# Platelet membrane involvement in blood coagulation

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# Platelet Membrane Involvement in Blood Coagulation

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ABSTRACT. **Intact platelets do not show procoagulant phospholipids on their exterior. These phospholipids are located at the inside leaf of the bilayer membrane. They become available by (a) disrupture of the platelets (mechanical, osmotical etc.), (b) by a mechanism specific for the platelets, that we call the membrane flip-flop.**

**Membrane flip-flop translocates procoagulant phospholipids (mainly phosphatidylserine) in the intact platelet from the inside to the outside. Thus the intact platelet becomes procoagulant. The trigger for the flip-flop mechanism is the simultaneous presence of small amounts of collagen *and* thrombin. The clotting factors IXa and VIIIa bind to the procoagulant lipids to form the factor X converting enzyme and factor Xa and factor Va to form prothrombinase.**

KEY WORDS: **Thrombocyte – Membrane asymmetry – Procoagulant phospholipids – Thrombin – Collagen**

## COFACTOR DEPENDENT REACTIONS IN THE BLOOD COAGULATION CASCADE

The sequence of physiological reactions that eventually leads to the formation of thrombin is known as the blood coagulation cascade. Each step in this cascade consists of the formation of an active proteolytic enzyme (a serine protease) from its pro-enzyme. Several reactions in the cascade require the presence of so-called non-enzymatic cofactors. These cofactors are divided in two classes:

a) protein cofactors,

b) negatively charged surfaces.

It is generally known that these non-enzymatic cofactors greatly enhance the rate of the clotting factor activation reactions in which they participate. The reactions and their cofactors are summarized in Table 1.

A quantitative study of these reactions has become possible

a) because the proteins involved can now be obtained in a pure state in large enough quantities and

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**Table 1.** Cofactor dependent reactions in blood coagulation

| Enzyme | Substrate | Protein cofactor | Surface |
|---|---|---|---|
| Factor $XII_a$ | Prekallikrein | High molecular weight kininogen | Negatively charged wettable surfaces, sulphatides etc. |
| Kallikrein | Factor XII | | |
| Factor $XII_a$ | Factor XI | | |
| Factor $VII_a$ | Factor IX | tissue factor-apoprotein | Negatively charged phospholipids + $Ca^{2+}$ (blood platelets). |
| Factor $VII_a$ | Factor $X^1$ | | |
| Factor $IX_a$ | Factor $X^2$ | factor VIII | |
| Factor $X_a$ | Factor II | factor V | |

[1] The extrinsic factor X activation
[2] The intrinsic factor X activation

**Table 2.** Effect of non-enzymatic cofactors on relative rates of prothrombin activation

| | Relative rate of prothrombin activation |
|---|---|
| Factor Xa | 1 |
| Factor Xa + $Ca^{2+}$ | 3 |
| Factor Xa + $Ca^{2+}$ + phospholipid | 68 |
| Factor Xa + $Ca^{2+}$ + factor Va | 560 |
| Factor Xa + $Ca^{2+}$ + phospholipid + factor Va | 20,000 |

b) because the products of the reactions can be determined spectrophotometrically with high accuracy due to the development of the so-called chromogenic substrates [1].

The effects of factor Va, $Ca^{2+}$ and phospholipid vesicles (containing the negatively charged phosphatidylserine) on the rate of prothrombin activation, are shown in Table 2. Factor Va and negatively charged phospholipids stimulate prothrombin activation by factor Xa independently and multiply their effects. With the complete prothrombinase complex, consisting of factor Xa, factor Va, $Ca^{2+}$ and phospholipid, a 20,000-fold rate enhancement is observed. Similar observations are made for the effect of cofactors in intrinsic and extrinsic factor X activation and the reactions of the contact activation system.

## MECHANISM OF ACTION OF NON-ENZYMATIC COFACTORS IN PROTHROMBIN AND FACTOR X ACTIVATION

Prothrombin and intrinsic factor X activation have many features in common. The substrates in both reactions (prothrombin and factor X) are vitamin *K* dependent proteins. They contain $\gamma$-carboxyglutamic acid residues, responsible for the $Ca^{2+}$ dependent binding to negatively charged phospholipid surfaces. The enzymes (factor Xa and factor IXa), are also vitamin K dependent proteins. The protein cofactors (factor Va and factor VIIIa)

