The role of platelet phospholipids in prothrombin and factor X activation

Citation for published version (APA):

van Rijn, J. L. M. L. (1984). *The role of platelet phospholipids in prothrombin and factor X activation*. [Doctoral Thesis, Maastricht University]. Rijksuniversiteit Limburg. <https://doi.org/10.26481/dis.19840913jr>

Document status and date:

Published: 01/01/1984

DOI:

[10.26481/dis.19840913jr](https://doi.org/10.26481/dis.19840913jr)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

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- The final author version and the galley proof are versions of the publication after peer review.
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THE ROLE OF PLATELET PHOSPHOLIPIDS
IN PROTHROMBIN AND FACTOR X ACTIVATION

krips repro meppel

THE ROLE OF PLATELET PHOSPHOLIPIDS IN PROTHROMBIN AND FACTOR X ACTIVATION

PROEFSCHRIFT

ter verkrijging van de graad van
Doctor in de Geneeskunde aan de
Rijksuniversiteit Limburg te Maastricht,
op gezag van de Rector Magnificus
Prof. Dr. H. C. Hemker,
volgens het besluit van het College van Dekanen
in het openbaar te verdedigen
in de Aula van de Universiteit
op donderdag 13 september 1984
des namiddags te 16.00 uur

door

Johannes Leonardus Maria Laurentius van Rijn

geboren te Haarlem

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Dit onderzoek werd uitgevoerd op de afdeling Biochemie van de Rijksuniversiteit Limburg in samenwerking met: Jan Rosing, Gerbrand van Dieijen, Rob Zwaal, Coen Hemker, Edouard Bevers, Paul Comfurius en José Govers-Riemslog. De financiële steun werd verleend door de Stichting voor Medisch Wetenschappelijk Onderzoek (FUNGO-ZWO).

Het proefschrift werd getypt door Mariet Molenaar.

Aan mijn ouders
Voor Eveline en Calijn

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ABBREVIATIONS

DEAE	diethylaminoethyl
EDTA	ethylenediamine tetraacetic acid
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Me ₂ SO	dimethylsulfoxide
PA	1,2-dioleoyl-sn-glycero-3-phosphate
PC	1,2-dioleoyl-sn-glycero-3-phosphocholine
PG	1,2-dioleoyl-sn-glycero-3-phosphoglycerol
PS	1,2-dioleoyl-sn-glycero-3-phosphoserine
phospholipase A ₂	phosphatide 2-acylhydrolase
p-NPGB	p-nitrophenyl-p'-guanidinobenzoate hydrochloride
QAE	quaternary aminoethyl
RVV-X	purified factor X activator from Russell's viper venom
SP	sulphopropyl
STI	soybean trypsin inhibitor
S2238	H.D.phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide hydrochloride
S2337	N-benzoyl-L-isoleucyl-L-glutamyl-(piperidyl)-glycyl-L-arginyl-p-nitroanilide hydrochloride
Tris	tris(hydroxymethyl)-aminomethane

CHAPTER 1

INTRODUCTION

Blood platelets from mammalian species are biconvex disks with a diameter of 2-3 μm and are formed in the bone marrow by fragmentation of megakaryocytes. The platelet count in peripheral blood is about 250,000/ μl and the life-span of human platelets is usually 7-10 days. Blood platelets participate in a number of physiological processes. In vitro and in vivo studies have demonstrated that platelets play an important role in a variety of events such as hemostasis, thrombosis, atherosclerosis, inflammation, phagocytosis, wound healing and transplant rejection.

Platelets are involved in the process of hemostasis which is initiated by vascular injury. When, as a result of vascular damage, the collagen in the subendothelial layer of the vessel wall is exposed to the blood, platelets instantaneously adhere to the exposed collagen fibres (fig. 1). The adhering platelets become activated to produce and release components which stimulate the aggregation of platelets from the blood stream with the adhered ones, thereby building up a fragile platelet plug (primary hemostatic plug). This platelet mass is consolidated by strands of fibrin, the ultimate product of the reactions of the coagulation cascade. In the end the fibrocellular mass, in which also other blood cells are trapped, retracts into the wound. As indicated in figure 1, platelets are not only involved in the formation of a primary hemostatic plug, but are also able to promote the activation of several clotting factors. Clotting factor activation is initiated by vascular damage and leads to the formation of thrombin that catalyses fibrin formation and activates platelets. Activated platelets form a procoagulant surface, at which clotting factor activation is facilitated.

Platelet functions in primary hemostatic plug formation. Apart from adhesion to the subendothelium of the vessel wall, all platelet functions required to form a primary plug are performed only after excessive changes in platelet shape and ultrastructure. The morphology of platelets, as viewed through the electron microscope, has advanced the understanding of their function in hemostasis (1). Being a fragment of the megakaryocyte's cytoplasm, the platelet has no nucleus. Endoplasmic reticulum and Golgi apparatus are seen only occasionally. The platelet membrane is rich in glycoproteins, which become

Fig.1. The role of platelets in the formation of a stable thrombus.
(Taken from reference 50)

visible at staining as a fuzzy surface coat, called glycocalyx. These glycoproteins have been found to function as receptors for platelet stimuli and are involved in platelet aggregation and the adhesion of platelets to factor VIII-von Willebrand factor on vessel wall subendothelium (2-5). Subjacent to the plasma membrane, a circumferential bundle of long microtubules supports the platelets asymmetric form (fig. 2). Many indentations of the plasma membrane penetrate deep in the platelet's interior and form the so-called surface connected- or open canalicular system. This system facilitates the interaction of the platelet membrane with intracellular organelles and with plasma-borne substances. Not connected with the open canalicular system is another system of narrow, membrane-limited tubules. This so-called dense tubular system has been shown to be the site where intracellular calcium, essential for platelet activation, is sequestered (6). In the platelet, at least two types of storage granules can be shown by electronmicroscopy; alpha granules and (electron) dense bodies, each surrounded by a membrane. Among the substances which are stored in the dense bodies are serotonin, calcium, ATP, ADP and pyrophosphate. Subcellular fractionation studies revealed that the alpha granule population is heterogeneous and consists of a lysosomal and a nonlysosomal fraction (7). Alpha granules of the nonlysosomal class contain a number of proteins several of which are involved in coagulation such as platelet factor 4 (a heparin-neutralizing protein), factor V, factor VIII related antigen, fibronectin and fibrinogen.

Activation of platelets. Platelets can be stimulated by a variety of compounds including collagen, thrombin, ADP, epinephrine, immune complexes, serotonin,

Fig. 2 Schematic view of the blood platelet. (Taken from reference 50)

arachidonic acid and prostanoids. For several of these stimulating agents specific receptors were found to be localized in the platelet membrane (8). With almost all platelet stimuli the first response observed after triggering is a rapid shape change of the platelet, consisting of a rounding up of the central body of the cell and an extrusion of thin pseudopodia (9). Shape change is accompanied by an internal centralization of the storage granules which become enclosed by a ring of microtubules (10). The timing of this movement precedes or coincides with secretion of granular contents into the open canalicular system occurring after fusion of the granular membranes with the plasma membrane. Release of nonlysosomal alpha-granule proteins is induced by all of the agents that evoke dense body release. Small differences between dense body and alpha-granule release were found in the time course of release after the addition of the stimulus and in the required stimulus concentration (11). As compared with the discharge of the other granular contents, the release of lysosomal proteins is delayed and less complete (12). Less information is available about the function of secreted compounds. Serotonin helps in the arrest of bleeding by its vasoconstricting property. In vitro, ADP and serotonin are able to stimulate platelets, which in vivo may contribute to the accumulation of platelets in the primary hemostatic plug. Although both are present in the plasma, the secreted factor V and fibrinogen may be required for the local process of coagulation. Fibrinogen is essential for platelet aggregation to occur (13). Aggregation requires extracellular Ca^{2+} and is dependent on the shear flow of the medium in which the platelets are present. Aggregation is not necessarily a prerequisite for the release reaction and vice versa. ADP and epinephrine at low concentrations induce primary aggregation without release, whereas platelets which are not subject to shear stress do not aggregate but release normally (14). Moreover platelets from patients

with Glanzmann's thrombosthenia fail to aggregate but do release in response to stimulation (3).

Intracellular responses upon platelet activation. Upon stimulation of platelets endogenous phospholipases are activated, resulting in the release of arachidonic acid from platelet phospholipids. Part of the arachidonic acid is converted into 12-hydroxyeicosatetraenoic acid by the platelet enzyme arachidonate lipoxygenase, an enzymatic pathway which is reported to be essential for both the platelet aggregation and the release of serotonin (15). Another pathway is the conversion of arachidonic acid by platelet cyclooxygenase into the endoperoxides prostaglandin G_2 and prostaglandin H_2 . These endoperoxides are further transformed to thromboxane A_2 , which is a powerful initiator of platelet aggregation (16). The relative importance of thromboxane A_2 in the sequence of selfperpetuating platelet aggregation is not yet established, although it is known that cyclooxygenase plays a central role in the activation of platelets. Platelets which are treated with aspirin show a depressed release and aggregation reaction due to inhibition of cyclooxygenase (17). The action of cyclooxygenase is controlled by the availability of free arachidonate. Although arachidonic acid is one of the most abundant fatty acids in the platelet, almost all of it is esterified to the glycerol backbone of phospholipids in the inner leaflet of the plasma membrane. The release of arachidonic acid from phospholipids occurs either by the direct action of phospholipase A_2 liberating the free acid or by the sequential action of the phosphatidylinositol specific phospholipase C and diglyceride lipase (18-20). Both phospholipase A_2 and phospholipase C require Ca^{2+} for their enzymatic activity, which led to the suggestion that the phospholipases are activated by the increased cytosolic Ca^{2+} concentration, which is found after platelet stimulation (21,22). Also for other platelet responses which occur upon stimulation, an increased cytosolic Ca^{2+} concentration is a prerequisite. Calcium is involved in the phosphorylation of proteins and increased contractile activity. Studies with the calcium ionophore A23187 suggest that both pseudopod formation and granule centralization results from a flux of intracellular calcium from the dense tubular system into the cytoplasm (23). Also prostaglandin G_2 and thromboxane A_2 are able to release calcium from this subcellular fraction, which led to the hypothesis that thromboxane A_2 triggers the platelet activation mechanism by acting as an ionophore (24).

Procoagulant activity. Apart from their function in primary plug formation, blood platelets are involved in several reactions of the coagulation cascade. Stimulated platelets form a so-called procoagulant surface at which the inter-

action between clotting factors is promoted and consequently the rate of clotting factor activation is increased. Platelets are reported to participate in four specific coagulation reactions:

1. Contact product-forming activity of ADP-stimulated platelets, which resides in the platelet surface and concerns the binding and subsequent activation of factor XII (25).
2. Collagen induced coagulant activity of platelets, which bind factor XI to high affinity binding sites at their surface, thereby promoting the activation of factor XI in the presence of kallikrein (26-28).
3. Procoagulant activity of collagen-stimulated platelets in the intrinsic factor Xa formation i.e. the activation of factor X by activated factor IX, factor VIII and Ca^{2+} (29).
4. Procoagulant activity of platelets in the activation of prothrombin by activated factor X, factor V and Ca^{2+} , often referred to as platelet factor 3 (29-31).

Whether phospholipids can account for the procoagulant activity of the platelets in prothrombin- and factor X activation, or that this activity arises from platelet proteins is a long-standing matter of debate. Although phospholipids highly promote both the factor X- and prothrombin activation (29), isolated platelet plasma membranes were found to be more procoagulant in a clotting assay (32). This was interpreted to mean that platelet factor 3 represents the activity of platelet membrane lipoprotein rather than membrane phospholipids alone (33). Miletich et al. (34-36) have demonstrated binding of human factor Xa to a receptor on isolated platelets, which was later identified as factor Va (37). Factor Va binds with high affinity to an equal number of binding sites on the surface of non-stimulated- and thrombin-stimulated platelets, which indicates that the release reaction is not a prerequisite for factor Xa, factor Va binding (38,39). Factor Xa bound to factor Va on the platelet surface, catalyzes the activation of prothrombin efficiently, comparable with the activation of prothrombin in the presence of phospholipid bilayers (35,40,41). To exhibit procoagulant activity, membranes must contain negatively charged phospholipids (42-45), which in the resting platelet are almost exclusively located in the platelet interior (46). The question arises whether negatively charged phospholipids are of importance in the reactions on the platelet surface. To answer this question more insight is required about the mechanism by which phospholipids act in the activation of prothrombin and factor X.

Fig. 3 Schematic model of the enzyme-substrate complexes for prothrombin and intrinsic factor X activation.
(Taken from ref. 45)

Activation of prothrombin and factor X. Prothrombin- and intrinsic factor X activation have many features in common (for a review see ref. 47). Both the substrates (prothrombin and factor X) and the enzymes (factor Xa and factor IXa) are vitamin K dependent proteins that contain γ -carboxyglutamic acid residues, which bind via Ca^{2+} -bridges to negatively charged phospholipid surfaces. Both the enzymes are serine proteases and the substrates are zymogens of serine proteases. The protein cofactors (factor Va and factor VIIIa) have no enzymatic activity and have a high affinity for negatively charged phospholipid surfaces, although they lack γ -carboxyglutamic acid residues. Figure 3 shows a representation of the active enzymatic complex consisting of enzyme, substrate and protein cofactor bound at a phospholipid bilayer. Bovine factor X is a zymogen which exists as a two chain disulfide-linked glycoprotein. The light chain of the molecule contains the γ -carboxyglutamic acids, and remains covalently linked with the protein after activation of factor X into its protease form called factor Xa. The heavy chain, when cleaved by factor IXa at position $\text{Arg}_{51}\text{-Ile}_{52}$, contains the active site for proteolytic activity of the factor Xa molecule. The conversion of the glycoprotein prothrombin into its activated form thrombin requires two reaction steps. First, factor Xa cleaves off an activation peptide (fragment 1.2) by splitting an arginine-threonine bond leaving prethrombin 2. Prethrombin 2, which lost its γ -carboxyglutamic acids and has no protease activity, is further converted into thrombin after cleaving an arginine-isoleucine bond by factor Xa. In the absence of accessory components, i.e. phospholipid and protein cofactors, prothrombin and factor X activation are very inefficient processes.

Kinetic studies revealed that without accessory components the V_{max} is less than one turnover per min and that the values of the K_m for prothrombin and factor X are considerably higher than their plasma concentrations of 2 μM and 0.2 μM respectively (41,48). The presence of phospholipid plus Ca^{2+} causes, in both complexes, a drastic drop in the K_m to values far below the factor X and prothrombin concentration in plasma, while the protein cofactors strongly increase the V_{max} (41,48). The increase of the V_{max} of prothrombin activation by factor Va is ascribed, for a great deal, to alterations in the catalytic pathway leading from the enzyme-substrate complex to the ultimate product (41). Although it is generally accepted that phospholipid bilayers act as surfaces that promote the assembly of enzyme-cofactor-substrate complexes, the mechanism by which they decrease the K_m is still matter of discussion (49).

The present investigation. This thesis describes studies on the procoagulant properties of platelets and phospholipids present in platelet membranes. In particular, the role of platelets and phospholipids in prothrombin- and factor X activation was investigated, using systems with highly purified coagulation factors and chromogenic substrates to measure rates of thrombin and factor Xa formation. Chapter II presents a study on the changes in phospholipid composition in the outer leaflet of the plasma membrane of platelets after stimulation. This was done in order to find a relationship between platelet activity in prothrombin activation and the presence of procoagulant (net negatively charged) phospholipids in the outer monolayer of the platelet membrane.

