothrombinase assay as described in detail earlier (Tans *et al*, 1979). 0·1 ml delipidated bovine plasma (diluted five-fold with 0·05 M Tris, pH 7·4, 0·12 M NaCl) was incubated for 30 s with 0·1 ml factor X-activating enzyme from Russell's Viper venom (RVV-X) and 0·1 ml $CaCl_2$ (15 mM) to achieve activation of factor X. 0·1 ml of a suspension of sonicated phospholipid vesicles was added in concentrations ranging from 1000 to 1 nmol/ml and the clotting time was measured. The concentration of RVV-X was used as adjustable parameter to set the coagulation time at about 225 s when Tris buffer was added instead of lipid vesicles. An identical range of phospholipid concentrations was used when lysed platelet suspension was added instead of phospholipid vesicles. This was effected by adjusting the platelet count of the suspension, prior to sonication, to 2×10^9 platelets/ml which corresponds to approx. 1000 nmol phospholipid/ml, assuming an average molecular weight of 800 for the phospholipids (compare also Table I). Platelets were lysed by sonication for 3 min in ice, using 15 s intervals with cooling periods of 15 s (Baenziger & Majerus, 1974).

Factor V was determined by a one-stage assay using factor V-deficient reagent (Borchgrevink *et al*, 1960). The factor V concentration was expressed as a percentage of the content in a normal bovine plasma pool. The factor V and factor Va preparations (activated by factor V-activating enzyme from Russell's Viper venom) were kindly supplied by Dr M. J. Lindhout. The factor X-deficient plasma (from a patient with congenital factor X deficiency) was kindly donated by Dr B. N. Bouma, Academic Hospital, State University of Utrecht, Utrecht, The Netherlands.

RESULTS

Lipid Composition of Bovine Blood Platelets

The number of platelets obtained from batches of 1 litre of bovine blood varied between 3 and 6×10^{11}. The lipid composition, expressed as ng per 10^5 platelets, is shown in Table I. Total lipid based on dry weight was found to be higher than the sum of phospholipid and cholesterol. The difference can presumably be accounted for by triglycerides and glycolipids (Marcus *et al*, 1969, 1972). The weight ratio of phospholipid to cholesterol was approximately 3, which corresponds to a molar ratio of 1·6.

The phospholipid composition of whole bovine platelets which is shown in Table II, appears to be rather similar to those reported for human and pig platelets (Marcus *et al*, 1969; Chap *et al*, 1977).

TABLE I. Bovine platelet lipids

Lipid	*ng/10⁵ platelets*
Total lipid (dry weight)	51·5 (49·8–52·3)
Cholesterol	11·6 (11·4–11·9)
Phospholipid	36·6 (36·1–37·7)
Cholesterol+phospholipid	48·2 (47·5–49·6)

The values given are the means of six experiments. The values in parentheses indicate the range.

TABLE II. Phospholipid composition of bovine platelets

Component	*Percentage of total phospholipid*
Sphingomyelin	25·8±1·9
Phosphatidylcholine	33·3±2·4
Phosphatidylserine	7·0±0·5
Phosphatidylinositol	4·3±0·3
Phosphatidylethanolamine	29·5±2·2

The values given are expressed as the mean±SD (calculated for six experiments).

Coagulant Activities of Platelet Lipid and Lysed Platelets

The clot-promoting activity of the bovine platelet lipids was compared with that of lysed platelets as a function of the phospholipid concentration. The results compiled in Fig 1 show that at low phospholipid concentration (between 1 and 50 nmol/ml of lipid added) there is a sharp decrease in clotting time which is identical for sonicated vesicles of extracted lipid and lysed platelets. At higher lipid concentrations the clot-promoting activities of both preparations start to deviate. For lipid vesicles a minimal clotting time of 21±0·4 s (mean±SD) was found at about 100 nmol/ml of lipid added. Lysed platelets had a minimal clotting time of 18±0·3 s at a phospholipid concentration of 750 nmol/ml. Above the most active phospholipid concentration, both preparations showed a gradual decrease in clotting activity, but lysed platelets were found to remain more active than the lipid extracts.