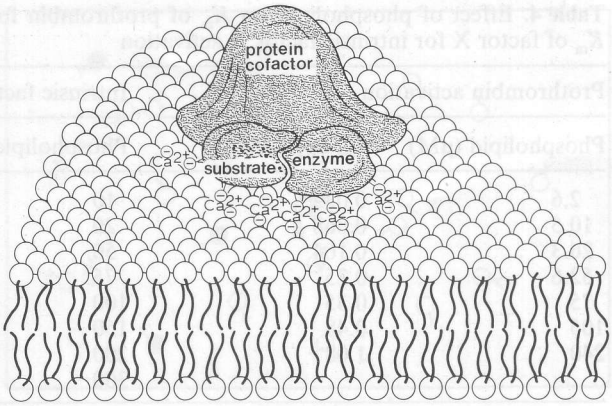


**Fig. 1.** General model for the prothrombin and factor X activating complexes. Phospholipid bilayers, containing negatively charged phospholipids, promote the assembly of the enzyme-cofactor-substrate complex

**Table 3.** Kinetic parameters of prothrombin and factor X activation

| Prothrombin activator | $K_m$ prothrombin (μM) | $V_{max}$ (IIa·min$^{-1}$·Xa$^{-1}$) |
|---|---|---|
| Xa, $Ca^{2+}$ | 84 | 0.68 |
| Xa, $Ca^{2+}$, phospholipid | 0.06 | 2.3 |
| Xa, $Ca^{2+}$, phospholipid, Va | 0.21 | 1,919 |
| **Factor X activator** | **$K_m$ factor X (μM)** | **$V_{max}$ (Xa·min$^{-1}$·IXa$^{-1}$)** |
| IXa, $Ca^{2+}$ | 181 | 0.01 |
| IXa, $Ca^{2+}$, phospholipid | 0.06 | 0.0025 |
| IXa, $Ca^{2+}$, phospholipid, VIIIa | 0.063 | 500 |

have no enzymatic activity and have a high affinity for negative phospholipid surfaces, although they lack γ-carboxyglutamic acids. A general model for the prothrombin and factor X activating complexes is shown in Figure 1. Phospholipids promote the assembly of the enzyme-cofactor-substrate complex (factor Xa- and factor Va- prothrombin and factor IXa- factor VIIIa- factor X) via the above described interactions of clotting factors with the phospholipid surface. Although it is clear that this type of enzyme complex functions much more efficiently than the enzyme without accessory factors (Table 2), the rate measurements do not permit conclusions about the mode of action of the accessory factors in the enzymatic mechanism of clotting factor activation. Therefore we determined the effects of accessory components on the kinetic parameters of prothrombin and intrinsic factor X activation [2, 3]. This enabled a precise quantitation of earlier observed rate enhancements and is the first step in elucidating the role of accessory components in the mechanism of coagulation factor activation. The kinetic parameters of prothrombin and intrinsic factor X activation, observed for enzyme complexes of varying composition, are summarized in Table 3. In the absence of accessory components, prothrombin and factor X activation are

**Table 4.** Effect of phospholipid on $K_m$ of prothrombin for prothrombin activation and $K_m$ of factor X for intrinsic factor X activation

| Prothrombin activation | | Intrinsic factor X activation | |
|---|---|---|---|
| Phospholipid (μM) | $K_m$ app (μM) | Phospholipid (μM) | $K_m$ app (μM) |
| 2.6 | 0.032 | 10 | 0.058 |
| 10.5 | 0.068 | 20 | 0.139 |
| 26.3 | 0.164 | 50 | 0.363 |
| 52.6 | 0.25 | 75 | 0.409 |
| 75 | 0.35 | 100 | 0.525 |
| 105 | 0.48 | 150 | 0.822 |
| 240 | 1.08 | 200 | 1.83 |
| | | 300 | 1.76 |

In this experiment no factor Va or factor $VIII_a$ was added

very inefficient processes. The $V_{max}$ (that is the turnover number of substrate molecules by the completely saturated enzyme) is very low and the $K_m$ (the substrate concentration causing half maximal enzyme saturation for prothrombin and factor X are considerably higher than the respective plasma concentrations (prothrombin 2 μ*M*, factor X 0.2 μ*M*). The presence of phospholipid plus $Ca^{2+}$ causes, in both complexes, a drastic drop in the $K_m$ to values below the plasma concentrations, while the protein cofactors greatly increase the $V_{max}$. The observed changes of the kinetic parameters explain the earlier observed rate enhancements caused by accessory components.