In a more extended investigation, presented in chapter III, we employed reaction systems for prothrombin- and intrinsic factor X activation, which were made suitable for the detection of procoagulant phospholipids in membranes. With these reaction systems the appearance of platelet-procoagulant activity was followed after stimulating the platelets with collagen plus thrombin. The same reaction systems were also applied to platelets which were stimulated by the calcium-ionophore A23187. The appearance of platelet procoagulant activity was compared with the dose dependent induction of platelet aggregation, release of ATP and release of factor V. The results of this study are presented in chapter IV. The study described in chapter V concerns the role of factor VIII in the assembly of the complex for intrinsic factor X activation on bilayers which are composed of phospholipids present in platelets. Parameters of binding of factor IXa to these bilayers were inferred from kinetic measurements in the absence and presence of factor VIIa. Chapter VI describes the relationship of the kinetic parameters of prothrombin activation with the

amount of acidic phospholipids in membrane bilayers. Special attention is given to the role of factor Va in the assembly of enzymatic complexes on membranes with minute amounts of acidic phospholipids. Evidence is presented for a model about the mode of action by which phospholipids decrease the K_m for prothrombin.

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CHAPTER II

GENERATION OF PROTHROMBIN-CONVERTING ACTIVITY AND THE EXPOSURE OF PHOSPHATIDYLSERINE AT THE OUTER SURFACE OF PLATELETS.

SUMMARY

In the present paper we compare prothrombin-converting activities of platelets non-activated, or activated by collagen, thrombin or collagen plus thrombin in the absence and presence of added factor Va. In all experiments described, the rate of thrombin formation for platelets activated by the combined action of collagen and thrombin is greater than that of platelets stimulated by collagen or thrombin alone. The presence of added factor Va enhanced the rate of thrombin formation in all cases, but the higher activity observed with platelets stimulated by collagen plus thrombin remains. When platelets are activated by collagen plus thrombin in the presence of factor Xa and prothrombin, a lag period of approximately 10 min is observed before the rate of thrombin formation reaches a steady state. Addition of an excess of factor Va in this experiment reduces the lag time to 3 min. This lag period is interpreted as the time required to generate extra binding sites for the prothrombinase complex at the platelet surface. These extra sites explain the difference in thrombin formation rate between these platelets and platelets activated by either collagen or thrombin only.

The exposure of phospholipids at the platelet outer surface was studied with phospholipases after various activations of the platelets. It is demonstrated that activation by collagen plus thrombin is accompanied by increased susceptibility of platelet phospholipids towards phospholipase A₂. Among these degradable phospholipids are 25% of the phosphatidylserine and 30% of the phosphatidylethanolamine. On the other hand, little or no phosphatidylserine is exposed at the membrane exterior of thrombin-treated or control platelets. We propose that the exposure of phosphatidylserine at the outer surface of platelets activated with thrombin plus collagen is essential for the rate enhancement of thrombin formation observed under these conditions. The possibility of a transbilayer movement of phospholipids in the platelet membrane as a result of the activation process will be discussed.

INTRODUCTION

One of the major functions of platelets in the process of hemostasis is to provide a catalytic surface for the formation of the intrinsic factor-X-activating complex and the prothrombinase complex (for a review, see (1)). Nesheim et al. (2) have shown that there is no appreciable difference in catalytic efficiency of the prothrombinase complex when phospholipid vesicles plus factor Va are substituted for activated platelets. Rosing et al. (3) showed

that the role of phospholipids in the prothrombinase complex is to decrease the K_m for prothrombin below its plasma concentration, whereas factor Va strongly increases the V_{max} of the reaction (2,3). Recently similar results were found for the role of phospholipids in the intrinsic factor -X-activating complex (4).

It has been demonstrated that the cytoplasmic surfaces of both erythrocytes and platelets, as well as liposomes prepared from phospholipids present in the inner leaflet of the plasma membrane of these cells, possess a strong procoagulant activity (5). In contrast, resting platelets and intact erythrocytes, exposing only the outer membrane surface, do not reduce the clotting time in a one-stage prothrombinase assay. Moreover, liposomes prepared from phospholipids present in the outer leaflet of these membranes are also inactive. The difference in clot-promoting activity between inner and outer surface of these blood cells can be explained by the asymmetric distribution of phospholipids over the membrane bilayer. The vitamin-K-dependent coagulation factors Xa and II require the presence of negatively charged phospholipids for the calcium-mediated binding via their γ -carboxyglutamic acid residues (6-10). The phospholipid orientation in erythrocyte and platelet plasma membranes has been established using phospholipases (11-17) and chemical labels (18-21) and appears to be rather similar for both types of membranes. In both membranes, the negatively charged phospholipids, phosphatidylserine and phosphatidylinositol, are almost exclusively located in the inner leaflet, which provides an explanation for the procoagulant activity of the inner surface of both membranes.

In contrast to erythrocytes platelets have the unique property of undergoing morphological and functional changes following addition of ADP, collagen, thrombin and several other agents, resulting in shape change, aggregation and release of their granule contents. This may be accompanied by exposure of a procoagulant lipid surface. It has been suggested, however, that the procoagulant behaviour of platelets, activated by thrombin is due to the release and activation of factor V, which then forms the receptor for factor Xa on the platelet surface (22-25). In fact, addition of factor Va to unstimulated platelets results in the same number of factor Xa molecules bound as observed for platelets activated by thrombin (26). Furthermore, a patient has been described whose platelets show decreased binding of factor Xa, although the platelet factor Va content and phospholipid composition seemed to be normal, suggesting the involvement of a membrane protein in the factor-Va-factor-Xa receptor on the platelet outer surface (27,28). Also, it has been shown that

IgM paraprotein from a patient with macroglobulinemia reacts with negatively charged phospholipids and inhibits Ca-dependent binding of radiolabeled factor X and II to phospholipid vesicles but not to platelets (29). These observations cast some doubt on whether or not phospholipids are involved in the formation of the prothrombinase complex at the platelet surface. The purpose of the experiments described in this paper was to study the exposure of phospholipids at the platelet outer surface after various activation procedures in order to attempt to correlate alterations in platelet prothrombin-converting activity with reorientations in the platelet membrane phospholipids. The data presented here have implications for the participation of negatively charged platelet phospholipids in prothrombinase activity occurring at the platelet surface.

EXPERIMENTAL PROCEDURES.

Materials. [14 C]Serotonin creatinine sulfate (58 Ci/mol) was obtained from Amersham Corp. From Sigma Chemical Co. (St. Louis) were purchased: fat-free human serum albumin, nicotinamide adenine dinucleotide, reduced form (NADH), *Naja naja* venom, bee venom (*Apis mellifera*) and Russell's viper venom. Adenosine diphosphate (ADP) was from Boehringer, Mannheim. Collagen was from Hormon Chemie, München. S2238 was purchased from AB Kabi Diagnostica (Stockholm, Sweden).

Protein preparations. Factor-X-activator from Russell's viper venom was purified as described by Schiffman et al. (30) and was free of phospholipase activity. Bovine prothrombin was prepared according to the method of Owen et al (31). Prothrombin concentrations were calculated from the absorbance at 280 nm, using $A_{280}^{1\%} = 15.5$ (31), and 72 000 for the molecular weight of prothrombin (31). Bovine factor X was prepared from factor X_2 using factor-X-activator according to the method of Fujikawa et al. (32). Factor Xa concentrations were calculated after active-site titration according to Smith (33). Factor V was isolated as described by Esmon (34) with minor modifications (unpublished results of Lindhout et al.). Factor V (0.3 mg/ml) was activated with thrombin and the specific activity of the activated preparation was 500 units/mg, measured according to Kappeler (35). One unit is defined as the amount of factor V present in 1 ml normal bovine plasma. The concentration of factor V was calculated assuming a molecular weight of 330 000 for factor V (36). Thrombin was purified from prothrombin after activation with factor Xa as described by Rosing et al. (3). The thrombin concentration was determined by

active-site titration according to Chase and Shaw (37). Phospholipase A₂ from N.naja venom and bee venom, as well as sphingomyelinase C from Staphylococcus aureus were purified as described elsewhere (15).

Isolation of platelets. Fresh blood was drawn from healthy male volunteers who had not taken any medication for at least a week. Acid citrate dextrose was used as an anticoagulant. Platelet-rich plasma, obtained after centrifugation at 120xg for 10 min, was incubated with 1 μ Ci/[¹⁴C]serotonin/100 ml plasma for 20 min at room temperature resulting in 95% uptake. Platelets were isolated by centrifugation (1400xg, 15 min) and the pellet was gently resuspended in a calcium-free Hepes buffer pH 6.2, containing 137 mM NaCl, 2.68 mM KCl, 10 mM Hepes, 1.7 mM MgCl₂, 25 mM glucose and 0.5 mg fat-free human serum albumin/ml. Cells were washed twice and finally resuspended in the same buffer at pH 7.4. Cell counts were carried out using a Coulter Counter. All platelet handling was done at room temperature.

Measurement of serotonin release and leakage of lactate dehydrogenase. Release of [¹⁴C]serotonin was determined in the platelet supernatant after centrifugation at 7000xg for 2 min in an Eppendorf microfuge. Leakage of platelet lactate dehydrogenase was determined according to the method of Wroblewski and la Due (38). Total lactate dehydrogenase content of the platelets was measured after complete lysis, which could be obtained either by sonication, or by freeze-thawing or by addition of 0.1% Triton X-100. All three methods resulted in the same total lactate dehydrogenase activity.

Measurement of platelet prothrombin-converting activity by a chromogenic substrate assay. Washed platelet suspensions (0.5 ml) with a platelet concentration of $5 \times 10^6 \text{ ml}^{-1}$ were activated in the presence of 15 μ l 0.1 M CaCl₂ by 10 μ l collagen (0.5 mg/ml) and/or 10 μ l thrombin (0.1 μ M)(final concentrations during the platelet activation are 10 μ g/ml collagen and 2 nM thrombin). Eight minutes after platelet activation was started, 25 μ l factor Xa (20 nM), and in some experiments 25 μ l factor Va (40 nM), were added to the incubation mixture. Two minutes later, 430 μ l of a buffer of pH 7.9 containing 50 mM Tris, 120 mM NaCl, 3 mM CaCl₂, 0.5 mg human serum albumin/ml and 2.6 μ M prothrombin were added to start the formation of thrombin. The final incubation mixture then consisted of 2.5×10^6 platelets/ml, 3 mM CaCl₂, 0.5 nM factor Xa, 1.0 nM factor Va (if added) and 1.1 μ M prothrombin. The activation of platelets as well as the thrombin formation were carried out at 37 °C under continuous stirring with a siliconized stirrer in plastic tubes. After various time intervals, samples of 25-100 μ l are taken from the incubation mixture and added to 2 ml buffer containing 50 mM Tris, 120 mM NaCl, 2 mM EDTA, pH 7.5 and

0.249 mM S2238. From the absorbance change at 405 nm the amount of thrombin formed was calculated from a calibration curve made with known amounts of active-site-titrated thrombin. In some experiments thrombin formation was measured immediately after the addition of platelet activator. In these cases, factor Xa (and factor Va) was added 2 min prior to the simultaneous addition of activator(s) and prothrombin. We did not use platelet suspensions at a physiological concentration of 10^8 ml^{-1} because too high rates of thrombin formation in these suspensions gave rise to deviations from linearity in thrombin formation as a function of time.

Phospholipase treatment of the platelets. 20 ml of activated platelet suspensions ($5 \times 10^8 \text{ ml}^{-1}$) or appropriate controls were incubated with a mixture of phospholipase A_2 from bee venom (35 IU) and N.naja (48 IU) in the presence of 10 mM CaCl_2 , 2 mM o-phenantroline and 1 μg indomethacin/ml. After 60 min, samples were taken to determine the amount of lysis. Phospholipase activity was inhibited by addition of 2 ml 0.2 M EDTA. The incubation mixture was centrifuged at 30 000xg for 30 min and the resuspended platelet pellet was extracted according to the method of Bligh and Dyer (39). Phospholipid analysis was carried out as described before (12). In order to detect phospholipid degradation in intact platelets only, corrections were made for phospholipid breakdown in the population of lysed cells.

RESULTS

Rate of thrombin formation in the absence of exogenous factor Va. The effect of activated and non-activated platelets on the conversion of prothrombin to thrombin by factor Xa was measured and the results are shown in Fig. 1. Initial rates of thrombin formation after activation of the platelets for 10 min with various stimulators are given in Table 1. In the absence of exogenous factor Va, the initial rate of thrombin formation found for unstimulated platelets is nil. However, as shown in Fig. 1A, thrombin is formed after a period of approx. 10 min. After this lag period, a steady-state rate of thrombin formation of $2 \text{ nM} \cdot \text{min}^{-1}$ is reached, being the equivalent of approx. $0.2 \text{ units} \cdot \text{ml}^{-1} \cdot \text{min}^{-1}$ (assuming that 1 mg thrombin corresponds to 2500 units) for 3×10^6 platelets/ml. This is in agreement with the observations by Dahlbäck et al. (25) and Miletich et al. (24) who found $2 \text{ units} \cdot \text{ml}^{-1} \cdot \text{min}^{-1}$ and $5 \text{ units} \cdot \text{ml}^{-1} \cdot \text{min}^{-1}$ respectively, for 10^8 platelets/ml. The initial velocity of prothrombin activation catalyzed by platelets that have been stimulated by thrombin amounts to $2 \text{ nM} \cdot \text{min}^{-1}$ and this rate of thrombin formation remains

Fig. 1. Platelet prothrombin-converting activity in the absence and presence of exogenous factor Va. Platelet activators were added 10 min prior to the initiation of the reaction by addition of prothrombin. Factors Xa (and Va) were added 2 min before the reaction was started. (A) Thrombin formation in the absence of exogenous factor Va. (B) Thrombin formation in the presence of exogenous factor Va. Platelets were activated by: collagen plus thrombin (○), collagen (△) and thrombin (●). Control platelets are represented by the closed circles (●). The dashed line in B represents thrombin formation on 1 % lysed platelets. Concentrations of coagulation factors and platelets are given in Experimental Procedures.

constant with time. When platelets are activated by a mixture of collagen and thrombin, the (initial) rate of thrombin formation is increased to 11 nM thrombin formed per min. This enhanced rate of thrombin formation could not be observed when platelets were activated by other stimulators such as ADP, adrenaline, kaolin or combinations of these stimulators (not shown). Activation of platelets by collagen results in an initial rate of thrombin formation of zero but after a lag period of several minutes thrombin is formed at a steady-state rate equal to that of platelets treated with collagen plus thrombin. An explanation for this observation will be given later in this section.

Rate of thrombin formation in the presence of exogenous factor Va. In the experiments described above, the rate of thrombin formation is at least partially determined by the release and activation of factor V present in the platelet granules. In order to see whether addition of exogenous factor Va influences the rate of prothrombin conversion we repeated the same experiments in the presence of added factor Va. The results of these experiments are shown in

Table 1. Rate of thrombin formation on activated human platelets in the presence and absence of exogenous factor Va. The values given in the table are initial velocities. The data are obtained from the experiments described in Fig. 1 A and B. Experimental details are given in the legend of Fig. 1 and in Experimental Procedures.