Factor V Activity of Gel-filtered Platelets

The results shown in Fig 1 and the finding that lysis of gel-filtered platelets produces release of factor V activity (Breederveld *et al*, 1975; Østerud *et al*, 1977) prompted us to investigate

TABLE III. Factor V activity of gel-filtered platelets

Material	Clotting time in factor V assay (s)	Percentage factor V
Whole platelets	48 ± 1·5	1
Sonicated platelets	32 ± 1·0	5
Once frozen-and-thawed platelets	32 ± 1·0	5
Buffer	205 ± 7·0	

The platelet suspensions used in the factor V assay contained 2×10^9 platelets/ml. Sonicated platelets were obtained as described in the text. The clotting times are expressed as the mean ± SD.

factor V (or Va) concentration on a lipid basis as in lysed platelets. For this purpose, 1000 nmol of lipid/ml were made 5% in factor V or 0·25% in factor Va and further diluted with Tris buffer for use in the coagulation assays. As shown in Fig 2, these additions of factor V (or Va) had no influence on the clotting times at phospholipid concentrations up to 50 nmol/ml of lipid added. At higher lipid concentrations, however, the effects of factor V or Va are clearly noticeable. For the mixtures of platelet lipid with both factor V or factor Va the minimal clotting time was found to be decreased to 20 ± 0·3 s and was reached at higher phospholipid concentrations (approx. 500 nmol/ml of lipid added) as compared to lipids alone. Nevertheless, lysed platelets were still found to be more active.

In subsequent experiments the amounts of factor V and factor Va added to 1000 nmol of sonicated lipid per ml were increased to 15% and 0·75%, respectively. As demonstrated in Fig 3, this three-fold increase in factor V (or Va) changed the clot-promoting behaviour of the lipid vesicles to become identical to that of lysed platelets over the whole range of phospholipid concentrations tested. Whereas platelet lipid alone had a minimal clotting time of 21 ± 0·4 s at a phospholipid concentration of about 100 nmol/ml (cf. Fig 1) platelet lipids with 15% of factor V or 0·75% of factor Va (both in 1000 nmol phospholipid per ml) as well as lysed platelets were found to have a minimal clotting time of about 18 ± 0·3 s at a lipid concentration of about 750 nmol/ml.

Experiments with Lower Factor X Concentrations in the Prothrombinase Assay

In the experiments described above, delipidated bovine plasma was used in the prothrombinase assay (compare Tans *et al*, 1979). In this plasma, factor X concentrations (assayed according to Hemker *et al*, 1972) were found to be normal whereas factor V was decreased to about 35% of the normal plasma value.

It can be argued that if the coagulant advantage of lysed platelets over extracted platelet lipids at higher phospholipid concentrations were due to platelet factor V, this difference should disappear on decreasing the factor X concentration in the prothrombinase assay to such an extent that factor V will never be rate-limiting. Therefore the clotting behaviour of lysed

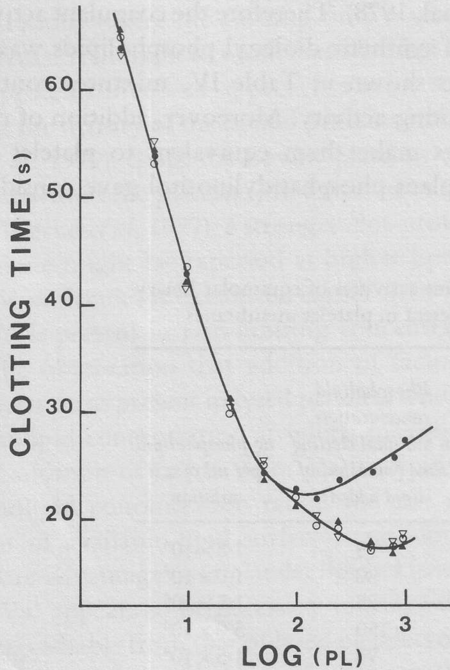

Fig 3

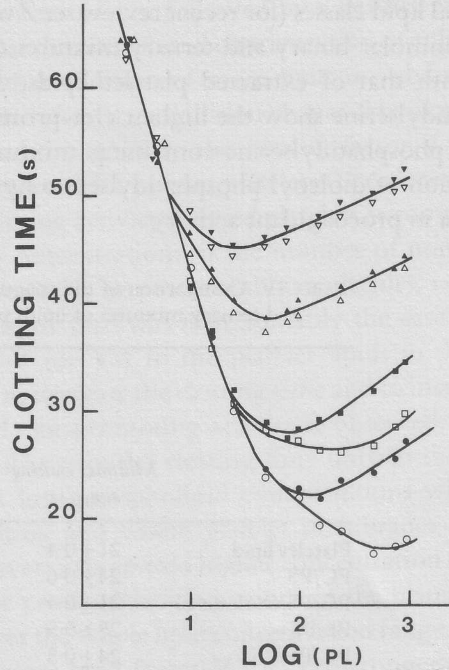

Fig 4

Fig 3. Clotting times as a function of the logarithm of concentration of lipid added, expressed in nmol/ml, for sonicated vesicles of lipid extracted from bovine platelets (●), for sonicated platelets (○), and for vesicles of platelet lipid prepared at a concentration of 1000 nmol/ml, then made either 15% of factor V(▲) or 0·75% of factor Va (▽) and further diluted with Tris buffer. Further conditions are the same as in Fig 1.

