The interaction between blood platelets and blood coagulation factors

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The Interaction between Blood Platelets and Blood Coagulation Factors

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Summary: *Prothrombin activation and intrinsic factor X activation are greatly stimulated by the presence of negatively charged phospholipids and non-enzymatic protein cofactors (factor Va for prothrombin- and factor VIIIa for factor X activation). Studies in model system show that phospholipids promote the assembly of the prothrombin- and factor X activating complexes and that the non-enzymatic protein cofactors factor Va and factor VIIIa enhance the catalytic activity of factor Xa and factor IXa, respectively. In vivo, blood platelets likely provide the procoagulant membrane surface required for coagulation factor activation. The effect of human platelets in prothrombin- and intrinsic factor X activation was determined in assay systems with purified coagulation factors. Compared with unstimulated platelets, platelets triggered with collagen plus thrombin showed a 10–20fold activity increase in prothrombin and factor X activation. Treatment of the stimulated platelets with phospholipase dA_2 abolished both activities. This indicates, that upon platelet stimulation, phospholipids (particularly phosphatidylserine) exposed at the platelet outer membrane are an essential component of the platelet procoagulant surface.*

Zusammenfassung: *Interaktion zwischen Blutplättchen und Blutgerinnungsfaktoren*

Die intrinsische Aktivierung von Faktor X und die Aktivierung von Prothrombin werden in Anwesenheit von negativ geladenen Phospholipiden und Protein-Kofaktoren (Faktor $VIII_a$ für die Aktivierung von Faktor X und Faktor V_a für die von Prothrombin) stark beschleunigt.

Modelluntersuchungen zeigten, daß Phospholipide die Bildung von Prothrombin- und Faktor X-aktivierenden Komplexen fördern und daß die Kofaktoren V_a und $VIII_a$ die katalytische Wirkung von Faktor X_a bzw. Faktor IX_a erhöhen. In vivo sorgen höchstwahrscheinlich Blutplättchen für die prokoagulierenden Membranstrukturen, welche für die Aktivierung von den Gerinnungsfaktoren benötigt werden.

Der Einfluß von Blutplättchen auf die Aktivierung von Prothrombin und Faktor X wurde mit gereinigtem Gerinnungsfaktoren getestet. In Vergleich zu den nichtstimulierten Blutplättchen, zeigten jene, die mit Kollagen und Thrombin stimuliert wurden, eine 10- bis 20fache höhere Aktivität bei der Aktivierung von Prothrombin und Faktor X. Werden die stimulierten Blutplättchen mit Phospholipase A_2 behandelt, so führt dies zu einer Zerstörung der Aktivität.

Dies zeigt, daß die Phospholipide (besonders Phosphatidylserin), welche sich nach Stimulierung bei den Plättchen auf der Außenmembran befinden, wesentliche Komponenten der prokoagulierenden Oberfläche bilden.

Key words: *Blood coagulation factors, interaction with platelets · Platelets, interaction with coagulation factors*

1. The blood coagulation cascade

Blood coagulation is caused by a series of sequential proenzyme-enzyme conversions. In a mixture of trypsinogen and chymotrypsinogen the addition of a small amount of enterokinase would cause the activation of one enzyme, that then, in its turn would activate the other, thus causing a primitive cascade. In the blood coagulation the mechanism is much more refined. In principle a three step cascade does explain thrombin formation: tissue thromboplastin converts factor VII into factor VII_a; factor VII_a activates factor X; factor X_a activates prothrombin to form thrombin. In this mechanism there is a reinforcement loop involved because factor VII_a also activates factor IX, and factor IX_a activates factor X. This loop causes appreciable amounts of factor X to be activated, even in the presence of only minute amounts of tissue thromboplastin (Fig. 1). Apart from the activation by factor VII_a, factor IX can also be activated by factor XI_a that itself is formed by the so called contact activation. This mechanism explains why platelet free plasma without a trace of tissue thromboplastin will clot in a glass tube. Its physiological significance is less clear.