Platelet activator	Initial rate of thrombin formation	
	without exogenous Va	with exogenous Va
	nM.min ⁻¹	nM.min ⁻¹
None	0	8
Collagen	0	7
Thrombin	2	9
Collagen + thrombin	11	61
1 % Lysed platelets	0	7

Fig. 1B. Addition of factor Va, factor Xa and prothrombin to unstimulated platelets initiates thrombin formation at a rate of 8 nM.min⁻¹. In contrast to the experiment in the absence of factor Va, no lag time is observed. An almost equal rate of thrombin formation of 9 nM.min⁻¹ is found after activation of the platelets with thrombin for 10 min. However, with platelets activated by the combined action of collagen and thrombin, the rate of thrombin formation is increased to 61 nM.min⁻¹. The effect of collagen alone is similar to that of collagen plus thrombin with the exception that a lag period of 2 min is observed before steady-state velocity is reached. An explanation for this phenomenon with collagen-activated platelets will be given below. The conclusion from this set of experiments is that platelets activated by a combination of collagen and thrombin form a more suitable catalytic surface for the prothrombinase complex than platelets treated only with thrombin. The difference in the rate of thrombin formation cannot be explained by a difference in extent of platelet secretion. No differences in [¹⁴C]serotonin release are observed between platelets stimulated with thrombin or the combined action of collagen and thrombin. A possible explanation could be a difference in platelet lysis due to a different activation of the cells. The inner leaflet of the platelet plasma membrane which will be exposed as a result of lysis of the cells has a much higher procoagulant activity compared to the outer leaflet of intact platelets (1,40). Therefore we measured the amount of lactate dehydrogenase, a cytoplasmic enzyme which is liberated upon cell lysis. Approximately

1% of this enzyme activity is present in the supernatant of platelets stimulated by both thrombin and collagen plus thrombin. The rate of thrombin formation using a preparation of 1% lysed platelets, obtained by a 1:100 dilution of a completely lysed platelet preparation, is found to be $7 \text{ nM} \cdot \text{min}^{-1}$ in the presence of exogenous factor Va (see Table 1). In the absence of exogenous factor Va no measurable amounts of thrombin can be detected. In as far as leakage of 1% lactate dehydrogenase really reflects 1% cell damage this cannot explain the rate of thrombin formation with a platelet suspension activated by a combination of collagen and thrombin. The maximal rate of thrombin formation found for platelets stimulated with collagen alone equals that of platelets stimulated by the combined action of collagen and thrombin, both in the absence and in the presence of exogenous factor Va. However, when thrombin formation is measured by incubating collagen-activated platelets with factor Xa, factor Va and prothrombin, the thrombin formed may react with the platelets producing a situation which reflects platelet activation by the combined action of collagen and thrombin. This may also explain why steady-state velocity of thrombin formation with collagen-activated platelets is observed after a lag time. Moreover, platelets activated for 10 min with collagen and subsequently 5 min with thrombin exhibit the same prothrombin-converting activity as with the simultaneous addition of collagen and thrombin (data not shown).

Generation of prothrombin-converting activity in platelets activated by collagen plus thrombin. Platelets stimulated by the combined action of collagen and thrombin exhibit a higher prothrombin-converting activity than thrombin-activated or non-activated platelets. This indicates that platelet activation by collagen plus thrombin generates a large number of additional binding sites for the prothrombinase complex, the more so as higher rates of thrombin formation are also apparent in the presence of exogenous factor Va. In order to measure the generation of these sites, platelets were activated by collagen plus thrombin in the presence of factor Xa and prothrombin and with or without exogenous factor Va. The amount of exogenous factor Va was chosen such that maximal rate of thrombin formation was observed, in order to avoid any effect of the factor Va released by the platelets. At different time intervals after platelet activation the reaction was stopped by EDTA and the amount of thrombin formed was measured as described in Experimental Procedures. The results are shown in Fig.2. In the absence of exogenous factor Va, it takes about 10 min before steady-state velocity of thrombin formation is reached. In the presence of exogenous factor Va, however, this lag period is reduced to approx. 3 min. No lag time is apparent when the same experiment is

Fig. 2. Effect of exogenous factor Va on the lag time observed in platelets activated by collagen plus thrombin. Platelets were activated by collagen (10 $\mu\text{g/ml}$) plus thrombin (2 nM) immediately followed by addition of prothrombin. Factor Xa (and factor Va) were added 2 min prior to the addition of collagen, thrombin and prothrombin. Experimental conditions are given in Experimental Procedures. Circles represent platelets in the absence of exogenous factor Va; triangles in the presence of 2 nM exogenous factor Va.

carried out with lysed platelets, or when platelets are activated first for 10 min before addition of the clotting factors (compare also Fig.1). This indicates that the lag period is not caused by the binding rate of the components of prothrombinase to the platelets. It is suggested that formation of additional binding sites by platelets activated by collagen plus thrombin is completed in about 3 min, whereas maximal release and activation of factor V from the platelets takes approx. 10 min. A similar time period for the release of factor Va by thrombin-treated platelets has also been observed by Kane et al. (26). Our data also indicate that the amount of factor Va coming from the platelets is not sufficient to saturate all the binding sites that are generated upon platelet activation by the combined action of collagen and thrombin.

Localisation of prothrombin converting activity of platelets. In order to establish whether the prothrombin-converting activity of activated platelets remains associated with the platelets or is released as a separate entity into the supernatant, platelet suspensions activated by collagen plus thrombin are centrifuged for 5 min at 7000xg. More than 85% of the activity is found in the platelet pellet. Moreover, when activated platelet suspensions are filtered through 0.45- μm millipore filters, no activity whatsoever is observed in the filtrate. It is concluded that the prothrombin-converting activity still forms an integral part of the platelet surface after stimulation.

Phospholipase treatment of platelets. Because it is known from model experiments with phospholipid vesicles that negatively charged phospholipids, and in particular phosphatidylserine molecules, are required to obtain a suitable surface for the prothrombinase complex, it was of interest to determine the exposure of the phospholipids at the outer surface of platelets after different activation procedures. In analogy to the work on erythrocytes (11,12,

15) and previous studies on platelets (16,17) we used phospholipases as a tool to establish the phospholipid localization in the platelet membrane before and after activation. Because of their size, phospholipases are unable to permeate the membrane and therefore their action is restricted to the lipids present in the outer monolayer of the membrane (1). An essential requirement for evaluation of the results is that membrane integrity is maintained during phospholipase treatment. This was obtained by addition of 2 mM o-phenantroline to the activated platelets prior to the addition of phospholipases. This reduced cell lysis caused by phospholipase action to less than 10%, an amount for which reliable corrections can be made. Action of phospholipase A_2 results in the liberation of free arachidonic acid which is rapidly converted by the platelet cyclo-oxygenase into thromboxane A_2 , which is a potent platelet activator (41). In order to prevent any effect of this compound both in control platelets and activated platelets, indomethacin was added prior to the addition of phospholipase. The data of these experiments are summarized in Table 2. Activation by collagen, thrombin or the combination of these stimulators did not result in a change in total phospholipid composition of the platelets. Previous observations by several workers (42-45) demonstrated transient changes in phospholipid composition (in particular phosphatidylinositol and phosphatidic acid) during the first minutes of platelet activation, but the composition is normalized within 15 min. Therefore, in the experiment to determine the exposure of phospholipids, phospholipases were added 15 min after the activation procedure.

Phospholipids from intact unstimulated platelets are hardly susceptible to a mixture of phospholipase A_2 from bee venom and N.naja. A mixture of these two phospholipases was used to eliminate differences in substrate specificity. Not more than 2.2% of the total phospholipid can be degraded in these platelets under non-lytic conditions. This is in agreement with the finding of Chap et al. (16) who explained this failure of phospholipid hydrolysis by the tight packing of the lipids in the membrane. A slight increase in total phospholipid hydrolysis is observed when thrombin-activated platelets were subjected to both phospholipases. Approx. 6% of the total phospholipid can be hydrolysed consisting mainly of phosphatidylcholine and phosphatidylethanolamine (Table 2). Whether phosphatidylserine or phosphatidylinositol are also hydrolysed is not clear since standard deviations are large. Changes in the orientation of phospholipids in the membrane of thrombin-treated platelets have been reported by Schick et al. (21) using the non-penetrating probe trinitrobenzenesulfonic acid. They found an increased exposure of phosphatidylethanolamine but not of

Table 2. Non-lytic degradation of platelet phospholipids by simultaneous action of phospholipases A_2 from *N. naja* and bee venoms. Platelets were activated for 15 min followed by addition of phospholipases. Final concentration for thrombin was 2 nM and for collagen 10 μ g/ml. Experimental details are given in Experimental Procedures. Phospholipid composition is expressed as percentage of total lipid phosphorus $\pm$ S.D. Hydrolysis is expressed as the percentage degradation $\pm$ S.D. of the corresponding phospholipid classes. The number in parentheses refer to the number of experiments from which these data were calculated. Data for phospholipid composition and phospholipid hydrolysis of platelets treated with collagen only, are similar to those for untreated platelets. Sphingomyelin is not hydrolysed because it is not a proper substrate for phospholipase A_2 .

Phospholipid composition (%)			
Phospholipid	untreated	activated by thrombin	activated by collagen + thrombin
Phosphatidylserine	9.8 $\pm$ 1.3	10.2 $\pm$ 0.2	10.2 $\pm$ 0.3
Phosphatidylinositol	4.2 $\pm$ 1.2	3.3 $\pm$ 0.2	2.7 $\pm$ 0.4
Sphingomyelin	19.1 $\pm$ 1.2	19.1 $\pm$ 0.3	19.8 $\pm$ 0.2
Phosphatidylcholine	36.9 $\pm$ 2.1	37.7 $\pm$ 0.5	37.9 $\pm$ 0.8
Phosphatidylethanolamine	27.6 $\pm$ 1.8 (n=12)	28.0 $\pm$ 0.5 (n=8)	27.5 $\pm$ 1.0 (n=8)
Hydrolysis (%)			
Phospholipid	untreated	activated by thrombin	activated by collagen + thrombin
Phosphatidylserine	0.4 $\pm$ 0.3	4.4 $\pm$ 5.0	24.6 $\pm$ 1.4
Phosphatidylinositol	0	1.2 $\pm$ 2.0	4.6 $\pm$ 5.1
Sphingomyelin	0	0	0
Phosphatidylcholine	4.0 $\pm$ 1.6	9.8 $\pm$ 3.3	25.0 $\pm$ 3.5
Phosphatidylethanolamine	1.2 $\pm$ 1.3	8.8 $\pm$ 2.5	30.0 $\pm$ 2.5
Total phospholipid	2.2 $\pm$ 1.4 (n=8)	5.8 $\pm$ 2.1 (n=10)	20.4 $\pm$ 2.1 (n=11)

phosphatidylserine at the platelet outer surface which is in agreement with our observations. In contrast, when platelets activated by collagen plus thrombin are treated with phospholipase A_2 , a maximum of about 20% of the total platelet phospholipids can be degraded without significant lysis of the cells. This increased susceptibility of platelet phospholipids indicates that changes in membrane structure have occurred as a result of the activation process. Micro-environmental changes in human platelet membranes during activation by thrombin have also been reported by Nathan et al.(46) using a fluorescent probe. The hydrolysed fraction of phospholipids consists of 24.6% of

the phosphatidylserine, 25% of the phosphatidylcholine and 30% of the phosphatidylethanolamine content of the platelets. When unstimulated platelets were subjected to a combined action of sphingomyelinase C from *Staphylococcus aureus* and phospholipase A₂ from *Naja naja* a degradation of approx. 25% of the total phospholipid was observed but less than 6% of the phosphatidylserine molecules were degraded (data not shown), confirming the findings of Chap et al. (16). However, using the same enzyme mixture on activated platelets which underwent the release reaction, results were obtained which were poorly reproducible and difficult to interpret because of an increased lysis of the platelets (more than 25% of the total lactate dehydrogenase activity is found in the supernatant at the end of this phospholipase treatment). The experiments with phospholipases indicate that during activation by collagen and thrombin micro-environmental alterations occur in the lipid bilayer membrane which are accompanied by an increased exposure of phosphatidylserine at the platelet outer surface. This phenomenon may be responsible for the increased prothrombin-converting activity of these platelets, since an increase of exposed phosphatidylserine would create more binding sites for the components of the prothrombinase complex.

DISCUSSION

It has been established recently that factor Va serves as the binding site for factor Xa at the platelet surface (2,22-28). Platelet procoagulant activity which appears after thrombin activation was explained by the release and activation of factor V from the platelet granules, thereby providing the receptor for factor Xa. Our observations on the prothrombin-converting activity of thrombin-treated and control platelets support this concept. In the absence of exogenous factor Va, non-activated platelets show the same steady state rate of thrombin formation after 10 min as thrombin-treated platelets, which can be explained by the fact that control platelets become activated by trace amounts of thrombin formed during the lag period, resulting in release and activation of factor V. Moreover, addition of exogenous factor Va to thrombin-stimulated or control platelets results in an equal rate of thrombin formation for both preparations. This is in full agreement with the results of Kane et al. (26) who demonstrated that the binding sites for factor Va-factor Xa are present on the surface of unstimulated platelets. Since addition of an excess of factor Va to thrombin-stimulated platelets results in a five-fold enhancement of the rate of thrombin formation, the amount of factor Va re-

leased from the platelets is apparently not sufficient to saturate all the binding sites. This seems to be in contrast to the observations by Miletich et al. (27) who showed that normal platelets release at least five times more factor Va activity than is required for maximal binding of factor Xa. We have no explanation for this discrepancy although it should be mentioned that these investigators have measured binding of factor Xa whereas we have measured the rate of thrombin formation which is also determined by the binding of the substrate prothrombin. Although factor Va has been suggested to form the receptor for factor Xa on the platelet surface (26) as well as on phospholipid vesicles (2), the nature of the factor Xa-factor Va binding sites on platelets is still obscure. It is generally known that the activation of prothrombin on phospholipid vesicles requires the presence of negatively charged phospholipids. Recently, Bloom et al. (47) found that the number of factor Va molecules bound per phospholipid vesicle increases with increasing mole fraction of phosphatidylserine. Moreover the binding constant of factor Xa for negatively charged phospholipids in the presence of factor Va is the same as that observed for factor Xa binding to thrombin-activated platelets (2). Whether or not small amounts of phosphatidylserine are involved in factor Va-factor Xa binding sites at the surface of thrombin-stimulated and control platelets remains uncertain. The present results with phospholipase A₂ treatment on non-activated platelets show too little phospholipid hydrolysis to exclude the possibility that a minor fraction of the phosphatidylserine is present at the membrane exterior. Perret et al. (17) found 9(+12)% hydrolysis of the phosphatidylserine in the plasma membrane after treatment of intact platelets with a mixture of phospholipase A₂ and sphingomyelinase. We observed 4.4 (+5)% breakdown of the phosphatidylserine when thrombin-activated platelets were treated with phospholipase A₂. No detectable phosphatidylserine can, however, be labelled by treatment with trinitrobenzenesulfonic acid of either control or thrombin-treated platelets (21). Kane et al. (26) do not exclude the possibility that indeed some specialized phospholipid structures are required for the binding sites. However, the finding of a patient deficient in these binding sites suggests that some protein component might be involved as well, also since no abnormalities in these patients' platelet phospholipid composition have been found (27,28). Since it has been observed in different laboratories (5,16,17,21) that considerable amounts of phosphatidylserine are present on the cytoplasmic surface of the platelet plasma membrane, it will be clear that lysed platelets provide an excellent surface for the formation of the prothrombinase complex. This indeed appears to be the case as shown by the