## THE ROLE OF PHOSPHOLIPIDS

Since this paper deals with platelet membrane (phospholipid) involvement we will not further discuss the effects of the protein cofactors, but focus on the mode of action of the phospholipid. Both in prothrombin and intrinsic factor X activation, phospholipids cause a drastic drop in the $K_m$ for prothrombin and factor X, respectively. Also the $K_m$ is dependent on the phospholipid concentration present in the reaction mixture (Table 4). When the phospholipid concentration is increased, the $K_m$ increases in parallel. So the $K_m$, measured in the presence of phospholipid, must be an apparent $K_m$ and is not, as such, a reaction constant of the activation under study. A model that can explain both the drop in $K_m$ and the apparent character observed in the presence of phospholipid is presented in Figure 2. In free solution, enzyme (factor Xa or factor IXa) and substrate (prothrombin or factor X) have a low affinity for each other, which explains the high $K_m$ measured under these conditions. When negatively charged phospholipids are included in the reaction mixture, both the enzymes and substrates

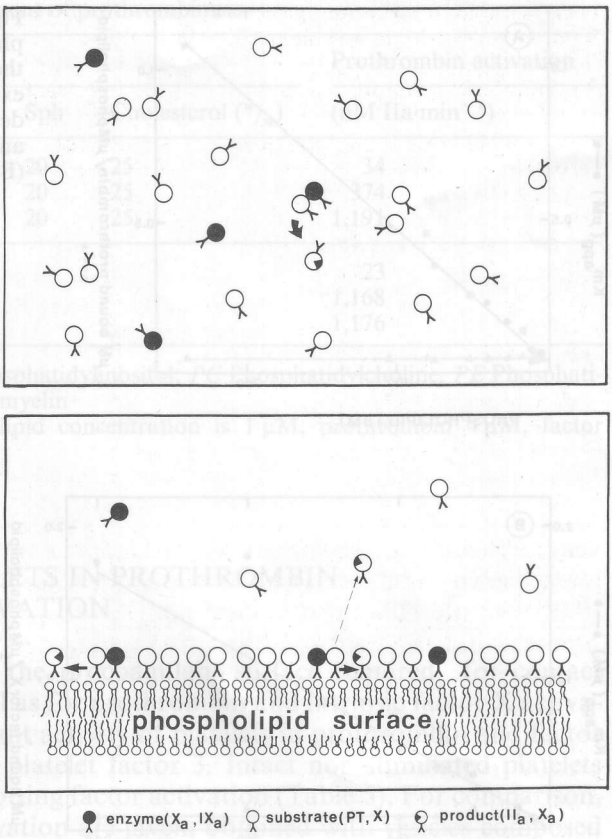


**Fig. 2.** Mode of action of phospholipids in prothrombin and factor X activation

bind to the phospholipid surface via calcium bridges between their $\gamma$-carboxyglutamic acid residues and negatively charged head groups of phospholipid molecules. This makes a reaction possible between phospholipid bound enzyme and phospholipid bound substrate and thus favours the number of collisions between enzyme and substrate. This is the same effect as an increase in concentration of the substrate would have. Because the real concentration in the reaction vessel does not change by the addition of phospholipid the concentration of substrate at the phospholipid surface expresses itself as a drop in $K_m$. Half maximal saturation of enzyme with substrate, in the presence of phospholipid, is reached at a much lower substrate concentration because it is determined by the prothrombin concentration at the phospholipid surface. To attain the same prothrombin density at the phospholipid surface at higher phospholipid concentrations, more prothrombin is required, which explains the observed increase in the apparent $K_m$ at increasing phospholipid concentrations. In this model, it is expected that although the apparent $K_m$ increases with the phospholipid concentration, a $K_m$ expressed in terms of concentration of phospholipid bound substrate,