Fig 4. Clotting times as a function of the logarithm of concentration of lipid added, expressed in nmol/ml. In the prothrombinase assay use was made of delipidated bovine plasma and vesicles of lipid from bovine platelets (●), delipidated bovine plasma and lysed bovine platelets (○), a 1:9 (v/v) mixture of delipidated bovine plasma and factor X-deficient plasma, and vesicles of lipid from bovine platelets (■), the same plasma mixture and lysed bovine platelets (□), a 1:19 (v/v) mixture of delipidated bovine plasma and factor X-deficient plasma, and either lipid vesicles (▲) or lysed platelets (△), and a 1:24 (v/v) mixture of delipidated bovine plasma and factor X-deficient plasma and either lipid vesicles (▼) or lysed platelets (▽). Further conditions are the same as in Fig 1.

platelets and lipid extracts was compared in mixtures of delipidated plasma and congenital factor X-deficient plasma, delipidated in the same manner.

As shown in Fig 4, decreasing factor X concentrations not only increased the coagulation time but also abolished the difference between lysed platelets and extracted platelet lipids. At 1:9 (v/v) mixtures of delipidated plasma and factor X-deficient plasma the higher clot-promoting activity of lysed platelets is smaller but still apparent, while this difference is completely diminished with mixtures of delipidated plasma and factor X-deficient plasma of 1:19 (v/v) or 1:24 (v/v).

Comparison of Procoagulant Activity of Platelet Lipids with Some Mixtures of Individual Lipids

It is generally accepted that lipid mixtures possess a higher clot-promoting activity than

individual lipid classes (for recent review see Zwaal, 1978). Therefore the coagulant activity of some equimolar binary and ternary mixtures of synthetic dioleoyl phospholipids was compared with that of extracted platelet lipids. As shown in Table IV, mixtures containing phosphatidylserine show the highest clot-promoting activity. Moreover, addition of cholesterol to phosphatidylserine-containing mixtures make them equivalent to platelet lipids. Substitution of dioleoyl phosphatidylserine by plant phosphatidylinositol gave considerable reduction in procoagulant activity.

TABLE IV. Comparison of the coagulant activities of equimolar binary and ternary mixtures of lipids present in platelet membranes

	Minimal clotting time (s)	*Phospholipid concentration at minimal clotting time (nmol/ml of lipid added)*	*ng phospholipid per ml of test solution*
Platelet lipid	$21 \pm 0{\cdot}4$	75	$1{\cdot}5 \times 10^4$
PC/PS	$24 \pm 0{\cdot}6$	50	$1{\cdot}0 \times 10^4$
PC/PS/cholesterol	$21 \pm 0{\cdot}4$	75	$1{\cdot}5 \times 10^4$
PE/PS	$25 \pm 0{\cdot}5$	150	$3{\cdot}0 \times 10^4$
PC/PE/PS	$24 \pm 0{\cdot}5$	75	$1{\cdot}5 \times 10^4$
PC/PE	$60 \pm 1{\cdot}5$	750	$15{\cdot}0 \times 10^4$
PC/PI	$33 \pm 1{\cdot}0$	750	$15{\cdot}0 \times 10^4$
Tris buffer	$225 \pm 7{\cdot}0$	—	—

Abbreviations: PC=dioleoyl phosphatidylcholine; PS=dioleoyl phosphatidylserine; PE=dioleoyl phosphatidylethanolamine; PI=phosphatidylinositol from plant.

The clotting times are expressed as the mean ± SD.

DISCUSSION

The influence of platelets or phospholipids on the clotting time of plasma in the presence of activation enzyme RVV-X provides direct information on their role in thrombin formation from prothrombin. Since factor X is activated by incubation with RVV-X and $CaCl_2$ *prior to addition* of platelets or lipids no contributions of their accelerating effect on the earlier steps in coagulation are to be expected.

The results clearly demonstrate that at low lipid concentrations (up to 50 nmol/ml of lipid added), there is no difference in procoagulant activity (platelet factor 3 activity) between vesicles of platelet lipids and lysed platelets.