fact that completely lysed platelets exhibit a much higher prothrombin-converting activity than intact platelets. Therefore, it is important to know whether platelet activation is accompanied by lysis of the cells. Some leakage of lactate dehydrogenase, often considered as a marker for platelet lysis, is observed during platelet activation which is in agreement with the findings of Joist et al. (48). This may indicate actual cell damage, but may also reflect leakage of lactate dehydrogenase as a result of the fusion process causing the platelet release reaction. Transient leakage of enclosed components during fusion of phospholipid vesicles has been demonstrated by Wilschut et al. (49). In as far as liberation of lactate dehydrogenase would indeed represent cell membrane damage, we cannot exclude the possibility that a minute amount of broken cells, which may be present in the platelet population, participates in the prothrombin-converting activity of platelets. Activation of platelets by a mixture of collagen and thrombin results in a much higher prothrombin-converting activity compared to platelets which are activated by thrombin or collagen alone. When thrombin formation is measured immediately after platelet activation by collagen plus thrombin in the presence of an excess of exogenous factor Va a minimal lag time of 3 min is observed before thrombin formation reaches a steady-state rate. This lag period can be interpreted as the time required to generate the extra sites for the prothrombinase complex in addition to those already present with non-activated or thrombin-activated platelets. In the latter two cases, no lag time in thrombin formation was observed (unpublished observation), suggesting that a limited number of binding sites are already present at the platelet outer surface. Data from phospholipase treatment of platelets activated with collagen plus thrombin reveal that approximately 25% of the phosphatidylserine has become exposed at the platelet outer surface. In analogy to the observation with negatively charged phospholipid vesicles, increased exposure of phosphatidylserine may create additional binding sites for the components of the prothrombinase complex, resulting in a higher rate of thrombin formation when platelets are activated by a mixture of collagen and thrombin. It is tempting to speculate that during activation by the combined action of collagen plus thrombin, phospholipids (and in particular phosphatidylserine) are transferred from the inner to the outer monolayer of the platelet membrane. Such a transbilayer movement of phospholipids, also referred to as 'flip-flop' was demonstrated to be an extremely slow process in lipid vesicles, composed of phosphatidylcholine only (50). More recently, however, evidence has become available for the existence of rapid flip-flop of phospholipid molecules both in artificial and natural membranes (for a review

see (51)). Different mechanisms have been proposed to explain the transbilayer movement of phospholipids. One possibility concerns the formation of intrabilayer inverted lipid micelles as shown by Cullis and de Kruijff (51-52). These structures, the formation of which is affected by Ca^{2+} , are suggested to play a role in endocytosis and exocytosis and may therefore also occur during the platelet release reaction. It should be emphasized, however, that no significant amounts of phosphatidylserine are exposed as a result of platelet activation by thrombin alone, although extensive secretion of granular components occurs. Alternative mechanisms for flip-flop have been proposed some of which include the involvement of membrane proteins (53). Whether one or more of the platelet membrane proteins are able to induce transbilayer movement of phospholipids, after platelet stimulation by the combined action of collagen and thrombin, is part of our current research. An alternative explanation for the exposure of phosphatidylserine is that these negatively charged phospholipids are already present in considerable quantities, but shielded by proteins in such a way that they are neither available for phospholipases nor for coagulation factors. From the present data it is difficult to refute this possibility. However, in unstimulated platelets approx. 50% of the plasma membrane phospholipids can be hydrolyzed without cell lysis by the combined action of phospholipase A_2 and sphingomyelinase, but no significant degradation of phosphatidylserine or phosphatidylinositol occurs. Assuming an equal distribution of phospholipids over both halves of the membrane it may be concluded that the negatively charged phospholipids of the platelet plasma membrane are predominantly confined to the cytoplasmic surface of unstimulated platelets. Also, with erythrocytes it has been established by a variety of methods that phosphatidylserine is localized only at the membrane interior. Therefore, we favour the possibility that rapid transbilayer movement of phospholipids occurs when platelets are activated by a combination of collagen and thrombin.

In conclusion, platelets can contribute two components to the prothrombinase complex: factor Va and binding sites for the components of the prothrombinase complex. The way in which factor V becomes available is clear from the literature (22,24,54,55). It appears as a result of the release reaction and small amounts of thrombin can convert it to factor Va. Once factor Va is formed it can create a few hundred binding sites for factor Xa on the platelet surface and appreciable thrombin formation can occur (24). Whether or not minor amounts of phosphatidylserine participate in the platelet receptor for factor-Va-factor-Xa is not clear at present. However, when collagen is also

present, additional binding sites for the components of the prothrombinase complex appear, due to an increased exposure of phosphatidylserine at the platelet outer surface. As a result increased amounts of factor-Va-factor-Xa as well as prothrombin can bind to the platelet surface leading to enhanced thrombin formation. This may represent an important physiological mechanism in hemostasis, particularly when the vessel wall is ruptured and collagen becomes exposed to circulating platelets.

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CHAPTER III

THE ROLE OF ACTIVATED HUMAN PLATELETS IN PROTHROMBIN AND FACTOR X ACTIVATION

SUMMARY

The role of activated human platelets in intrinsic factor X activation was compared with their effect in prothrombin activation. Compared with unstimulated platelets, platelets triggered by the combined action of collagen plus thrombin showed a 10-fold activity increase in prothrombin activation, and a 20-fold rate enhancement in factor X activation. Treatment of collagen plus thrombin stimulated platelets with N.naja phospholipase A₂ almost completely abolished their activity in prothrombin and factor X activation. Since no significant cell lysis occurs during phospholipase treatment, this indicates that platelet phospholipids, exposed at the membrane exterior, play an essential role in the interaction of platelets with the proteins of the prothrombin and factor X activating complexes.

The time course of generation of the procoagulant platelet surface was different when the amount of coagulation factors present in the assay systems was varied. At suboptimal concentrations of coagulation factors maximum platelet activity was reached after a shorter time period than at saturating concentrations. When measured at suboptimal amounts of coagulation factors the platelet activity in prothrombin and factor X activation is also more sensitive to phospholipase treatment. Experiments with synthetic phospholipid mixtures show that prothrombin and factor X activation are optimal at low mole % phosphatidylserine when high concentrations of factor Va and factor VIIIa are employed. The optimal mole % phosphatidylserine increases when the concentrations of non-enzymatic protein cofactors is lowered. These findings are discussed in relation to a model in which phosphatidylserine, exposed at the outer surface of activated platelets plays an essential role in prothrombin and factor X activation. It is proposed that this phosphatidylserine is not homogeneously distributed in the platelet outer membrane, but that areas with different phosphatidylserine density participate in coagulation factor activation.

INTRODUCTION

The interaction between blood platelets and coagulation factors is essential for haemostatic plug formation. Activated platelets promote the catalysis of two sequential reactions of the blood coagulation cascade: the activation of factor X into factor Xa by a complex of factor IXa, factor VIIIa and Ca²⁺ and the conversion of prothrombin into thrombin by a complex of factor Xa, factor Va and Ca²⁺ (1,2).

Detailed information is available on the interactions of proteins of the

prothrombinase complex with the platelet membrane. Unstimulated bovine and human platelets reversibly bind factor Va with high affinity (3,4). Platelet bound factor Va, either derived from added factor Va or released from platelets upon activation with thrombin, is required for high affinity factor Xa binding (5-8). Kinetic experiments suggest that the platelet bound factor Xa-factor Va complex participates in prothrombin activation.

The nature of the components that comprise the binding site for factor Va and factor Xa on the platelet membrane is not yet defined. Similarities between the effects of platelets and negatively charged synthetic phospholipid vesicles in prothrombin activation led to the assumption that negatively charged phospholipids might also be involved in the assembly of the prothrombinase complex at the platelet membrane. Negatively charged (procoagulant) phospholipids are, however virtually absent from the outer surface of unstimulated platelets. They are almost exclusively located at the inner surface of the platelet plasma membrane and in intracellular membranes (9-12). This explains why intact unstimulated platelets show little procoagulant activity compared with lysed platelets. However, when platelets are stimulated by the combined action of collagen and thrombin, about 25% of the platelet phosphatidylserine molecules become exposed at the platelet outer surface, presumably by a mechanism involving transbilayer movement of phosphatidylserine (13,14). This process is accompanied by an increase of activity of platelets in prothrombin activation.

Since negatively charged phospholipids have similar effects in factor X and prothrombin activation (15,16), it could be anticipated that the appearance of phosphatidylserine at the platelet outer surface would stimulate both activities. In the present study we compared the effect of platelets, stimulated with various activators, in prothrombin and factor X activation. We report here that those platelet activators that increase the activity of platelets in prothrombin activation also increase the capacity of platelets to enhance intrinsic factor X activation. The data support the concept that the exposure of phosphatidylserine at the outer surface of platelets is essential to obtain an optimal activity of platelets in prothrombin and factor X activation.

EXPERIMENTAL PROCEDURES

Materials. S2238 H-D-Phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide hydrochloride and S2337 N-benzoyl-L-isoleucyl-L-glutamyl-(γ -piperidyl)-glycyl-L-arginine-p-nitroanilide hydrochloride were purchased from AB Kabi Diagnostica, Stockholm, Sweden. p-NPGB (p-nitrophenyl-p'-guanidinobenzoate hydrochloride) was from Nutritional Biochemicals. DEAE-Sephadex A-50, QAE-Sephadex A-50 and Sephadex G-100 and G-200 were products of Pharmacia. Factor VIII deficient plasma was from George King Biomedical, Overland, KS, USA. Echis carinatus, Russell's viper, soy bean trypsin inhibitor (STI) and fat-free human serum albumin were from Sigma. Naja naja venom was from Koch Light. Reaction tubes were 2 ml plastic flatbottom tubes obtained from Sterilin Ltd. Teddington Middlesex England. Teflon coated magnetic spinning bars, 7 x 2 mm were purchased from Bel-Art Products USA. Phosphatidylinositol and sphingomyelin were purchased from Koch Light. Phosphatidylcholine (18:1_{cis}/18:1_{cis} phosphatidylcholine) was from Sigma, USA. [¹⁴C] serotonin creatine sulfate (58 Ci/mol) was obtained from Amersham Corp. Lactate dehydrogenase reagents were from Boehringer Mannheim, Germany. All reagents used were of the highest grade commercially available.

Phospholipid and phospholipid vesicle preparations. Phosphatidylethanolamine and phosphatidylserine (both 18:1_{cis}/18:1_{cis}) were prepared from phosphatidylcholine by enzymatic synthesis by the method of Comfurius and Zwaal (17). Single bilayer vesicle solutions were prepared according to de Kruijff (18) as described earlier (16). Phospholipid concentrations were determined by phosphate analysis according to Böttcher et al. (19). Extraction of total phospholipid from platelets was done according to Reed et al. (20). Lipid analysis was carried out as described previously (13).

Proteins. Phospholipase A₂ was purified from Naja naja venom according to Zwaal et al. (21). One IU is defined as that amount of enzyme which degrades 1 μ Mol of egg phosphatidylcholine per min at 37°C. In the purified phospholipase A₂, which had a specific activity of 700 IU/mg, no proteolytic activity could be detected (21). The vitamin K dependent factors prothrombin, factor IX and factors X₁ and X₂ were purified from bovine blood according to established procedures (22) as described previously (15,16). The factor X activator from Russell's viper venom was purified as described by Schiffmann et al. (23). The purifications of thrombin, factor Xa, factor IXa and factor XIa (contact product) were described earlier (15,16). Bovine factor V was purified according a method similar to that described by Kane et al. (7) which is published in de-

tail (24). Factor VIII:C was purified from bovine blood according to the method of Vehar and Davie (25) with slight modifications (26). Factor V and factor VIII were activated with thrombin as described earlier (26). The specific clotting activities of the coagulation factors were: 6 U/mg (prothrombin), 30 U/mg (factor V), 100 U/mg (factor X), 145 U/mg (Factor IX) and 324 U/mg (factor VIII). One clotting unit is defined as the amount of coagulation factor present in 1 ml normal bovine plasma. Thrombin had a specific activity of 2360 NIH units/mg (1 nM thrombin is 0.085 NIH units/ml). Factor V was stored at -70°C in 50 mM Tris, 175 mM NaCl and 10 mM CaCl_2 (pH 7.9) and factor VIII in 10 mM Mes (morpholino ethanesulfonic acid), 175 mM NaCl and 10 mM CaCl_2 adjusted to pH 6.5 with 1 M Tris. The other protein preparations were stored at -70°C after dialysis against 50 mM Tris-HCl, 175 mM NaCl at pH 7.9. Before storage at -70°C , prothrombin and the factor X_1 , X_2 and IX preparations were passed through a column of STI-agarose (0.5 x 10 cm) to reduce the small amounts of factor Xa that might be present in these preparations. No thrombin or factor Xa could be detected in our protein preparations as determined with the chromogenic substrates S2238 and S2337.

Protein concentrations. Thrombin, factor Xa and factor IXa concentrations were determined by active site titration with p-NPGB (27-29). Prothrombin concentrations were determined after complete activation with Echis carinatus venom followed by active site titration with p-NPGB. Similarly, factor X concentrations were obtained by active site titration after complete activation of factors X_1 or X_2 by RVV-X. Factor Va concentrations were determined by kinetic analysis (24). The molar concentration of factor VIIa was determined by kinetic analysis similar to that employed for factor Va (30).

Isolation of platelets. Human platelets were isolated by differential centrifugation as described earlier (26). Platelets were kept at room temperature in a calcium free buffer pH 6.7 containing 136 mM NaCl, 2.68 mM KCl, 2 mM MgCl_2 , 10 mM Hepes, 5 mM glucose and 0.05% fatty acid free human serum albumin, conditions at which no loss of platelet activity was found for at least 15 hours. Platelet concentrations were determined with a Coulter counter. Bovine platelets were isolated according the same procedure.

Platelet stimulation and determination of prothrombin and factor X converting activity. Platelet activity in prothrombin and factor X activation was measured in 2 ml flatbottom plastic tubes, in which the reaction mixtures were stirred at 350 rev/min with teflon stirring bars. To a reaction tube containing 292.5 μl of a platelet suspension 13 μl of 75 mM CaCl_2 was added. The tube content was warmed and stirred for 5 min at 37°C and platelet activation

was started by addition of a platelet stimulator resulting in a final volume of 325 μ l and a CaCl_2 concentration of 3 mM. After a variable time period at 37°C and stirring, referred to as platelet activation time, the components for either prothrombin activation or for factor X activation were added, to determine the effect of platelets on the rates of thrombin or factor Xa formation.

Assay of platelet activity in prothrombin activation. Two minutes before measuring the effect of platelets in prothrombin activation, 25 μ l factor Va and 25 μ l factor Xa were added to the platelet suspension to allow equilibration with the platelets. Prothrombin activation was started by adding 125 μ l of a prewarmed mixture containing prothrombin in 50 mM Tris-HCl, 175 mM NaCl, 14.2 mM CaCl_2 and 0.5 mg/ml human serum albumin at pH 7.9. The final reaction mixture (500 μ l) contained: 20 mM Tris, 6 mM Hepes, 150 mM NaCl, 1.6 mM KCl; 2.9 mM glucose, 1.2 mM MgCl_2 , 6 mM CaCl_2 , 0.5 mg/ml human serum albumin at pH 7.9 and amounts of platelets, factor Xa, factor Va and prothrombin indicated in the legends of the tables and figures. 15 and 30 seconds after initiating prothrombin activation aliquots (usually 5 or 10 μ l) were taken from the reaction mixture and transferred to cuvettes to determine the amount of thrombin formed. The cuvettes (thermostated at 37°C) contained a buffer of 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml ovalbumin and 20 mM EDTA at pH 7.9 in such amounts that the final volume was 2 ml; 335 μ M of the thrombin specific chromogenic substrate S2238 was also present. Further activation of prothrombin is prevented by dilution and the presence of EDTA. The absorbance change recorded at 405 minus 500 nm on an Aminco DW2 spectrophotometer (set in the dual wavelength mode) is a measure of the amount of thrombin present in the aliquots. From a calibration curve made with known amounts of active site titrated thrombin, determined under the same conditions as described above, the amount of thrombin present in the reaction mixture is calculated. Under the conditions used, the formation of thrombin in the reaction mixtures was linear in time, and the rate of thrombin formation was calculated from the amount of thrombin present after 15 and 30 seconds.