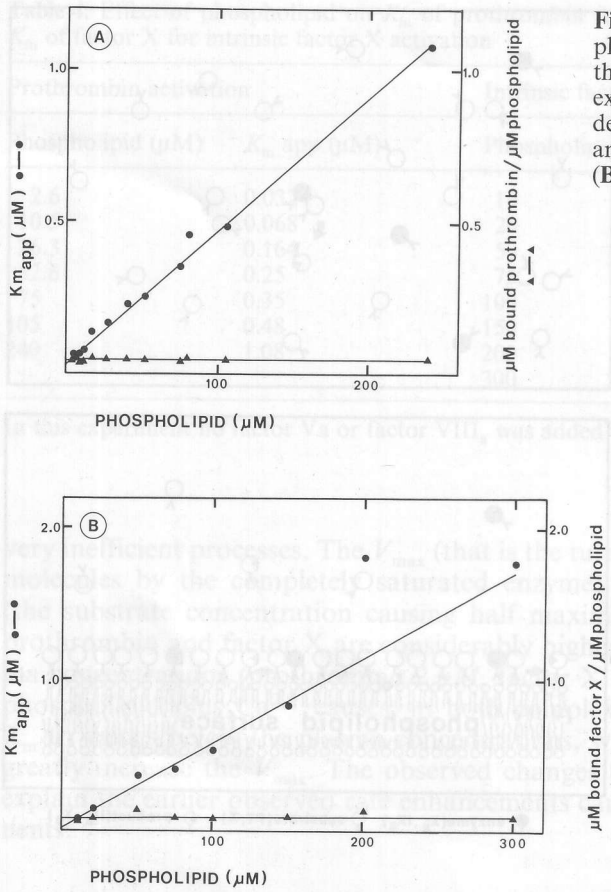


**Fig. 3A, B.** Effect of phospholipid concentration on the apparent $K_m$ and the $K_m$ expressed in substrate surface density for prothrombin (**A**) and intrinsic factor X activation (**B**)

would be constant. Using binding parameters (dissociation constant ($K_d$) and concentration of binding sites) for prothrombin and factor X binding to phospholipid vesicles reported by Nelsestuen [4], we calculated the concentration of substrate (prothrombin and factor X) bound per μM phospholipid, at the $K_m$'s presented in Table 4. Figure 3 shows the measured $K_m$ for prothrombin and factor X activation and the amount of prothrombin and factor X bound per μM phospholipid at varying phospholipid concentrations. Indeed the $K_m$ expressed in terms of phospholipid bound substrate is independent of the phospholipid concentration, which supports but does not prove the proposed model.

Not all phospholipids function equally well in prothrombin and factor X activation. In procoagulant membranes net negatively charged phospholipids have to be present; natural phospholipids are inactive. Furthermore a liquid crystalline state of the membrane is required for maximal procoagulant activity [5].

**Table 5.** Phospholipid requirement of prothrombinase

| Vesicle composition (mole %) | | | | | | Prothrombin activation |
|---|---|---|---|---|---|---|
| PS | PI | PC | PE | Sph | Cholesterol ($^{w}/_{w}$) | (nM IIa·min$^{-1}$) |
| – | – | 50 | 30 | 20 | 25 | 34 |
| – | 3 | 47 | 30 | 20 | 25 | 374 |
| 10 | 3 | 37 | 30 | 20 | 25 | 1,191 |
| Intact platelets | | | | | | 23 |
| Platelet phospholipid extract | | | | | | 1,168 |
| Platelet sonicate | | | | | | 1,176 |

*PS* Phosphatidylserine, *PI* Phosphatidylinositol, *PC* Phosphatidylcholine, *PE* Phosphatidylethanolamine, *Sph* Sphingomyelin
Vesicle and platelet phospholipid concentration is 1 μM, prothrombin 4 μM, factor Va 30 nM, factor Xa 15 nM

## THE ROLE OF PLATELETS IN PROTHROMBIN AND FACTOR X ACTIVATION

In vivo platelets provide the procoagulant surface required for contact activation reactions as well as for prothrombin and intrinsic factor X activation [6, 7]. The procoagulant activity of platelets in prothrombin and factor X activation is known as platelet factor 3. Intact non-stimulated platelets are however inactive in clotting factor activation (Table 5). For comparison, rates of prothrombin activation are given, obtained with vesicles composed of the phospholipids present in the platelet membrane. When the net negatively charged phospholipids (phosphatidylserine and phosphatidylinositol) are omitted from the vesicles in which the phospholipid composition of the platelet plasma membrane is mimicked, a low rate of prothrombin activation is measured. In contrast, artificial vesicles with a complete phospholipid composition as found in platelets and also vesicles made from a complete platelet phospholipid extract, give equally high rates of prothrombin activation. Table 5 therefore shows that the net negatively charged phosphatidylserine is essential in producing full prothrombin activation. Phosphatidylserine is however almost exclusively located in the inner monolayer of the platelet membrane [8]. It can for instance be exposed to clotting factors in the reaction mixture if the platelets are lysed by ultrasonication (Table 5). Lysed platelets exhibit the same prothrombin activation rates as vesicles composed of platelet phospholipids. This suggests that only phosphatidylserine contributes to the enhancement of prothrombin activation by lysed platelets. When intact platelets are stimulated with the physiological platelet triggers, collagen or thrombin, at concentrations which give maximal platelet aggregation and serotonin release, small rate enhancements are measured in assay systems for prothrombin and intrinsic factor X activation