Apparently, in this concentration range the amount of available lipid surface is rate-limiting, indicating that the more phospholipid membrane is provided the more prothrombinase complexes will be formed with a resultant decrease in coagulation time (Tans *et al,* 1979). If platelets were to contain a more active lipoprotein component not present in isolated platelet lipids, a procoagulant advantage of sonicated platelets would be present over the whole lipid concentration range. Nevertheless, an advantage becomes only apparent at higher lipid

concentrations where the clotting time reaches a minimum followed by a gradual increase. This increase is thought to result from the fact that the non-bound concentration of at least one of the proteins involved approaches zero, so that addition of more lipid membranes will decrease the density of the bound proteins and therefore their collision chance (Hemker, 1975; Tans *et al,* 1979). Since the bovine platelets used in this study also contain factor V activity localized within the platelet, just as has been observed for human platelets (Breederveld *et al,* 1975; Østerud *et al,* 1977), a stronger clot-promoting activity of lysed platelets as compared to lipids alone might be expected at higher lipid concentrations, if the number of non-bound factor V molecules is exhausted earlier than factor Xa or prothrombin molecules, i.e. when factor V is present in rate-limiting concentrations. That this is presumably the case follows from the observation that addition of factor V (or Va) to the platelet lipids in the same concentration as present in lysed platelets tends to decrease the clotting time and to increase the phospholipid concentration at which maximal clot-promoting activity is observed. Significantly, addition of factor V (or Va) to lipids decreases the clotting time only in the higher phospholipid concentration range and not at low phospholipid concentrations where the amount of available lipid surface is rate-limiting and where platelet membranes have no coagulant advantage over platelet lipids. However, a three-fold higher concentration of factor V (or Va) appears to be necessary to make the procoagulant behaviour of the platelet lipids indistinguishable from that of lysed platelets over the whole lipid concentration range. Such an effect is to be expected when platelet membranes bind factor V (or Va) stronger than the isolated phospholipids.

Various plausible explanations can be given to sustain this view: (a) The cytoplasmic surface of the platelet plasma membrane has been shown to have a much more efficient procoagulant phospholipid composition than the exterior surface (Chap *et al,* 1977; Zwaal *et al,* 1977). This asymmetric orientation will be (partially) retained in a certain fraction of the vesicles obtained from lysed platelets whereas in reconstituted vesicles all phospholipids will be homogeneously partitioned, which is a less efficient situation. (b) Platelet membrane fragments may have a size and a structure different from that of phospholipid vesicles which might affect the binding properties for factor V (or Va).

Even if platelet membranes have a stronger affinity for factor V, the amount of bound factor V to platelets differs insignificantly from that to lipids when the number of binding sites, i.e. the amount of available lipid membrane surface, is small since the surface will be saturated. Platelet factor Va is presumably identical to the factor Xa receptor site in activated or lysed platelets (Miletich *et al,* 1977, 1978a, b), but factor Xa also binds to phospholipids (Papahadjopoulos & Hanahan, 1964). When the number of factor Xa molecules is drastically reduced, it can be expected that factor Xa becomes the rate-limiting coagulation factor when the phospholipid concentration is increased in the prothrombinase assay. The number of active prothrombinase complexes decreases, which explains why the clotting time increases and less phospholipid membrane is required for maximal activity. The observation that the difference in coagulation behaviour between platelet membranes and platelet lipids disappears at lower factor Xa concentrations strongly suggests that this difference can be attributed to additional platelet factor V (or Va) and not to some unknown platelet lipoprotein with higher platelet factor 3 activity than phospholipid alone. If this were the case, platelet membranes would still have a higher clot-promoting activity than phospholipids at lowered factor Xa concentrations.

Therefore, in our opinion there is no need to postulate a lipoprotein with higher platelet factor 3 activity than phospholipids (Marcus, 1969, 1978; Walsh, 1978).

Finally, the experiments with synthetic lipids confirm the general notion that phosphatidylserine is indeed the most prominent procoagulant phospholipid in platelet membranes. Equimolar mixtures of phosphatidylcholine, phosphatidylserine and cholesterol show the same clot-promoting activity as extracted platelet lipids. In human and pig platelet plasma membranes, phosphatidylserine has been shown to be almost exclusively located at the cytoplasmic side of the plasma membrane (Schick *et al*, 1976; Chap *et al*, 1977). At present there is little reason to suppose that the situation will be much different in bovine platelets. The present results strongly suggest that phosphatidylserine is an essential component of platelet factor 3. Therefore, increase in platelet factor 3 activity during platelet activation requires, in addition to the appearance of platelet factor Va on the outer surface, exposure of phosphatidylserine to interacting plasma coagulation factors. Experiments are in progress in our laboratory to elucidate the mechanism by which this occurs.

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