Assay of platelet activity in factor X activation. Forty five seconds before measuring the effect of platelets in factor X activation 25 μ l factor IXa and 33.4 μ l CaCl_2 (60.6 mM) were added to the platelet suspension. Factor X activation was started with 16.6 μ l factor X_2 and 100 μ l activated factor VIII. The final reaction mixture (500 μ l) had the same composition as described above for prothrombin activation and contained amounts of platelets, factor IXa, factor VIIla and factor X indicated in the legends of the tables and figures. 45 and 90 seconds after initiating factor X activation aliquots from

the reaction mixture were transferred to cuvettes to determine the amount of factor Xa formed. The experimental set up to measure factor Xa is essentially the same as described for the measurement of thrombin formation, with the exception that the absorbance change at 405 minus 500 nm was measured using the factor Xa specific chromogenic substrate S2337 (final concentration 192 μ M). Factor Xa formation is linear in time after a lag period of about 15 seconds after starting the reaction. Therefore the interval between 45 and 90 seconds after initiating factor X activation was used to calculate the rate of factor Xa formation. Control experiments were carried out to check whether the concentration of factor Xa is adequately measured in the presence of activated platelets or artificial phospholipid vesicles. A known amount of factor Xa was added to artificial phospholipid vesicles plus Ca^{2+} or to platelets that were subsequently activated with thrombin plus collagen. All added factor Xa is recovered in the chromogenic assay described above, which indicates that the binding of factor Xa to platelets and phospholipids is either reversible (EDTA is present in the assay system) or does not interfere with the chromogenic assay.

Stimulation of platelets by collagen. The collagen used in most of the experiments presented in this paper, was horse tendon collagen obtained from Hormon Chemie, München, Germany. Also collagen prepared from bovine achilles tendon obtained from Sigma and Merck, Germany as well as highly purified (type I) collagen from calfskin (a gift from Prof. J. Caen) were employed in these studies. Collagens were added in the non-fibrillar form dissolved in acidic dilution buffer obtained from Hormon Chemie.

Platelet aggregation. Platelet aggregation studies were carried out in a lumi-aggregometer from Chronolog Corporation. Platelets suspended in a Hepes buffer at pH 7.5 (see above) were brought in a cylindrical siliconized glass cuvette in a volume of 614 μ l and stirred with a teflon coated spinning bar. This cuvette was placed in the aggregometer thermostated at 37°C and 26 μ l of a 75 mM CaCl_2 solution was added. The platelets were prewarmed for 5 min and stirred. Then 10 μ l platelet stimulator was added and the aggregation pattern was recorded.

Measurement of serotonin release and leakage of lactate dehydrogenase. The conditions for platelet stimulation are the same as described by the determination of platelet prothrombin and factor X converting activity. Release of [^{14}C] serotonin was determined in the platelet supernatant after centrifugation at 7000 x g for 2 min. in an Eppendorf microfuge. Leakage of lactate dehydrogenase was measured as described earlier (13).

RESULTS

Activity of stimulated platelets in prothrombin and factor X activation. In a previous paper we have shown that activation of platelets with thrombin plus collagen resulted in the formation of a procoagulant surface highly active in prothrombin activation (13). It was proposed that the exposure of the negatively charged phospholipid phosphatidylserine at the outer surface of the platelets, which accompanied the generation of the procoagulant surface, is essential for the observed increase of platelet activity in prothrombin activation. In order to compare the activity of platelets in prothrombin and intrinsic factor X activation, assay systems were developed in which rates of thrombin and factor Xa formation were proportional with the amount of procoagulant phospholipid present. At high concentrations of coagulation factors this condition was satisfied. Fig.1A shows that with 15 nM factor Xa, 30 nM factor Va and 4 μ M prothrombin present in a reaction mixture, rates of prothrombin activation are proportional with the amount of procoagulant phospholipid when a platelet lysate, a platelet lipid extract or synthetic vesicles, containing 2.5 or 40 mole % phosphatidylserine, are used as a phospholipid source. At phospholipid concentrations below 2.5 μ M rates of prothrombin activation are linear with the amount of phospholipid present. A further increase of the concentration of coagulation factors had no effect on the rate of thrombin formation, which indicates that all available sites on the phospholipid surface participate in prothrombin activation. For intrinsic factor X activation saturation with coagulation factors and a linear relation between rates of factor Xa formation and the phospholipid concentration was obtained at 150 nM factor IXa, 15 nM factor VIIla and 0.5 μ M factor X. Due to a limited supply of factor VIII it was not possible to regularly use the saturating factor VIIla concentrations. We succeeded, however, in selecting coagulation factor concentrations at which rates of factor X activation, though not saturated with respect to factor VIIla, are still proportional with the amount of phospholipid present (Fig.1B).

Table I shows the effect of human platelets, stimulated with various platelet activators, in prothrombin and factor X activation. Human platelets (5×10^6 platelets/ml), stimulated for 15 min with optimal concentrations of different platelet activators, were used as a source of phospholipid in the assay systems described above. In this experiment factor X activation is measured at saturating factor VIIla concentration (15 nM). Release of serotonin and platelet aggregation was determined in a separate experiment. Platelets stimulated

Fig.1A,B. Effect of the concentration of procoagulant phospholipids on the rate of prothrombin (A) and factor X activation (B).

The concentrations of phospholipid given are the final concentrations present in the assay systems for prothrombin and factor X activation. (●-●) vesicles composed of (mole%) 2.5% phosphatidylserine, 4% phosphatidylinositol, 47.5% phosphatidylcholine, 28% phosphatidylethanolamine, 18% sphingomyelin and cholesterol in a 1:1 molar ratio to total phospholipid; (▲-▲) vesicles composed of 40% phosphatidylserine, 4% phosphatidylinositol, 10% phosphatidylcholine, 28% phosphatidylethanolamine, 18% sphingomyelin and cholesterol in a 1:1 molar ratio to total phospholipid; (■-■) vesicles made from a total phospholipid extract from platelets; (△-△) sonicated platelets. Prothrombin activation was measured at 4 μM prothrombin, 15 nM factor Xa and 30 nM factor Va and factor X activation at 0.5 μM factor X, 50 nM factor IXa and 0.1 nM factor VIIa. The total lipid extract from platelets had the following phospholipid composition 11% phosphatidylserine, 4% phosphatidylinositol, 39% phosphatidylcholine, 28% phosphatidylethanolamine, 18% sphingomyelin and cholesterol in a 1:1 molar ratio to phospholipid. Lipid extraction from platelets, lipid analysis and the assay systems for prothrombin and factor X activation are described in the experimental procedures.

by the combined action of 10 $\mu\text{g/ml}$ collagen and 1.3 nM thrombin exhibited a 10-fold activity increase in prothrombin activation and were 20 times more active in factor X activation than unstimulated platelets. Platelets stimulated only with thrombin show little rise in activity, but platelet stimulation with collagen alone resulted in activities that were approximately 30% of that observed with platelets stimulated by collagen plus thrombin. In a recent paper Bevers et al. (14) have shown that there is a limited exposure of phosphatidylserine when platelets are stimulated with collagen alone. Although the standard deviation for the determination of the amount of phosphatidylserine exposed was relatively high, it is possible that it accounts for the procoa-

Table 1. Effect of platelets in prothrombin and intrinsic factor X activation. A comparison with platelet aggregation and serotonin release. Human platelets at a concentration of 5×10^6 /ml were stimulated for 15 min with 1.3 nM thrombin and/or 10 μ g/ml collagen. In the experiment in which phospholipase was used platelet stimulation was followed by a 15 min incubation with 3 IU/ml phospholipase A_2 (*Naja naja*). Platelet aggregation was measured in a separate experiment at 5×10^7 platelets/ml. Platelet aggregation, serotonin release and rates of prothrombin and factor X activation were determined as described in the experimental procedures.

Platelet stimulator	Prothrombin activation (nM IIa/min)		Factor X activation (nM Xa/min)		Aggregation	Serotonin release
	Xa ^a	Xa-Va ^b	IXa ^c	IXa-VIIIa ^d		
None	0	34.4	<0.1	2.3	-	0%
thrombin	1.9	40.3	<0.1	3.1	+	72%
collagen	7.9	98.7	<0.1	18.6	+	60%
thrombin+collagen	24.5	351.5	<0.1	47.3	+	74%
thrombin+collagen+ phospholipase A_2	0.4	17.4	<0.1	1.1	n.d	n.d

a. 0.5 nM factor Xa, no factor Va, 4 μ M prothrombin

b. 15 nM factor Xa, 30 nM factor Va, 4 μ M prothrombin

c. 150 nM factor IXa, no factor VIIIa, 0.5 μ M factor X

d. 150 nM factor IXa, 15 nM factor VIIIa, 0.5 μ M factor X

gulant activity observed for collagen stimulated platelets. However, it can also not be ruled out that activities found with collagen stimulated platelets were in fact the result of a combined action of collagen and small amounts of thrombin present in the assay system. In the prothrombinase assay 2 nM thrombin is formed in less than 5 seconds, while thrombin is also present in the assay system in which factor X activation is measured because the factor VIIIa preparation still contained the small amounts of thrombin that were used for the activation of factor VIII. With other platelet activators (ADP, serotonin, epinephrine either alone or in combination) activities similar to unstimulated platelets were observed (data not shown). It is essential that platelets are stirred during the activation process. Non-stirred platelets incubated with thrombin plus collagen show no activity increase in prothrombin and factor X activation. It should be emphasized, that the prothrombin and factor X converting activities mainly reside with the stimulated platelets since less than 10% of the activity remained in the supernatant after sedimenting the platelets by centrifugation at 7000xg.

A possible explanation for the effect of collagen plus thrombin could be that platelets stimulated with this trigger are subject to increased cell lysis.

This could explain the increased prothrombin and factor X converting activities since lysed platelets are highly active in prothrombin and factor X activation (see Fig.1A,B). We used leakage of lactate dehydrogenase, a cytoplasmic platelet enzyme, as a marker for cell lysis during platelet activation. Approximately 1-2% of the total lactate dehydrogenase present leaks out of platelets that were stimulated with thrombin or collagen plus thrombin. The rates of prothrombin and factor X activation for a preparation of 2% lysed platelets, either obtained by 1:50 dilution of completely lysed platelets or by limited sonication that liberated 2% of the lactate-dehydrogenase present in platelets (cf. ref. 26), were 58 nM thrombin/min and 3.8 nM factor Xa/min, respectively. Since this is far below the rates observed with collagen plus thrombin stimulated platelets we conclude that the increased activity of these platelets is not due to cell lysis.

To stress the importance of the presence of added non-enzymatic cofactors factor Va and factor VIIIa experiments are included in table I in which the activity of stimulated platelets in prothrombin and factor X activation is measured in the absence of added factor Va or factor VIIIa. The presence of added factor VIIIa is essential to observe activity of collagen plus thrombin stimulated platelets in factor X activation. This indicates that there is no release of factor VIII:C from stimulated platelets. The lack of activity of platelet lysates (up to 10^7 lysed platelets/ml) in factor X activation measured in the absence of factor VIIIa suggests that platelets do not contain detectable amounts of factor VIII:C. The activity of thrombin and collagen plus thrombin stimulated platelets in prothrombin activation measured in the absence of added factor Va is presumably enhanced by factor V liberated from the platelets during the release reaction. Also in the absence of added factor Va platelets stimulated with collagen plus thrombin had the highest activity in prothrombin activation.

To test the proposal that the procoagulant surface of activated platelets has a "phospholipid-like" nature, platelets were first stimulated with collagen plus thrombin and subsequently treated with N.naja phospholipase A_2 , an enzyme able to degrade phospholipid molecules exposed at the outer platelet surface (13,31). Under these conditions, both the prothrombin and factor X converting activities were almost completely abolished and activities were observed that were even below those of unstimulated platelets (Table I). The activity of unstimulated platelets is also drastically lowered upon treatment with phospholipase A_2 (cf.ref.31). The absence of leakage of lactate dehydrogenase from the platelets indicates that the platelet membrane remains intact during

Fig.2. Stimulatory effect of varying amounts of collagen either in the absence or presence of thrombin on the activity of platelets in prothrombin and factor X activation. Platelets (0.9×10^9 /ml) were stimulated for 10 min with 0-40 µg/ml collagen (open symbols) or 5 nM thrombin plus 0-40 µg/ml collagen (closed symbols). Prothrombin (Δ - Δ , $\blacktriangle$ - $\blacktriangle$) and factor X ($\circ$ - $\circ$, $\bullet$ - $\bullet$) activation were determined as described in the experimental procedures. The concentrations of coagulation factors present in the assay systems were: 4 µM prothrombin, 15 nM factor Xa and 30 nM factor Va (prothrombin activation) or 0.5 µM factor X, 50 nM factor IXa and 0.1 nM factor VIIIa (factor X activation). 100% activity in prothrombin activation was 670 nM IIA/min and in factor X activation 15.1 nM Xa/min.

treatment with phospholipase A_2 . The experiment presented in Table I was also carried out with bovine platelets. The effects of thrombin, collagen and collagen plus thrombin on the prothrombin and factor X converting activities of bovine platelets were the same as on human platelets.

Dependence of platelet activation on the concentration of thrombin, collagen and the source of collagen. The activity of platelets in prothrombin and factor X activation was measured after stimulation with variable amounts of collagen either in the absence of thrombin or at a constant thrombin concentration (5 nM). As shown in Fig.2 both activities rise parallel when the amount of collagen is increased and reach a maximum at a collagen concentration of 8 µg/ml. The collagen used in this experiment was in the nonfibrillar form. When collagen was first allowed to form fibrils at neutral pH and subsequently used for platelet stimulation the same activity in prothrombin and factor X activation was found. The collagen was obtained from horse tendon (Hormon Chemie). Collagens prepared from bovine achilles tendon (Sigma and Merck) and a highly purified type I collagen from calfskin are equally active in generating platelet prothrombin and factor X converting activities (data

Fig.3. Stimulatory effect of varying thrombin concentrations on the activity of platelets in prothrombin and factor X activation. Platelets ($0.9 \times 10^9/\text{ml}$) were stimulated for 10 min with varying concentrations of thrombin in the absence (open symbols) and presence of $4 \mu\text{g}/\text{ml}$ collagen (closed symbols). The concentration of thrombin given in this figure is nM present in the platelet activation mixture (1 nM thrombin is approx. 0.085 NIH units thrombin/ml). Prothrombin (Δ - Δ , $\blacktriangle$ - $\blacktriangle$) and factor X ($\circ$ - $\circ$, $\bullet$ - $\bullet$) activation were determined as described in the experimental procedures. The concentrations of coagulation factors in the assay systems were $4 \mu\text{M}$ prothrombin, 15 nM factor Xa, 30 nM factor Va (prothrombin activation) or $0.5 \mu\text{M}$ factor X, 50 nM factor IXa, 0.1 nM factor VIIa (factor X activation). 100% activity in prothrombin activation was 600 nM Ila/min and in factor X activation 16.6 nM Xa/min.

not shown). The effect of variable thrombin concentrations is shown in Fig.3. In the presence of $4 \mu\text{g}/\text{ml}$ collagen, 0.1 nM thrombin is already sufficient to evoke maximal platelet activity in prothrombin and factor X activation. In the absence of collagen there is a slow rise of both activities when the amount of thrombin is increased. At high thrombin concentrations the platelet activity is still appreciably lower than that of platelets stimulated by the combined action of thrombin plus collagen.

The concentrations of thrombin and collagen required to expose the procoagulant surface are similar to those required to induce platelet aggregation and release. Table II summarizes the concentrations of thrombin or collagen that produce threshold, half-maximal and maximal effect in platelet aggregation and release. The combined effect of thrombin plus collagen on these platelet functions is synergistic. Combination of concentrations of collagen and thrombin below the threshold concentrations for the individual components already produce half maximal platelet aggregation and release.

Table II. Collagen and thrombin requirements for platelet aggregation and release. Platelets were activated by thrombin and/or collagen at a concentration of 5×10^7 platelets/ml and the platelet aggregation and release reaction was measured as described in the experimental procedures.