**Table 6.** Effect of platelets in prothrombin and intrinsic factor X activation

| Platelets stimulated by: | Prothrombin activation (nM IIa·min$^{-1}$) | Factor X activation (nM $X_a$·min$^{-1}$) |
|---|---|---|
| – | 34 | 2.3 |
| Thrombin (1.3 nM) | 40 | 3.1 |
| Collagen (10 g/ml) | 98 | 18.6 |
| Thrombin (1.3 nM) plus collagen (10 g/ml) | 352 | 47.3 |
| Concentrations: | 4 μM prothrombin, 15 nM Xa, 30 nM Va for prothrombin activation 0.5 μM X, 150 nM IXa, 15 nM VIIIa for factor X activation | |

**Table 7.** Sites for prothrombin and factor X activation on human platelet membranes

| Platelet stimulator | Prothrombin activation (sites/platelet) | Factor X activation (sites/platelet) |
|---|---|---|
| – | 2,500 | 900 |
| Thrombin | 3,000 | 1,200 |
| Thrombin + collagen | 26,000 | 19,000 |

(Table 6). The increase of both activities can be explained by the small amount of platelet lysis brought about by the admixture of the reagents. Thrombin *plus* collagen will not cause more lysis than each reagent alone. When compared with unstimulated platelets, platelets triggered by the combined action of collagen plus thrombin however show a tenfold increase of activity in prothrombin activation, and a 20-fold rate enhancement in factor X activation. Bevers et al. [8] found that platelets, which were triggered by the combined action of collagen plus thrombin, exposed their internally localized phosphatidylserine to the membrane exterior. This introduction of phosphatidylserine in the outer monolayer of the platelet membrane produces a platelet surface which promotes the assembly of both the prothrombin and intrinsic factor X activating complex by binding of vitamin K dependent clotting factors. This explains the observed rate enhancement of thrombin plus collagen stimulated platelets in prothrombin and factor X activation (Table 6). Experiments as presented in Table 6 can be used to quantitate the number of prothrombin and factor X activating complexes on the platelet surface. In order to do this, the coagulation factors were added in saturating amounts so as to occupy all functional sites on the platelets surface and moreover to measure under $V_{max}$ conditions. $V_{max}$ values obtained with phospholipid vesicles as a procoagulant surface (2,700 min for prothrombin and 500 min for factor X activation) are used in these calculations. The amounts of calculated sites (Table 7) for unstimulated and thrombin activated platelets are relatively low as compared with

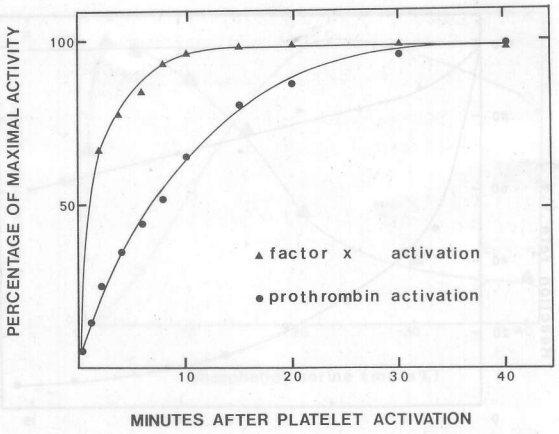


**Fig. 4.** Time course of appearance of platelet activity in prothrombin and factor X activation. Coagulation factor concentrations: for prothrombin activation, 4 μM prothrombin, 15 nM factor Xa, 30 nM Factor Va, for factor X activation, 0.5 μM factor X, 50 nM factor IXa and 0.1 nM factor VIIIa. 100% activity in prothrombin activation is 800 $nM \cdot min^{-1}$. IIa formed, 100% activity in factor X activation is 17 $nM \cdot min^{-1}$ factor Xa formed. Platelets were stimulated with thrombin plus collagen

platelets stimulated by collagen plus thrombin together. It is not clear whether unstimulated platelets, under physiological circumstances, indeed have this low but significant amount of functional binding sites or that they are partially activated. Also a part of this activity must be attributed to the inevitable lysis that occurs during platelet isolation procedures. For the collagen plus thrombin stimulated platelet, approximately 26,000 functional binding sites for the prothrombinase complex and approximately 20,000 for the factor X activating complex have been calculated. These numbers are so close to each other that they strongly suggest that at saturating coagulation factor concentrations, the same sites function in prothrombin and factor X activation at the surface of stimulated platelets. This observation suggests that the essential component for both the binding of the proteins of the prothrombin and the intrinsic factor X activating complexes is the phosphatidylserine, that after platelet triggering becomes exposed in the outer monolayer of the membrane.