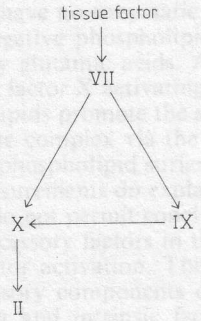

Fig. 1: The basic blood coagulation sequence.

Several reactions in the blood coagulation reaction sequence require the presence of so-called non-enzymatic cofactors. These cofactors are divided in two classes: 1, protein cofactors, 2, 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. Their place in the coagulation scheme is indicated in Fig. 2.

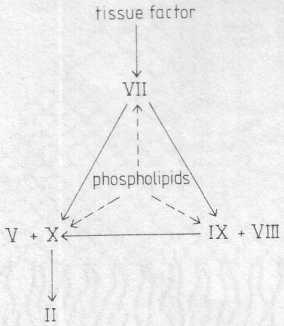

Fig. 2: Blood coagulation sequence with modulators.

Table 1: Phospholipid dependent reactions in blood coagulation.

Enzyme	Substrate	Protein cofactor
Factor VII_a	Factor IX	Tissue factor apoprotein
Factor VII_a	Factor X[a)]	
Factor IX_a	Factor X[b)]	Factor $VIII_a$
Factor X_a	Factor II	Factor V_a

a) The extrinsic factor X activation.
b) The intrinsic factor X activation.

Table 2: Effect of cofactors on the rate of thrombin formation.

	Rate of thrombin formation
Factor Xa	1
Factor Xa + Ca^{2+}	3
Factor Xa + Ca^{2+} + PL	68
Factor Xa + CA^{2+} + factor Va	560
Factor Xa + Ca^{2+} + PL + factor VA	20000

PL = phospholipid. The velocity obtained by factor X_a alone is arbitrarily defined as unity.

A quantitative study of these reactions has become possible because the proteins involved can now be obtained in a pure state in large enough quantities and because the products of the reactions can be determined spectrophotometrically with high accuracy due to the development of the so called chromogenic substrates (ref. [1]).

Table 2 shows the effect of factor Va, Ca^{2+} and phospholipid vesicles on the rate of prothrombin activation. 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 20000fold 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.

There exist extensive similarities between prothrombin and intrinsic factor X activation. The substrates in both reactions (prothrombin and factor X) are highly homologous vitamin K dependent proteins. At their N-terminal end they contain γ-carboxy glutamic acid residues, responsible for the Ca^{2+} dependent binding to negatively charged phospholipid surfaces. The enzymes (factor Xa and factor IXa), are also homologous vitamin K dependent proteins. The protein cofactors (factor Va and factor VIIIa) have no enzymatic activity and have a high affinity for negative phospholipid surfaces, although they lack γ-carboxy glutamic acids. A general model for the prothrombin and factor X activating complexes is shown in Fig. 3. Phospholipids promote the assembly of the enzyme-cofactor-substrate complex via the interactions of clotting factors with the phospholipid surface and via direct interaction. The rate measurements do explain the necessity of the cofactors but they 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

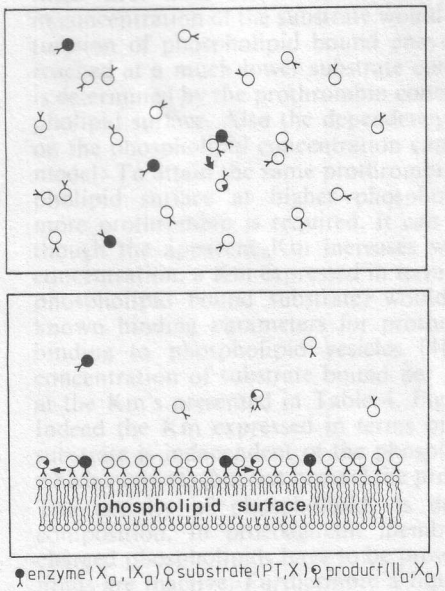

Fig. 3: Model for the prothrombin and factor X activating complexes. The phospholipid is represented as a bilayer and contains negatively charged headgroups.