Platelet activator	Aggregation/Release		
	threshold effect	half-maximal effect	maximal effect
thrombin	0.5 nM	0.75 nM	1.2 nM
collagen	1.0 μ g/ml	3.3 μ g/ml	5.0 μ g/ml
thrombin plus collagen		0.4 nM plus 0.5 μ g/ml	

Time course of appearance of platelet activity in prothrombin and factor X activation. The generation of a procoagulant surface was followed in time for platelets stimulated with thrombin or with collagen plus thrombin. Since the effect of collagen alone could not be singled out from the combined collagen and thrombin effect (see above), collagen stimulated platelets were not further considered. The activity of platelets in prothrombin activation was measured with different concentrations of factor Xa and factor Va present in the prothrombinase assay. The prothrombin activating mixtures contained platelets, 4 μ M prothrombin and 0.5 nM factor Xa, no added factor Va (Fig.4A), 0.5 nM factor Xa, 1 nM factor Va (Fig.4B) or 15 nM factor Xa, 30 nM factor Va (Fig.4C). In all assay systems platelets became active upon stimulation with collagen plus thrombin. As expected, the highest activities were found at saturating factor Xa and factor Va concentrations (Fig.4C). Unstimulated platelets exhibited the lowest prothrombin converting activity. Stimulation of platelets with thrombin alone resulted in a slow rise of prothrombin converting activity when added factor Va is present in the prothrombinase assay mixture (Fig.4B,C). When prothrombin activation is measured in the absence of added factor Va the situation is somewhat different (Fig.4A). Unstimulated platelets are completely inactive in prothrombin activation, presumably due to the virtual absence of both a procoagulant surface and factor Va. Thrombin stimulation of platelets gives rise to an appreciable activity of platelets in prothrombin activation when this is measured in the absence of added factor Va and compared with the activity of unstimulated platelets. However, also in the absence of added factor Va the highest activity was observed with platelets stimulated by thrombin plus collagen. We propose that in platelet experiments carried out in the presence of a saturating concentration factor Va the rate of prothrombin activation is determined by the procoagulant surface already

Fig.4A-D. Time course of appearance of platelet activity in prothrombin and factor X activation. The platelet concentration during stimulation was $0.9 \times 10^7/\text{ml}$. Platelets were not stimulated (■-■), stimulated with 1.3 nM thrombin (▲-▲) or stimulated with 1.3 nM thrombin plus 10 $\mu\text{g/ml}$ collagen (●-●). At different time intervals after stimulation coagulation factors were added and prothrombin (A,B,C) and factor X (D) activation were measured as described in the experimental procedures. The amounts of coagulation factors present in the assay systems were: (A) 4 μM prothrombin, 0.5 nM factor Xa, no factor Va, (B) 4 μM prothrombin, 0.5 nM factor Xa, 1.0 nM factor Va, (C) 4 μM prothrombin, 15 nM factor Xa, 30 nM factor Va or (D) 0.5 μM factor X, 50 nM factor IXa and 0.1 nM factor VIIIa.

present in case of unstimulated platelets or the procoagulant surface generated in case of platelets stimulated with thrombin plus collagen. In the absence of added factor Va both the presence or appearance of the procoagulant surface and the release of platelet factor Va is probed. We have no explanation for the origin of the procoagulant surface of unstimulated platelets. It is either present on platelets circulating in the blood or exposed upon platelet lysis or platelet activation during venepuncture of the blood or the isolation of the platelets.

One other feature of the time generation curves has to be mentioned here. The appearance of platelet prothrombin converting activity is apparently more rapid when prothrombinase activity is assayed in a system containing low amounts of factor Xa and factor Va. Half maximal activities were obtained after 2½ min (Fig.4A), 4 min (Fig.4B) and 9 min (Fig.4C). Compared with prothrombin converting activity, factor X converting activity appeared even more rapidly when platelets were stimulated by thrombin plus collagen (Fig.4D). Within two minutes after platelet activation half maximal factor X converting activity was observed in an assay system, in which suboptimal factor VIII concentrations were used. Also in factor X activation unstimulated platelets exhibit a constant and low activity and stimulation with thrombin alone results in a slow rise of platelet activity.

The effect of platelet concentration in prothrombin and factor X activation is shown in Fig.5A,B. Collagen plus thrombin stimulated platelets show a linear increase of both activities with the platelet concentration, until rates of thrombin and factor Xa formation were reached at which clotting factor activation in these assay systems is no longer linearly proportional with the amount of phospholipid present (cf. Fig.1). Thrombin stimulated and unstimulated platelets also show a linear increase of factor X activation when the platelet concentration in the assay system is increased. For the slight deviation from linearity with thrombin stimulated platelets at high platelet concentrations we have no explanation.

Effect of phospholipase A₂ on the activity of stimulated platelets in prothrombin and factor X activation. The results presented in Table I show that the activity of platelets in prothrombin and factor X activation could be completely abolished by treating the collagen plus thrombin stimulated platelets with phospholipase A₂. The time course of disappearance of platelet prothrombin and factor X converting activities was followed when platelets stimulated for 30 min with thrombin plus collagen were subsequently treated with a small amount of phospholipase A₂ (0.04 IU/ml). After different time intervals

Fig.5A,B. Effect of the concentration of stimulated platelets on the rates of prothrombin and factor X activation.

Platelet stimulation and the assay systems for prothrombin activation (A) and factor X activation (B) are described in the experimental procedures. Platelets were not stimulated (■-■), stimulated for 15 min. with 1.3 nM thrombin (▲-▲) or stimulated for 15 min. with 1.3 nM thrombin plus 10 μg/ml collagen (●-●).

Prothrombin activation was measured at 4 μM prothrombin, 15 nM factor Xa, 30 nM factor Va (Fig.5A). Factor X activation was measured at 0.5 μM factor X, 50 nM factor IXa and 0.1 nM factor VIIa (Fig.5B). The amount of platelets indicated in the figure is that present in the final assay mixture.

of phospholipase treatment coagulation factors were added to measure the remaining activity of platelets in prothrombin and factor X activation. Fig.6 shows that the prothrombinase activity of stimulated platelets is more sensitive to phospholipase treatment when measured in the absence of factor Va than at suboptimal and saturating factor Xa and factor Va concentrations. It seems that part of the prothrombinase activity measured at saturating factor Xa and factor Va is less sensitive to phospholipase treatment. The prothrombinase activity measured at saturating factor Xa and factor Va could, however, be completely abolished when the stimulated platelets were treated with higher amounts of phospholipase A₂ (2IU/ml). Fig.6 also shows that the factor X converting activity of stimulated platelets measured at suboptimal factor VIIa is highly sensitive to treatment with low amounts of phospholipase A₂. The results shown in Fig.6 cannot be due to a detrimental effect of phospholipase A₂ on the coagulation factors since their activity was not affected by prolonged incubation with phospholipase. This can actually also be concluded from the experiment itself. For each data point in the figure there is an

Fig.6. Effect of phospholipase A_2 on the activity of stimulated platelets in prothrombin and factor X activation. Platelets (0.9×10^7 /ml) were stimulated for 30 min with 1.3 nM thrombin plus 10 μ g/ml collagen. Then phospholipase A_2 was added to a final concentration at 0.04 IU/ml. After the time intervals indicated, the remaining activity of platelets in prothrombin and factor X activation was measured as described in the experimental procedures. Prothrombin activation was determined at: 4 μ M prothrombin, 0.5 nM factor Xa, no added factor Va (Δ - Δ); 4 μ M prothrombin, 0.5 nM factor Xa, 1 nM factor Va ($\blacksquare$ - $\blacksquare$); 4 μ M prothrombin, 15 nM factor Xa and 30 nM factor Va ($\bullet$ - $\bullet$). Factor X activation was measured at 0.5 μ M factor X, 50 nM factor IXa and 0.1 nM factor VIIIa ($\circ$ - $\circ$). Rates obtained with 30 min stimulated platelets, set at 100%, were respectively 750 nM IIa/min, 423 nM IIa/min, 44.2 nM IIa/min and 18.5 nM Xa/min. When no phospholipase was added the activity of the platelets remained virtually constant. The dotted line represents the effect of the extra addition of 2 IU/ml phospholipase A_2 .

equal time of incubation of coagulation factors with phospholipase A_2 . This enzyme is added at time 0 (after activation of platelets for 30 min with thrombin plus collagen) and the coagulation factors are added at 2,4,6,8,10 or 15 min to probe the remaining procoagulant surface. Also the fact that phospholipase treated control platelets after proper dilution still become active in prothrombin and factor X activation, and show a normal response in aggregation and release upon stimulation with thrombin and/or collagen demonstrates that the action of phospholipase A_2 is restricted to phospholipids exposed at the membrane exterior (Table III). Platelet aggregation and release are even not affected when control platelets are treated at a 10-fold higher phospholipase A_2 concentration.

Effect of different synthetic phospholipid mixtures in prothrombin and factor X activation. It was investigated whether differences in time course of gene-

Table III. The effect of phospholipase A₂ treatment of unstimulated platelets on various platelet functions.

Human platelets at a concentration of 5×10^8 /ml were incubated at 37°C for 70 min with 3 mM CaCl₂ either in the absence or presence of 0.1 I.U./ml phospholipase A₂ (Naja naja). After treatment, the platelets were diluted to 5×10^7 /ml (for measurement of platelet aggregation, release) or 5×10^6 /ml (for measurement of their procoagulant properties). The diluted platelet suspensions were stimulated for 15 min with 1.3 nM thrombin and/or 10 µg/ml collagen. The prothrombin and factor X converting activities were measured as described in the experimental procedures at 15 nM factor Xa, 30 nM factor Va, 4 µM prothrombin and 50 nM factor IXa, 0.1 nM factor VIIa and 0.5 µM factor X, respectively.

<u>Non treated platelets</u>				
Platelet stimulator	Prothrombin activation (nM IIa/min)	Factor X activation (nM Xa/min)	Aggregation	Serotonin release
none	46.0	0.42	-	0%
thrombin	50.2	0.60	+	69%
thrombin plus collagen	385.4	9.45	+	72%
<u>Phospholipase A₂ treated platelets</u>				
Platelet stimulator	Prothrombin activation (nM IIa/min)	Factor X activation (nM Xa/min)	Aggregation	Serotonin release
none	16.2	<0.1	-	0%
thrombin	23.4	<0.1	+	65%
thrombin plus collagen	297.6	7.8	+	75%

ration of a procoagulant platelet surface probed at varying coagulation factor concentrations and the accompanying different sensitivities to phospholipase A₂ treatment could be related to differences in phospholipid requirement. Therefore, prothrombin and factor X activation were measured in the presence of phospholipid vesicles with varying lipid composition. These vesicles were composed of lipids present in platelet membranes. The mole fraction of each phospholipid was fixed at the same value as found in a total lipid extract of platelets, with the exception of phosphatidylserine and phosphatidylcholine. Prothrombin activation was measured in the absence of factor Va and at the suboptimal and saturating factor Xa and factor Va concentrations also employed in Fig.4. Factor X activation was measured in the absence of factor VIIa and at the same coagulation factor concentrations as used in Fig.4D.

Large differences between prothrombin and factor X activation at different coagulation factor concentrations were observed when the mole fraction phosphatidylserine in a vesicle was varied at the expense of phosphatidylcholine

Fig.7A,B. The effect of phospholipid vesicles with varying mole percentage phosphatidylserine in prothrombin and factor X activation. The phospholipid composition of the vesicles in mole % of total phospholipid was: 28% phosphatidylethanolamine, 19.6% sphingomyelin, phosphatidylserine as indicated in the figure, supplemented to 100% with phosphatidylcholine. Cholesterol was present in a 1:1 molar ratio to total phospholipid. The preparation of vesicles and the assay systems for prothrombin and factor X activation are described in the experimental procedures.

- A: Prothrombin activation: (▲-▲) 2 μ M phospholipid, 4 μ M prothrombin, 15 nM factor Xa, no factor Va, 100% = 4.2 nM IIa/min, (●-●) 2 μ M phospholipid, 4 μ M prothrombin, 0.5 nM factor Xa, 1 nM factor Va, 100% = 803 nM IIa/min, (■-■) 2 μ M phospholipid, 4 μ M prothrombin, 15 nM factor Xa, 30 nM factor Va, 100% = 2900 nM IIa/min.
- B: Factor X activation: (△-△) 25 μ M phospholipid, 0.5 μ M factor X, 50 nM factor IXa, no factor VIIIa, 100% = 1.5 nM Xa/min, (○-○) 2 μ M phospholipid, 0.5 μ M factor X, 50 nM factor IXa, 0.1 nM factor VIIIa, 100% = 71.5 nM Xa/min, (□-□) 2 μ M phospholipid, 0.5 μ M factor X, 150 nM factor IXa, 15 nM factor VIIIa, 100% = 250 nM Xa/min.

(Fig.7A,B). The mole fraction phosphatidylserine required for maximal stimulation of prothrombin and factor X activation varied at different coagulation factor concentrations. In prothrombin activation the optimal phosphatidylserine mole fraction varied from 2.5 mole % at saturating factor Xa and factor Va concentration to more than 40 mole% when prothrombin activation was measured in the absence of factor Va (Fig.7A). Phospholipid vesicles with more than 40 mole% phosphatidylserine were not used in this experiment since they aggregate in the presence of 10 mM CaCl_2 . For factor X activation the same phenomenon was observed. The phosphatidylserine optimum was dependent on the

amounts of factor IXa and factor VIIIa present in the assay system for factor X activation (Fig.7B). At high concentrations of factor IXa and factor VIIIa the optimum is observed at 10 mole% phosphatidylserine, while similar to prothrombinase the optimum in the absence of the non-enzymatic cofactor factor VIIIa is at 40 mole% phosphatidylserine or higher. These differences in phosphatidylserine requirement at different coagulation factor concentrations can explain the observed differences in time course of generation of a procoagulant platelet surface and sensitivity to phospholipase treatment measured under the same conditions (see discussion).

DISCUSSION

The experiments presented in this paper show that platelets stimulated with thrombin plus collagen expose a procoagulant surface that highly promotes prothrombin and intrinsic factor X activation. With unstimulated and thrombin-stimulated platelets markedly lower activities were observed. In previous papers (13,14) we have shown that the negatively charged phospholipid phosphatidylserine, which in unstimulated platelets is localized in the inner membrane monolayer, becomes exposed in the outer membrane monolayer when platelets are activated with collagen plus thrombin. Negatively charged phospholipids are required in synthetic phospholipid vesicles that are used as a procoagulant surface in prothrombin and factor X activation. We therefore propose that the phosphatidylserine exposed at the outer surface of activated platelets plays an important role in the interactions of the platelet membrane with the proteins of the prothrombin and factor X activating complexes and that these interactions are essential for the observed rate enhancements of prothrombin and factor X activation. This concept is supported by the finding that the activity of stimulated platelets in prothrombin and factor X activation is almost completely abolished when the collagen plus thrombin stimulated platelets are treated with phospholipase A₂ (Table I). In this experiment phospholipase action was limited to phospholipids of the outer membrane monolayer and the membrane integrity was left intact.