The time course of the appearance of procoagulant activity in stimulated platelets can be followed in the assay systems for prothrombin and intrinsic factor X activation. In Figure 4 the generation of a procoagulant surface is followed at saturating factor Xa, Va and prothrombin concentrations in the prothrombin activating assay, and at sub-saturating factor IXa and VIIIa concentrations in the factor X activating system. Half maximal factor X converting activity is observed within 2 min after addition of collagen plus thrombin, whereas prothrombin converting activity reaches the half maximal value after 9 min. The observed differences between the time courses seem to contradict the idea that the essential component of the procoagulant surface of stimulated platelets is exclusively phosphatidylserine. This difference can be explained however without postulating other procoagulant components.

The requirement of procoagulant phospholipid as a component of the

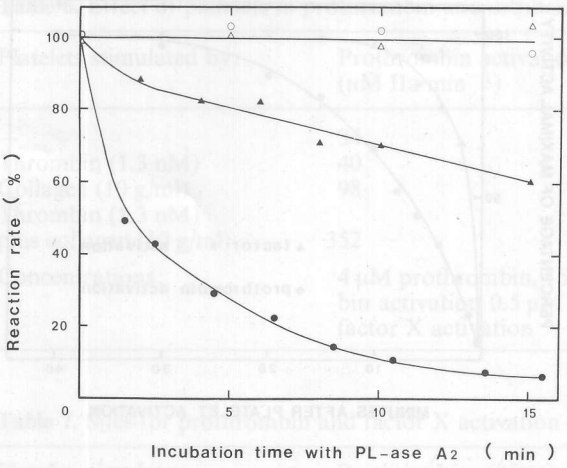


**Fig. 5.** Effect of phospholipase $A_2$ on the activity of collagen plus thrombin stimulated platelets in prothrombin and factor X activation. Coagulation factor concentrations were as described in Fig. 4. Stimulated platelets were either treated (*closed symbols*) or not (*open symbols*) with phospholipase $A_2$. The *triangles* represent prothrombin activation, the *circles* factor X activation

functional sites for the prothrombin and factor X activating complexes could be determined in phospholipase digestion experiments. Platelets are stimulated with collagen plus thrombin until maximal procoagulant activity is reached and subsequently treated with *Naja naja* phospholipase $A_2$, an enzyme known to degrade phospholipid molecules exposed at the outer platelet surface [7]. Using high phospholipase $A_2$ concentrations, both prothrombin and intrinsic factor X converting activities of the stimulated platelets are completely abolished within 10 min. The absence of leakage of lactate dehydrogenase from the platelets during incubation with the phospholipase, indicates that the platelet membrane remains intact. So the conclusion can be drawn that procoagulant phospholipids are an essential component in the functional sites for prothrombin and factor X activation on the stimulated platelet. When small amounts of phospholipase $A_2$ are used, the time course of decay of prothrombin and factor X converting activity of the stimulated platelets could be followed. After different time intervals of phospholipase treatment, the coagulation factors are added to measure the remaining procoagulant activity. Figure 5 shows that the factor X converting activity of platelets is more sensitive to phospholipase treatment than the platelet prothrombin converting activity. It was investigated whether the differences in time course of generation of a procoagulant platelet surface and the sensitivity to phospholipase $A_2$ treatment, as estimated with prothrombin and factor X activation, could be related to different phospholipid requirements. Therefore, prothrombin and factor X activation are measured in model systems, using phospholipid vesicles with varying phospholipid composition. Prothrombin and factor X converting activities are measured, using the same coagulation factor concentrations as in the experiments with platelets. The vesicles are composed of lipids at the same molar concentration as found in platelet membranes, with the exception of phosphatidylserine and phosphatidylcholine. The mole fraction phosphatidylserine is varied

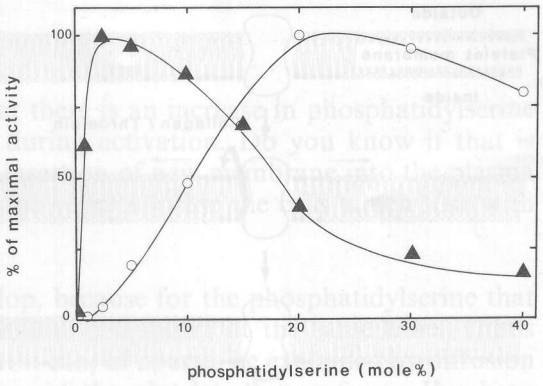


**Fig. 6.** The effect of phospholipid vesicles with varying mole percentage phosphatidylserine in prothrombin (*closed symbols*) and factor X activation (*open symbols*). 100% activity in prothrombin activation is 2,900 $nM \cdot min^{-1}$, 100% activity in factor X activation is 71.5 $nM \cdot min^{-1}$