Table 3: Enzyme kinetic constants of prothrombin and factor X activation.

Prothrombin activator	Km prothrombin (μmol/l)	Vmax ($IIa \cdot min^{-1} \cdot Xa^{-1}$)
Xa, Ca^{2+}	84	0.68
Xa, Ca^{2+}, phospholipid	0.06	2.3
Xa, Ca^{2+}, phospholipid, Va	0.21	1919
Factor X activator	**Km factor X (μmol/l)**	**Vmax ($Xa \cdot min^{-1} \cdot IXa^{-1}$)**
IXa, Ca^{2+}	181	0.01
IXa, Ca^{2+}, phospholipid	0.06	0.0025
IXa, Ca^{2+}, phospholipid, VIIIa	0.063	500

activation [2, 3]. This 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. Prothrombin and factor X activation are very inefficient processes. The turnover number of substrate molecules by the completely saturated enzyme (Vmax) is very low and the substrate concentration causing half maximal reaction velocity Km for prothrombin and factor X are considerably higher than the respective plasma concentrations (prothrombin 2 μmol/l, factor X 0.2 μmol/l). The presence of phospholipid plus Ca^{2+} causes, in both complexes, a drastic drop of the Km to values below the plasma concentrations, while the protein cofactors greatly increase the Vmax.

2. Why are phospholipids necessary?

Since this paper deals with platelet involvement in blood coagulation we will not further discuss the effects of the protein cofactors. Both in prothrombin and intrinsic factor X activation, phospholipids cause a drastic drop of the Km for prothrombin and factor X, respectively. Furthermore the Km shows an increase with a rise of the phospholipid concentration present in the reaction mixture (Table 4). So the Km, measured in the presence of phospholipid, must be an apparent Km and is not, as such, a reaction constant of the activation under study. A model that can explain both the drop of Km and the apparent character observed in the presence of phospholipid is presented in Fig. 4. In free solution, enzyme (factor Xa or factor IXa) and substrate (prothrombin or factor X) have a low affinity for each other, and therefore show a high Km. With negatively charged phospholipids present in the reaction mixture, both the enzymes

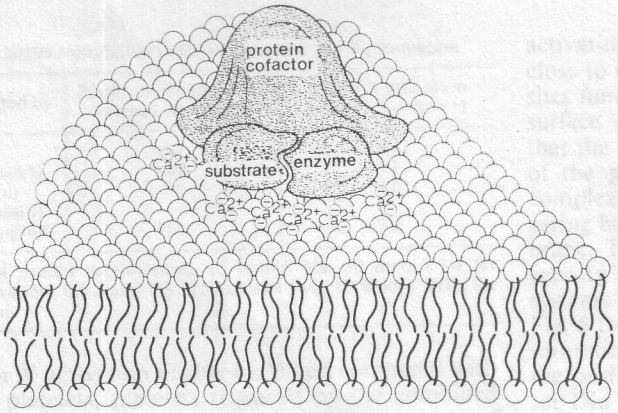

Fig. 4: Possible mode of action of phospholipids in blood coagulation.

Table 4: Effect of phospholipid concentration on Michaelis constants.

Prothrombin activation		Intrinsic factor X activation	
Phospholipid (μmol/l)	Km app (μmol/l)	Phospholipid (μmol/l)	Km app (μmol/l)
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

app = apparent.
In this experiment no factor Va or factor VIII$_a$ was added.