Our experiments do not rule out an involvement of platelet membrane proteins in the interactions with the coagulation factors. Majerus and co-workers (32) described a patient with a bleeding disorder whose platelets lack a surface component required for the binding of the factor Xa-Va complex. In a recent paper (4) they proposed that this component presumably is a protein. The group

of Mann reported that unstimulated bovine platelets bind factor Va three orders of magnitude tighter than synthetic phospholipid vesicles (3). Although "a highly specialized phospholipid structure" at the platelet surface can cause this affinity difference, it can also be visualized that a platelet membrane protein is responsible for this phenomenon. Tuszynski et al. (33) demonstrated that platelet-bound factor Va is associated with components from the platelet cytoskeleton. The ability of the cytoskeleton to promote factor Xa-catalyzed prothrombin activation is, however, still dependent on the presence of lipid. Our experiments were designed in such a way that rates of prothrombin and factor X activation are limited by the availability of a negatively charged procoagulant phospholipid surface. Saturating coagulation factor concentrations were employed, so that the binding affinities of the membrane for the coagulation factors became less important. Therefore, we cannot rule out the possibility that besides phosphatidylserine other membrane components play a role in the assembly of the prothrombin and factor X activating complexes at the surface of platelets stimulated with collagen plus thrombin.

Kinetic experiments have shown that the activation of prothrombin and factor X in the absence of phospholipids are very inefficient processes (15,16). Phospholipids dramatically stimulate these reactions since they promote the assembly of the prothrombin and factor X activating complexes in such a way that phospholipid bound substrates (prothrombin or factor X) are activated by phospholipid bound enzymes (factor Xa-Va or factor IXa-VIIIa). In experiments with thrombin plus collagen stimulated platelets such high rates of prothrombin and factor X activation were observed that it seems justified to assume that the activation of coagulation factors takes place at the platelet membrane surface. This would mean, that in analogy with the model system (15,16), platelet bound prothrombin and factor X are activated by a platelet bound factor Xa-Va and factor IXa-VIIIa complex, respectively. Also the fact that descarboxyprothrombin, a prothrombin derivative that lacks the γ -carboxyglutamic acid residues responsible for the Ca^{2+} -dependent binding of prothrombin to phospholipids, is activated in the presence of platelets with a 50-fold lower catalytic efficiency than prothrombin (34) indicates that the prothrombin which becomes activated is bound to negatively charged platelet phospholipids. Binding of prothrombin to the platelet membrane, however, has not yet been demonstrated. If the binding affinity of platelets for prothrombin is similar to that of synthetic phospholipids it is possible that it escaped detection since the affinity would then be 3-4 orders of magnitude lower than observed

for factor Xa and Va.

Both bovine and human platelets possess high affinity binding sites for factor Va (3,4). 1000-2000 binding sites with an approximate K_d of $10^{-10}M$ are detected per platelet, independent whether the platelets are stimulated with thrombin or not. Platelet bound factor Va is the receptor for factor Xa. 200-300 binding sites for factor Xa are present per platelet either from human (5) or bovine source (3,6). In these studies there was no investigation of platelets stimulated with collagen plus thrombin. The platelet bound factor Xa participates in prothrombin activation. From reports of other laboratories, it can be calculated that thrombin stimulated human (5) and bovine platelets (34), in which factor V released from platelets is the source of factor Va, activate prothrombin at a rate of 4-8 nM thrombin formed/min with 10^7 platelets/ml. This correlates very well with the 1.9 nM thrombin formed/min that we observed with 0.3×10^7 thrombin stimulated platelets/ml when we measured prothrombin activation in the absence of added factor Va (Table I). Platelets have, however, a much higher capacity to promote prothrombin activation. Table I also shows that platelets stimulated with collagen plus thrombin exhibit a 170-fold higher activity. In this experiment saturating amounts of factor Xa and factor Va were present. It is essential that a limited amount of platelets is used in these experiments. At high platelet concentrations (5×10^7 /ml) such a large number of procoagulant sites is exposed, that even if enough factor Xa and factor Va could be added to saturate these sites the prothrombin available in the assay systems would be completely converted within a few seconds. It should also be emphasized that platelets have to be stirred during stimulation in order to obtain procoagulant activity. Stirring of platelets is also essential for the expression of other platelet functions. Non-stirred platelets activated with the proper stimuli do not aggregate. Also the release of serotonin is drastically diminished or even absent when non-stirred platelets are activated with collagen (2), ADP (35) or the ionophore A23187 (36). We have ruled out possible artefacts like platelet lysis and platelet fragmentation. Activation of stirred platelets with thrombin plus collagen does not induce liberation of cytoplasmic lactate dehydrogenase and fragmentation of platelets is unlikely since more than 90% of the procoagulant activity sediments with platelets upon centrifugation. We do not know why stirring of platelets is required for the expression of procoagulant activity. It is possible that an efficient interaction of platelets with collagen fibers requires stirring or that a minimal shear stress is essential for the generation of a procoagulant surface. The latter would be an important biological phenomenon since circula-

Table IV. Procoagulant sites on human platelet membranes.

The number of procoagulant sites per platelet is calculated with the formula: number of sites/platelet = $v/V_{\max} \times 10^{-9} \times \text{Avogadro's number/platelets.l}^{-1}$, in which v is the rate of prothrombin or factor X activation determined in the experiments presented in table I and $V_{\max}$ is the maximal rate of prothrombin activation (16) or factor X activation (15).

Platelet stimulator	Prothrombin activation (sites/platelet) ^a	Factor X activation	Factor Xa (sites/platelet)	Factor Va
none	2400	850	< 10 ^b	2000-3000 ^c
thrombin	2800	1150	200-300 ^b	n.d.
thrombin+collagen	24100	17500	n.d.	n.d.

n.d. = not determined

a. functional sites/platelet (this paper)

b. binding sites, from ref.5.

c. binding sites, from ref.4.

ting platelets in vivo are also subject to shear stress. Since the flow regime of a solution stirred with a stirring bar consists of very complex time varying laminar flow streams it is, however, not possible to express procoagulant activities as a function of the hydrodynamic force on the platelets.

The data presented in Table I can be used to quantitate the number of functional sites that participate in prothrombin and factor X activation at the platelet surface. Since the coagulation factors were present in saturating amounts both activities were measured at $V_{\max}$ conditions. For this calculation the $V_{\max}$ of prothrombin activation is taken as 2700 mole thrombin/min/mole factor Xa (16) and the $V_{\max}$ of factor X activation as 500 mole factor Xa/min/mole factor IXa (14) and it is assumed that the platelet-bound factor Xa-Va and factor IXa-VIIIa complexes have the same activity as found in the model systems. The latter assumption seems to be justified since so far we never found a procoagulant surface (either from synthetic or platelet origin) that gave $V_{\max}$ values higher than those published earlier (15,16). The $V_{\max}$ of prothrombin activation by factor Xa in the presence of synthetic phospholipids and a saturating concentration of factor Va is constant when the amount of phosphatidylserine present in the phospholipid vesicles is varied between 2 and 40 mole % (37). The results of these calculations are presented in Table IV. Unstimulated and thrombin-stimulated platelets have a low number of functional binding sites for the prothrombin- and factor X activating complexes (850-2800/platelet). This number correlates very well with the number of factor Va binding sites (2000, ref. 4) determined in a binding study under these conditions. We do not know, however, whether this correlation is purely

accidental or that it means that the factor Va binding sites detected on unstimulated platelets are functional sites, that participate in prothrombin activation. For collagen plus thrombin stimulated platelets approximately 24000 functional binding sites for the prothrombinase complex and approximately 18000 for the factor X activating complex are calculated. An identical number of functional sites for prothrombin and factor X activation is to be expected when the phosphatidylserine exposed is an essential component of the procoagulant site. (Also the fact that the collagen and thrombin requirements for generation platelet activity in prothrombin and factor X activation are the same supports this).

It cannot be excluded that phosphatidylserine is also part of the procoagulant site (factor Xa-Va receptor) at the surface of unstimulated platelets. This possibility is supported by the following observations: a) a small amount of phosphatidylserine (probably sufficient to explain the low rate of prothrombin activation) is determined in the outer monolayer of unstimulated human platelets (9). This phosphatidylserine can even be present in domains with high density (see below). b) prothrombin converting activity of unstimulated platelets is abolished upon treatment with phospholipases (31) c) des (1-44) factor Xa, a factor Xa derivative that lacks γ -carboxyglutamyl residues, does not bind to the platelet factor Xa receptor (4). We do not feel that the experiments in which a monoclonal antibody against negatively charged phospholipids does not block platelet prothrombin activation and factor Xa binding (38) can be used as an argument against phospholipid involvement. This antibody only blocks prothrombin activation on synthetic phospholipid vesicles and does not interact with sonicated platelets, for which it can hardly be argued that negatively charged phospholipids are absent. The authors suggest that the phosphatidylserine in platelet membranes may be relatively inaccessible to the antibody.

Two other phenomena observed during our studies on the properties of the procoagulant sites of platelets need further discussion:

- a) The time course of generation of the procoagulant surface on collagen plus thrombin stimulated platelets is different when prothrombin and factor X activation are measured at different coagulation factor concentrations (Fig.4).
- b) Phospholipase treatment of thrombin plus collagen stimulated platelets more rapidly destroys the procoagulant surface when this is measured at low coagulation factor concentrations, than at high concentrations.

The results obtained with synthetic phospholipid vesicles show that there are

remarkable differences in phosphatidylserine requirement in the assay systems employed in the above mentioned experiments. At low concentrations of factor Xa/factor Va or factor IXa/factor VIIa procoagulant surfaces containing low amounts of phosphatidylserine hardly stimulate prothrombin and factor X activation. Optimal rates are observed at 40 mole % phosphatidylserine or higher. At high concentrations of coagulation factors the optimal mole % phosphatidylserine required is much lower (as low as 2.5% for prothrombin activation at saturating factor Xa and factor Va concentrations). Under these conditions procoagulant surfaces with low phosphatidylserine still have an appreciable activity. Based on these observations it can be hypothesized that the phosphatidylserine exposed at the surface of collagen plus thrombin stimulated platelets is not homogeneously distributed in the outer lipid leaflet of the membrane bilayer. Domains with high phosphatidylserine density, which rapidly appear at the surface of stimulated platelets are measured at low coagulation factor concentrations. Domains with low phosphatidylserine density which appear slower, can only be probed at high coagulation factor concentrations. This would also explain why the activity of the procoagulant surface measured at low coagulation factor concentrations is more sensitive to phospholipase treatment. It has been shown, that phosphatidylserine monolayers, at surface pressures found in the platelet membrane (34 dyne/cm, ref. 10), are more rapidly degraded by phospholipase A_2 than monolayers of phosphatidylcholine (39,40). It is therefore likely that platelet membrane surface areas containing high local phosphatidylserine concentrations are more susceptible to phospholipase A_2 than surface areas containing low phosphatidylserine concentrations.

If such lateral phase separations occur in the outer leaflet of the platelet plasma membrane after or during platelet activation, it presumably requires other components to induce or maintain them. An involvement of extra-cellular calcium in this process cannot be excluded, but it is also possible that membrane proteins participate in the properties of activated platelets to produce at least two different lipid domains with different phospholipid compositions.

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CHAPTER IV

ACTIVITY OF HUMAN BLOOD PLATELETS IN PROTHROMBIN AND IN FACTOR X ACTIVATION INDUCED BY IONOPHORE A23187

SUMMARY

Two reactions of the blood coagulation cascade are highly stimulated by membranes containing negatively charged phospholipids. These are the activation of prothrombin to thrombin by factor Xa with factor Va as cofactor, and the conversion of factor X into Xa by factor IXa with factor VIIIa as cofactor. It is thought that under physiological conditions these reactions take place with the clotting factors adsorbed at the surface of blood platelets.

The role of blood platelets in the two reactions was studied using two assay systems consisting of highly purified clotting factors. The rates of formation of thrombin and factor Xa, measured with specific chromogenic substrates, were dependent on the quantity of negatively charged phospholipid present in the reaction mixture. Phospholipid vesicles with platelet phospholipid composition were highly active in the two reaction systems. When the purified lipids were substituted by disrupted platelets, containing the same quantity of lipids, the same high activities were found. With intact human blood platelets a low but significant activity was observed. Incubation of the platelets with the calcium ionophore A23187, resulted in a 50-60 fold activity increase in formation of thrombin and factor Xa. Platelet disruption increases procoagulant activity, since the negatively charged phospholipids, originally situated in the inner leaflet of the lipid bilayer of the platelet membrane become exposed upon platelet disruption. Ionophore activation of platelets was however not due to lysis since discharge of platelet lactate dehydrogenase was less than 3%. The activity of A23187-activated platelets in formation of thrombin and factor Xa was completely abolished after incubation with phospholipase A₂, which is to be expected if negatively charged lipids are present at the platelet outer surface. In contrast to platelets, erythrocytes could not be activated by the ionophore.

As found by other workers, A23187 induces release of ATP and factor V(a) and results in platelet aggregation. The appearance of procoagulant activity, ATP and factor V(a) release were all dependent on the A23187 concentration and were too rapid to study the time course. All platelet activities were induced between 0.2 μ M and 0.7 μ M A23187, suggesting a relationship between the release processes and the appearance of procoagulant activity.

INTRODUCTION

The formation of the haemostatic plug which stops bleeding upon injury, involves platelet reactions like adhesion, release and aggregation, as well as activation reactions of the plasma clotting factors that result in fibrin formation (1,2).

Methods to purify the clotting factors are now available, and this has promoted detailed study of the function of these factors in the sequence of activation reactions that results in fibrin formation (3). Two of these reactions, the activation of prothrombin to thrombin by factor Xa and the activation of factor X to factor Xa by factor IXa are strongly accelerated in the presence of the respective cofactors Va and VIIIa and in the presence of membranes containing negatively charged phospholipids. Detailed kinetic analysis of these reactions resulted in the finding that the cofactors Va and factor VIIIa respectively increase the V_{max} of formation of thrombin and factor Xa, whereas the presence of phospholipid lowers the K_m values for the substrates factor II and X (4,5). The results support the concept that during activation, enzyme, cofactor and substrate are complexed at a phospholipid surface.

Miletich et al. found that after stimulation of platelets by thrombin, 200-300 high affinity receptors for factor Xa appear at the platelet surface (6). The receptor was identified as factor Va released from the platelet by thrombin (7) and from the results by the groups of Majerus (6-8), of Stenflo (9,10), and of Mann (11,12) obtained with human and bovine platelets, it can be concluded that platelets, independent of thrombin stimulation, have 200-900 binding sites for factor Xa-Va complexes. The chemical nature of the binding sites has not been elucidated but is most likely comprised of negatively charged phospholipid (13) which is in agreement with older proposals that platelets *in vivo* provide procoagulant phospholipids (14,15). Paradoxically, in platelets, as in most other eukaryotic cells, the negatively charged phospholipids, phosphatidylserine and phosphatidylinositol are nearly all localized at the inner leaflet of the lipid bilayer of the platelet membrane and therefore inaccessible for participation in clotting reactions (16,17). Results from our laboratory indicate that after incubation of platelets with collagen plus thrombin about 25% of the platelet phosphatidylserine is exposed at the platelet surface, presumably by a flip-flop or transbilayer transport mechanism (13). It was further shown that collagen + thrombin-activated platelets accelerate prothrombin activation, by factors Xa and Va (13,18), and intrinsic factor X activation by factors IXa and VIIIa (Rosing et al., unpublished results).

The divalent cation ionophore A23187 activates several platelet functions and is known to induce release of ATP, serotonin and factor V and to cause platelet aggregation (6,19-21). Moreover, Bevers et al. (22) found that 50% of the platelet phosphatidylserine is exposed at the surface of A23187-activated

platelets and that A23187 stimulation leads to highly increased activity of the platelets in a platelet factor-3 clotting assay (23). It was therefore of interest to study the properties of ionophore-stimulated platelets in prothrombin- and factor X activation, using reaction systems consisting of highly purified proteins. We also studied release of platelet factor V, ATP release and aggregation in order to detect possible relationships between the various platelet activities.