at the expense of phosphatidylcholine and the overall phospholipid concentration is kept at 2 μM. The rates, given as percentage of the maximal activity, measured for prothrombin and factor X activation at varying mole percentage phosphatidylserine, are given in Figure 6. Large differences in phosphatidylserine requirement for prothrombin activation and factor X activation are observed. For the intrinsic factor X activation, a mole fraction of 20% phosphatidylserine is required for maximal activity, whereas for prothrombin activation 2.5% phosphatidylserine is optimal. At a surface containing less than 2.5% phosphatidylserine, factor X activation rates are negligible, while prothrombin activation still proceeds at a considerable rate. This phenomenon provides the explanation for the observed differences in phospholipase sensitivity of stimulated platelets in prothrombin and factor X activation. If phosphatidylserine in the outer membrane is degraded by phospholipase $A_2$, such that the mole fraction phosphatidylserine in the membrane becomes less than 2.5%, still a considerable rate of prothrombin activation can be measured, whereas factor X activation is almost completely abolished.

Platelets stimulated with collagen plus thrombin expose 25% of their phosphatidylserine at the platelet outer surface [8], it can be calculated that the overall mole fraction phosphatidylserine in the outer monolayer of the platelet is at most 5%. A model phospholipid vesicle containing 5 mole % phosphatidylserine would show a ratio of the rates of prothrombin activation and factor X activation of 200:1. Platelets stimulated for 2 min with thrombin plus collagen give a rate ratio of 15:1. So the phosphatidylserine exposed at the outer surface of the collagen plus thrombin stimulated platelet, cannot be randomly distributed in the outer monolayer of the platelet membrane. Domains with higher mole percentage phosphatidylserine must exist in the outer monolayer of the platelet membrane after stimulation with collagen plus thrombin. Otherwise the observed ratio of prothrombin and factor X activation rates cannot be explained. Most of these phosphatidylserine enriched domains are formed within 3 min after platelet stimulation, as can be concluded from the time course of generating factor X

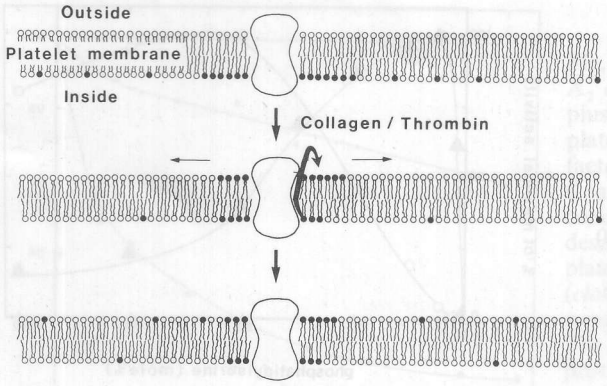


**Fig. 7.** Model for the generation of a platelet procoagulant surface. Phosphatidylserine molecules are indicated by *closed dots*

converting activity. After 3 min platelet stimulation, most of the procoagulant surface for prothrombin activation has still to be made (Fig. 4). Based on these observations, we propose that the phosphatidylserine, exposed at the surface of collagen plus thrombin stimulated platelets, is not homogeneously distributed in the outer monolayer of the platelet membrane. Domains with high phosphatidylserine density, which rapidly appear at the surface of stimulated platelets, are measured predominantly with the factor X activating system and to a minor extend with the prothrombin activating system. Domains with low phosphatidylserine, which appear slower, can only be measured by the prothrombin activating system. If these lateral phase separations in the outer monolayer of the membrane occur during platelet activation, other components may be required to induce or maintain them. In Figure 7 a model is proposed, in which after triggering with collagen plus thrombin the first phosphatidylserine molecules that appear at the platelet surface, are clustered by membrane proteins. The progressive transbilayer movement after prolonged activation saturates the clustering proteins with phosphatidylserine. Phosphatidylserine molecules appearing thereafter are diluted over the platelet membrane, so forming a domain with low phosphatidylserine density favourable for factor II activation.

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# Discussion

ÖBRINK: You have shown that there is an increase in phosphatidylserine on the outside of the platelets during activation. Do you know if that is a function of flip-flop or if it is insertion of new membrane into the plasma membrane? It could be membrane vesicles inside the cells which fuse with the plasma membrane.