and substrates bind to the phospholipid surface. This makes a reaction possible between bound enzyme and bound substrate and thus favours the number of collisions between these two. This causes the same effect as an increase in concentration of the substrate would do. Half maximal saturation of phospholipid bound enzyme with substrate, is reached at a much lower substrate concentration because it is determined by the prothrombin concentration at the phospholipid surface. Also the dependency of the apparent Km on the phospholipid concentration can be explained by this model: To attain the same prothrombin density at the phospholipid surface at higher phospholipid concentrations, more prothrombin is required. It can be expected that, although the apparent Km increases with the phospholipid concentration, a Km expressed in terms of surface density of phospholipid bound substrate, would be constant. Using known binding parameters for prothrombin and factor X binding to phospholipid vesicles [4], we calculated the concentration of substrate bound per μmol/l phospholipid, at the Km's presented in Table 4. Fig. 5 shows the results. Indeed the Km expressed in terms of phospholipid bound substrate is independent of the phospholipid concentration, which supports (but not proves) the proposed model.

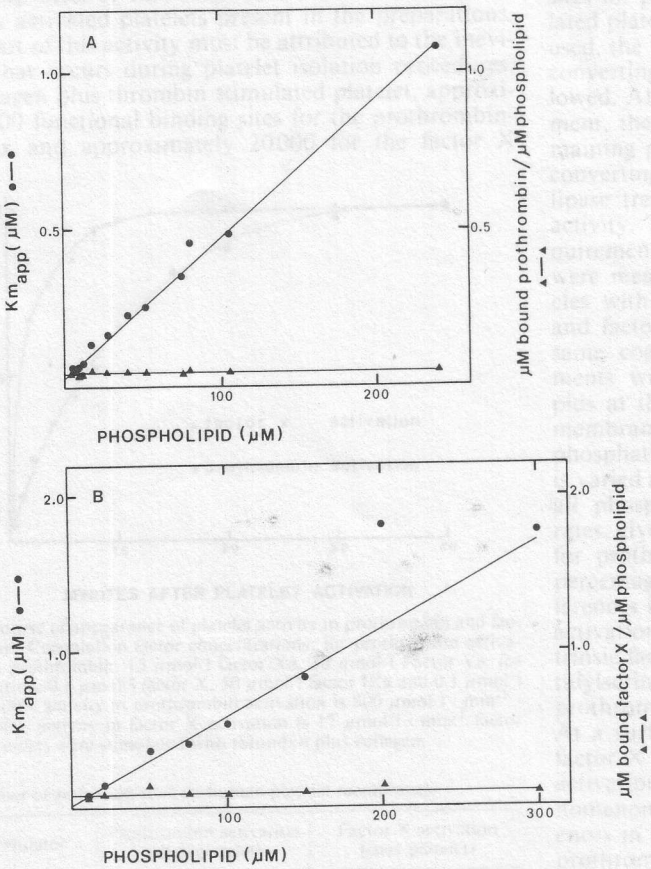

Fig. 5: Effect of phospholipid concentration on the apparent Km, and the Km expressed in substrate surface density for prothrombin (**A**) and intrinsic factor X activation (**B**).

The activity of phospholipids is dependent upon their composition. In procoagulant membranes, net negatively charged phospholipids have to be present. Neutral phospholipids are inactive. Furthermore a liquid crystalline state of the membrane is required for an optimal function in blood coagulation activity [5].

3. Platelets as a source of procoagulant surface

Under physiological conditions wounded cells and intact platelets provide the phospholipid surface required for prothrombin and intrinsic factor X activation. The procoagulant activity of platelets is known as platelet factor 3. We will not discuss here the possibility that platelets initiate contact activation [6, 7]. Intact non-stimulated platelets are however inactive in clotting factor activation (Table 5). In model experiments, rates of prothrombin activation were determined 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.

Table 5: Phospholipid composition in prothrombinase.

Vesicle composition (mol %)						Thrombin formation (nmol/l IIa · min^{-1})
PS	PI	PC	PE	Sph	Cholesterol (w/w)	
–	–	50	30	20	25	34
–	3	47	30	20	25	374
10	3	37	30	20	25	1191
Intact platelets						23
Platelet phospholipid extract						1168
Platelet sonicate						1176

PS = Phosphatidylserine, PI = Phosphatidylinositol, PC = Phosphatidylcholine, PE = Phosphatidylethanolamine, Sph = Sphingomyelin. Vesicle and platelet phospholipid concentration is 1 μmol/l, prothrombin 4 μmol/l, factor Va 30 nmol/l, factor Xa 15 nmol/l.