HEMKER: We think it's flip-flop, because for the phosphatidylserine that appears outside, phosphatidylcholine disappears at the same time. That's one thing. The other thing is that it can, of course, be explained by diffusion of vesicles with the outer surface of the platelet. But as far as I've been told, the asymmetry of the vesicles in the platelets is just the other way round from the asymmetry of the membrane of the platelet itself. So if this is the outer membrane and this is the vesicle, then the phosphatidylserine will be at the inside of the outer membrane and at the outside of the vesicle. So upon fusion, you will have the same situation as if there were no flip-flop. This of course is what we also find, because we don't find phosphatidylserine at the outside if we trigger the platelets with thrombin, in which case there is fusion.

FROJMOVIC: Two small points. One is that Neville CRAWFORD has been publishing papers on sonication of platelets to essentially try to fractionate membranes, and they hypothese that they may be separating surface connecting system from the surface itself, and that is showing very distinct biochemical properties. I just leave this to you as a possible thought, that instead of flip-flop, perhaps you could have exchange form the surface connecting system to the membrane which is being induced. Can you rule that out? Part B, is there a flop-flip?

HEMKER: I'm not quite sure that I got the gist of your first question. As far as we know, we have no reason to assume that the surface connecting system has a different phospholipid composition than the overall membrane. Secondly, indeed there is flop-flip.

CAEN: You mentioned no effect whatsoever when using indomethacin. Have you tried the collagen-thrombin interaction with some compound which may affect calcium and even phosphatidic acid?

HEMKER: No, we didn't try.

NURDEN: Let me see if I remember correctly. Both Ken MANN and Dr MAJERUS were proposing that there was a protease activity in platelets that may be responsible for at least some transformation of Factor V into Factor V-a, and it was hypothesized this might be the calcium-dependent protease. Now, one possible explanation for the deficit of glycoprotein I and V and

the 17,000 glycoprotein in these platelets is that there is an abnormal, at least focal, protease activity in Bernard-Soulier megakaryocytes or platelets. It could be this protease. It would therefore be very interesting to look at the Factor V in the platelets and its release from Bernard-Soulier platelets to see if there's any abnormality there.

HEMKER: That was precisely the reason why yesterday I asked if you know anything about the release of Factor V in Bernard-Soulier platelets.

SOULIER: May I ask you something, Dr. HEMKER? In 1975 WALSH said that in Bernard-Soulier syndrome there was no collagen activation at the platelet level. Perhaps collagen and thrombin are inadequate when there is a lack GPI. But he also said that there was no Factor XI at all on the platelet, and when mentioning other proteins, you didn't make any allusion to that. What is your opinion about that?

HEMKER: In fact, we have been doing these experiments with purified coagulation factors, and we don't have any purified Factor XI around, so that's the reason I didn't study it.

MILTON: Presumably the reason why an asymmetry of lipids exists in the membrane is that there is an energy barrier there that stops the lipids going across to other side. I want to know if that energy barrier is a function of the conformation of the membrane. I'm just wondering if you completely change the conformation of the membrane, it may be preferable for the membrane lipids to come back over, just from the energy point of view.

HEMKER: A membrane that is symmetrical is a more likely one than one that is asymmetrical. I keep asking membrane biochemists, what is the source of the asymmetry? I get all kinds of answers, but not very strict ones up to this moment. It might well be, it's very probable indeed, that there is an interaction between the inside of the membrane and the proteins that are described just lying against the inside of the membrane. We see all kinds of reactions in activated platelets, and among them might be this interaction between internal proteins and membrane disruption, followed specifically by phosphatidylserine externalization. But on the other hand, that wouldn't explain why it's a reversible process.

WHITE: In that respect, certainly there are changes in the submembrane area and submembrane proteins. But the interesting aspect of that is in fact, they're quite reversible. We've done a number of studies on activating platelets usually by aggregating them with ADP and taking apart the aggregated platelets with something like prostacyclin, and then, those platelets completely recover their shape, but they're refractory. As soon as you dissociate the platelets, the phosphorylation will disappear, and if you activate them again, it'll redevelop along with the now triton resistant cores, and

then I would suspect that the cycling of those proteins and protein events will correlate with the flip-flop or flop-flip of the membrane.

HEMKER: I think that's a distinct possibility, and I would just like to suggest that if you do this type of experiment again, you should not only try collagen and thrombin but also a combination of the two, and whenever you come across situations in which the combination of the two does something which is absolutely different from one of the two alone, then you are very probably in the region where the phenomena you observe have something to do with the flip-flop.