In contrast, artificial vesicles with a phospholipid composition identical to that found in platelets and also vesicles made from a complete platelet phospholipid extract, give high rates of prothrombin activation. Table 5 therefore shows that the net negatively charged phosphatidylserine is essential prothrombin activation. It is important to note however that phosphatidylserine is 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, small rate enhancements are measured in assay systems for prothrombin and

6: Role of platelet stimulation in thrombin and factor X_a formation.

Platelets stimulated by	Prothrombin activation (nmol/l IIa · min^{-1}	Factor X activation (nmol/l X_a · min^{-1})
–	34	2.3
Thrombin (1.3 nmol/l)	40	3.1
Collagen (10 μg/ml)	98	18.6
Thrombin (1.3 nmol/l) plus collagen (10 μg/ml)	352	47.3

Concentrations: 4 μmol/l prothrombin, 15 nmol/l Xa, 30 nmol/l Va for prothrombin activation; 0.5 μmol/l X, 150 nmol/l IXa, 15 nmol/l VIIIa for factor X activation.

intrinsic factor X activation (Table 6). When compared with unstimulated platelets, platelets triggered by the combined action of collagen plus thrombin show a 10fold increase of activity in prothrombin activation and 20fold 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 (flip-flop) 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. The amounts of calculated sites (Table 7) for unstimulated and thrombin activated platelets are relatively low as compared to the number found with collagen plus thrombin stimulated platelets. It is not clear whether unstimulated platelets, under physiological circumstances, indeed have a low (but significant) amount of functional binding sites, or that they have none, but that there are partially activated platelets present in the preparations. At least 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 26000 functional binding sites for the prothrombinase complex and approximately 20000 for the factor X

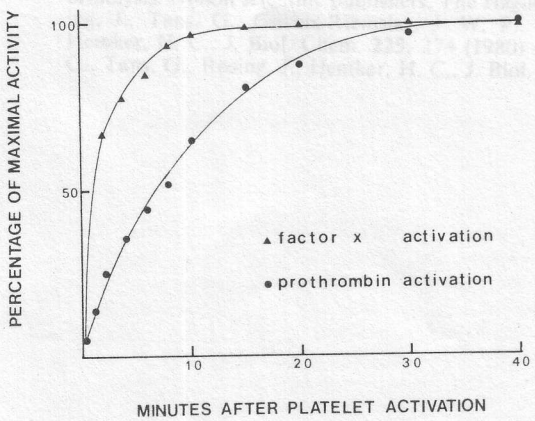

Fig. 6: Time course of appearance of platelet activity in prothrombin and factor X activation. Coagulation factor concentrations: for prothrombin activation, 4 μmol/l prothrombin, 15 μmol/l factor Xa, 30 μmol/l Factor Va, for factor X activation, 0.5 μmol/l factor X, 50 μmol/l factor IXa and 0.1 μmol/l factor VIIIa. 100% activity in prothrombin activation is 800 μmol/l · min^{-1} · IIa formed. 100% activity in factor X activation is 17 μmol/l · min^{-1} factor Xa formed. Platelets were stimulated with thrombin plus collagen.

Table 7: Number of activation sites on human platelet membranes.

Platelet stimulator	Prothrombin activation (sites/platelet)	Factor X activation (sites/platelet)
–	2500	900
Thrombin	3000	1200
Thrombin + collagen	26000	19000

activating complex are calculated. These numbers are so close to each other, that they strongly suggest that, the same sites function in prothrombin and factor X activation at the surface of stimulated platelets. This observation suggests that the essential component for 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 Fig. 6 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 maximum value after 9 min. The observed differences between the time courses at first sight seem to contradict the idea, that the essential component of the procoagulant surface of stimulated platelets is exclusively phosphatidylserine. We do think that they can be easily accomodated in our model. This follows a.o. from phospholipase digestion experiments. Platelets were stimulated with collagen plus thrombin till maximal procoagulant activity is reached and subsequently treated with N. naja phospholipase A_2, an enzyme known to degrade phospholiped molecules exposed at the outer platelet surface. 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. It showed that the factor X converting activity of platelets is more sensitive to phospholipase treatment than the platelet prothrombin converting activity. This could be related to different phospholipid requirements. Therefore, prothrombin and factor X activation were 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 were 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 at the expense of phosphatidylcholine and the overall phospholipid concentration is kept at 2 μmol/l. The rates, given as percentage of the maximal activity, measured for prothrombin and factor X activation at varying mole percentage phosphatidylserine, are given in Fig. 6. Large differences in phosphatidylserine requirement for prothrombin activation and factor X activation are observed. For the intrinsic factor X activation, a molar 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. 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 mol% 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 show a valued of 15 : 1 for this ratio. Therefore 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 in order to make the observed ratio of prothrombin and factor X activation rates possible. 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 converting activity. After 3 min platelet stimulation, most of the procoagulant surface for prothrombin activation has still to be made (cf. Fig. 6). 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 density, which appear slower, can only be measured by the prothrombin activating system. It is not yet known what mechanisms and components govern the lateral distribution of phospholipid species in the outer surface of the platelet membrane. The model shown in Fig. 7 shows a membrane protein that induces a flip-flop reaction for phosphatidylserine (PS) molecules and thus acts as a source of PS at the outer surface. Such a protein also might induce a clustering of PS molecules around the source. In later stages of the process the PS might diffuse more freely in the outer platelet surface, and in this way form areas favourable for the prothrombin activation. Since the factor X_a generated near the source acts as an enzyme, it is a possibility that this enzyme does not leave the platelet atmosphere.

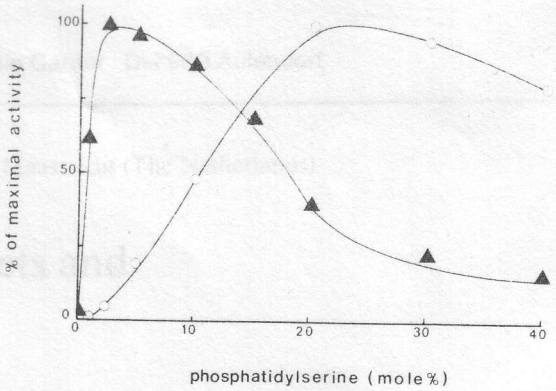

Fig. 7: The effect of phospholipid vesicles with varying mole percentage phosphatidylserine in prothrombin (closed symbols) and factor X activation (open symbols). Hundred percent activity in prothrombin activation is 2900 nmol/l · min^{-1}, 100% activity in factor X activation is 71.5 nmol/l · min^{-1}.

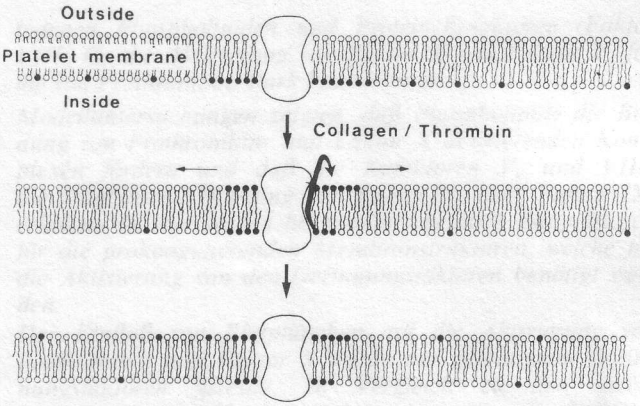

Fig. 8: Model for the generation of a platelet procoagulant surface. Phosphatidylserine molecules are indicated by closed dots.

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