MATERIALS AND METHODS

S2238 and S2337 were purchased from AB Kabi Diagnostica, Stockholm, Sweden. DEAE-Sephadex A-50, QAE-Sephadex A-50 and Sephadex G-100 and G-200 were products of Pharmacia. Ionophore A23187 was obtained from Calbiochem-Hoechst, USA. Factor VIII-deficient plasma was from Georg King Biomedical (Overland, KS, USA). Echis Carinatus and RVV venoms were from Sigma. Naja naja venom from Koch Light. Reaction tubes were 2-ml plastic flatbottom tubes obtained from Sterilin Ltd.(Teddington Middlesex, England). Teflon-coated magnetic spinning bars, 7 x 2 mm were from Bel-Art Products USA. Phosphatidylinositol and sphingomyeline were purchased from Koch Light. Phosphatidylcholine (18:1_{cis}/-18:1_{cis},dioleoylglycerophosphocholine) was from Sigma (USA), ATP-monitoring reagent containing luciferin and luciferase was from LKB, Sweden. Lactate dehydrogenase reagents were from Boehringer (Mannheim, FRG). All reagents used were of the highest grade commercially available.

Preparations of phospholipids and phospholipid vesicles. Phosphatidylethanolamine and phosphatidylserine (both 18:1_{cis}/18:1_{cis}) were prepared from phosphatidylcholine by enzymatic synthesis according to the method of Comfurius and Zwaal (24). Single bilayer vesicle solutions were prepared according to the method described by de Kruffy (25) by sonication of liposomes for 10 minutes in 50 mM Tris-HCl and 175 mM NaCl buffer at pH 7.9 at 0°C. Sonication was performed by using a MSE Mark II 150 W ultrasonic desintegrator set at 6 micron peak to peak amplitude. Phospholipid concentrations were determined by phosphate analysis according to Böttcher et al. (26).

Proteins. Phospholipase A₂ was purified from Naja naja venom according to Zwaal et al (27). Prothrombin, thrombin, factor X, factor Xa, factor IX, factor IXa and contactproduct (factor XIa) were purified according to established procedures (28) as described previously (4,5). RVV-X was purified from the crude venom as described by Schiffmann et al. (29). Bovine factor V was purified according to Lindhout et al (30) by a method similar as described by

Kane et al (8). Factor V was activated by incubation with thrombin and stored in a buffer containing 50 mM Tris, 175 mM NaCl and 10 mM CaCl_2 , pH 7.9. No changes in activity were found when activated factor V solutions were kept at 0°C for 24h or when stored at -70°C for a one year period. Factor VIII was purified from bovine blood according to the method of Vehar and Davie (31). The method was followed up to and including the sulfate-sepharose step. After this step the factor VIII preparation had a specific activity in a clotting assay of 324 units/mg. Factor VIII stock solutions were dialysed against a buffer containing 10 mM Mes, 175 mM NaCl, 10 mM CaCl_2 adjusted to pH 6.5 with 1 M Tris. Factor VIII was diluted with the same buffer containing 10% glycerol and 10 mg/ml human serum albumin. Factor VIII activities were determined with a one-stage coagulation assay using factor VIII-deficient human plasma. Activities of factor VIII were expressed in units/ml, assuming 1 unit/ml present in normal bovine plasma.

The protein preparations were stored at -70°C after dialysis against 50 mM Tris-HCl 175 mM NaCl at pH 7.9. Before storage at -70°C, prothrombin and the preparations of factor X_1 , X_2 and IX were passed through a column of soybean trypsin inhibitor coupled to agarose (0.5 x 10 cm) to reduce the small amounts of factor Xa that might be present in these preparations. No thrombin or factor Xa could be detected in our protein preparations as determined with the chromogenic substrates S2238 and S2337. Factor Va and factor VIII were stored at -70°C in Ca^{2+} -containing buffers as indicated above.

Protein concentrations. Concentrations of thrombin, factor Xa and factor IXa concentrations were determined by active site titration with p-nitrophenyl-p-guanidinobenzoate hydrochloride (32-34). Prothrombin concentrations were determined after complete activation with Echis carinatus venom followed by active site titration. Similarly, factor X concentrations were obtained by active site titration after complete activation of factors X_1 or X_2 by RVV-X. Factor Va concentrations were determined by kinetic analysis (30). When protein concentrations are expressed in mg/ml, they were calculated from values for the absorbance at 280 nm using an $A_{280}^{1\%}$ of 15.5 for factor II (35) an $A_{280}^{1\%}$ of 14.9 and 14.5 for factors IX and IXa (36) and an $A_{280}^{1\%}$ of 12.4 for factors X_1 and X_2 (37). The molar concentration of factor VIII was determined by kinetic analysis, similar to the method described for factor V (37a).

Activation of factor VIII. Earlier we reported the conditions for activation of factor VIII complex by thrombin (5). In the present work factor VIII:C is used prepared according to Vehar and Davie (31) which does not contain von Willebrand factor. The optimal conditions for activation of factor VIII:C by

thrombin were determined, by direct measurement of the effect of factor VIIIa on the rate of factor X activation. Incubation of factor VIII:C with thrombin, dependent on concentration and incubation time, caused a rapid increase in factor VIIIa activity followed by a decrease very similar to the thrombin effects on factor VIII complex (cf ref 5). Based on these results and considering the amounts of thrombin required for platelet stimulation, we decided to activate factor VIII:C for 1 min at 37°C at a thrombin concentration of 3.65 nM (0.13 µg/ml).

Isolation of human platelets. Blood was drawn by venepuncture from healthy male volunteers, who had not taken any medication for at least one week. As anticoagulant, one volume of acid/citrate/dextrose (0.18 M glucose/0.08 M trisodium citrate/0.052 M citric acid) was added to five volumes of whole blood. Platelet-rich plasma was obtained after centrifugation at 220 x g for 15 min. Platelets were isolated by centrifugation 1000 x g for 20 min and the pellet was gently resuspended in a calcium-free buffer pH 6.7 containing 136 mM NaCl, 2.68 mM KCl, 2 mM MgCl₂, 10 mM Hepes, 5 mM glucose and 0.05% fatty-acid-free human serum albumin. The platelets were washed twice in this buffer by centrifuging 600 x g, 15 min. Before centrifugation acid/citrate/dextrose was added to the suspension (1 volume to 14 volumes). Finally the platelets were resuspended in Hepes buffer at pH 7.5. The entire procedure for platelet preparation took about 2h and was carried out at room temperature (22°C). Platelet concentration was determined with a Coulter counter. Platelets were kept at room temperature and no loss of platelet functions (prothrombin- and factor X activation, release of ATP and factor V) was found for at least 15h.

Platelet stimulation and determination of prothrombin- and factor X converting activity was carried out in 2-ml flatbottom plastic tubes, that were stirred at 350 rev/min with teflon stirring bars. A stirring assembly was constructed such that 8 reaction tubes could be simultaneously stirred at 37°C. To a reaction tube containing 307 µl of a platelet suspension was added 13 µl of 75 mM CaCl₂. The tube content was stirred for 5 min at 37°C and platelet stimulation was started by adding 5 µl of A23187 dissolved in Me₂SO resulting in a final volume of 325 µl and a CaCl₂ concentration of 3 mM. After a variable time period at 37°C and stirring, termed platelet activation time, the components for either prothrombin activation or for factor X activation were added, to determine rates of thrombin- or factor Xa formation.

Reaction system for prothrombin activation used to assay platelet procoagulant activity. Two minutes before the start of the thrombin forming reaction, 25 μ l factor Va (600 nM) and 25 μ l factor Xa (300 nM) were added to the triggered platelet suspension to allow equilibration with the platelets. Prothrombin activation was started with the addition of 125 μ l of a prewarmed mixture containing 16 μ M prothrombin and 14.2 mM CaCl_2 in 50 mM Tris-HCl, 175 mM NaCl and 0.5 mg/ml of human serum albumin, pH 7.9, to result in a final volume of 500 μ l. The final concentrations of the reactants are: 15 nM factor Xa, 30 nM factor Va, 4 μ M prothrombin and 6 mM CaCl_2 at a pH of 7.9. After 15 and 30 seconds aliquots (usually 5 or 10 μ l) were taken from the reaction mixture to determine the amount of thrombin formed.

Reaction system for factor X activation for the assay of platelet procoagulant activity. Forty five seconds before the start of the factor Xa forming reaction 25 μ l IXa (1 μ M) and 33.4 μ l CaCl_2 (60.6 mM) were added to the triggered platelet suspension and the reaction was started with 16.6 μ l factor X_2 (15 μ M) and 100 μ l of activated factor VIII to result in a final volume of 500 μ l. The final reaction concentrations of the reactants are 50 nM factor IXa, 0.1 nM factor VIIa, 0.5 μ M factor X_2 and 6 mM CaCl_2 at pH 7.9. Forty five and 90 seconds after starting the reaction aliquots were taken from the reaction mixture to determine the amount of factor Xa formed.

Measurement of rate of thrombin formation. Aliquots of the reaction mixture for prothrombin activation were brought into cuvettes (thermostated at 37°C) containing a buffer of 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml ovalbumin and 20 mM EDTA at pH 7.9 in such amounts that the final volume was 2 ml; 235 μ M of the thrombin specific chromogenic substrate S2238 was also present. Further activation of prothrombin is prevented by dilution and the presence of EDTA. The absorbance change recorded at 405 nm minus 500 nm on an Aminco DW2 spectrophotometer (set in the dual wavelength mode) is a measure of the amount of thrombin present in the aliquots. From a calibration curve made with known amounts of active site-titrated thrombin, determined under the same conditions as described above, the amount of thrombin present in the reaction mixture is calculated. Under the conditions used, the formation of thrombin in the reaction mixtures was linear in time, the rate of thrombin formation was calculated from the amount of thrombin present after 15 and 30 seconds.

Measurement of the rate of factor Xa formation. The experimental set up is essentially the same as described by measurement of thrombin formation, with the exception that the absorbance change at 405 nm minus 500 nm was measured

using the factor Xa specific chromogenic substrate S2337 (concentration 192 μM). The factor Xa forming reaction is linear in time after a lag period of about 15 seconds. Therefore the interval between 45 and 90 seconds after starting was used to calculate the rate of factor Xa formation.

Platelet aggregation. Platelet aggregation studies were carried out in a Lumi-aggregometer (Chronolog Corporation) as follows. Platelets suspended in a Hepes buffer at pH 7.5 (see above) were brought to a cylindrical siliconized glass cuvette in a volume of 614 μl and stirred with a teflon-coated spinning bar. This cuvette was placed in the aggregometer thermostated at 37° C, and 26 μl of a 75 mM CaCl_2 solution was added. The platelets were prewarmed for 5 min and stirred. Then 10 μl of the ionophore A23187 dissolved in Me_2SO was added and the aggregation pattern was recorded.

Measurement ATP release of platelets. Platelets were stimulated as described for factor X and prothrombin activation. After stimulation for 5 min with ionophore, 300 μl of the activated platelet suspension was drawn and centrifuged in an Eppendorf 5412 centrifuge in 1.5 ml tubes during 5 min at room temperature, 7000 x g. 200 μl of the supernatant was added to 250 μl Hepes buffer pH 7.5 (see above) in a round glass cuvette placed in the holder of the lumi-aggregometer and prewarmed for 5 min. Then under stirring with a spinning bar 50 μl of a firefly lantern extract, containing luciferin-luciferase, was added and light emission was quantified.

Measurement of lactate dehydrogenase. From the activated platelet suspension 475 μl was added to a cuvette containing 250 μl Hepes buffer (pH 7.5 see above), 50 μl pyruvic acid (30 mg/ml) and 25 μl NADH disodiumsalt (3 mg/ml). The absorbance change at 340 nm minus 400 nm, determined on an Aminco DW-2 spectrophotometer in the dual wavelength mode, is a measure for the lactate dehydrogenase activity.

Determination of the release of factor V. Platelets in presence of Ca^{2+} were stirred and stimulated with ionophore exactly as described for determination of the activity in prothrombin- and factor X activation. After the desired stimulation time were added in rapid succession: 25 μl factor Xa (300 nM). 25 μl of sonicated phospholipid vesicles (25% phosphatidylserine, 75% phosphatidylcholine, 0.400 mM) and 125 μl of a prewarmed mixture containing prothrombin and CaCl_2 as used in the reaction system for prothrombin activation. The final concentration of reactants were: 15 nM factor Xa, 4 μM prothrombin, 20 μM phospholipid vesicles and 6 mM CaCl_2 at pH 7.9. After 15 and 30 seconds samples of the reaction mixture, were transferred to cuvettes containing S2238, to determine the amounts of thrombin present.

RESULTS

Properties of two reaction systems for assay of synthetic and platelet phospholipids based on prothrombin- and on intrinsic factor X activation.

The detection and quantification of procoagulant phospholipids at the platelet surface requires sensitive assays. We have used the phospholipid requirement for prothrombin activation by factor Xa in presence of factor Va, and intrinsic factor X activation by factors IXa and VIIla, to develop two assay systems that contain highly purified bovine clotting factors, in which rates of prothrombin- and factor X activation, are proportional with the amount of procoagulant phospholipid present. Both reaction systems are described in detail in Materials and Methods. The final concentration of proteins in the prothrombin activating system are: 15 nM factor Xa, 30 nM factor Va, 4 μ M prothrombin. For the factor X activation system: 50 nM factor IXa, 0.1 nM factor VIIla and 0.5 μ M factor X. Under these conditions it is found that the rates of thrombin and factor Xa formation are completely dependent on and directly proportional to the amount of procoagulant lipid present. All activation rates shown in this work were determined with the lipids or activated platelets present within the linear range (compare fig.4A,B).

The dependence of the rates of thrombin- and factor Xa formation on increased enrichment of phospholipid membranes with phosphatidylserine (such a process may take place during platelet activation) was studied with vesicles of platelet lipid composition that contain varying mole fractions of phosphatidylserine. The results presented in table 1 indicate that the presence of phosphatidylserine is essential for prothrombin- and factor X activation. At low mole fractions phosphatidylserine, high rates of thrombin formation are found in the prothrombin activation system. Rates of thrombin formation are optimal at 2.5 - 5% phosphatidylserine and lower activation rates are found at higher mole fractions phosphatidylserine. In contrast, the rates of factor Xa formation in the reaction system for factor X activation, increase with increased phosphatidylserine content of the vesicles. Thus the two measuring systems used here, show a different requirement for the presence of phosphatidylserine in the membranes. When 4% phosphatidylinositol was included in vesicles containing 10-11% phosphatidylserine, to simulate platelet lipid composition, no increase in the rates of thrombin- and factor Xa formation was found.

Table 1. Effect of increasing phosphatidylserine content of phospholipid vesicles on the rates of prothrombin- and factor X activation under our reaction conditions.

The phospholipid composition of the vesicles was expressed in mole percentage of total phospholipid: 28% phosphatidylethanolamine, 19% sphingomyelin, phosphatidylserine as given in the table, supplemented up to 100% with phosphatidylcholine. Cholesterol was present in the same (1:1) molar concentration as total phospholipid. The total phospholipid concentration in the reaction mixture was 0.5 μM . The composition of the reaction mixtures and determination of the rates of thrombin and factor Xa formation were exactly as described in Materials and Methods.

Phosphatidylserine in vesicle	Rate of thrombin formation	Rate of factor Xa formation
mole%	(nM IIa.min ⁻¹)	(nM Xa.min ⁻¹)
0	47	0.1
1	417	0.1
2.5	843	0.2
5	931	2.6
10	804	13.0
15	467	23.0

Effect of intact washed platelets, sonicated platelets, ionophore-stimulated platelets and similarly treated erythrocytes on rates of thrombin-and factor Xa formation in our assay systems.

The lipids of the human blood platelet membrane are asymmetrically distributed over the two leaflets of the lipid bilayer (16,17). The negatively charged phospholipids, phosphatidylserine and phosphatidylinositol (respectively 11 and 4% of total platelet phospholipids), that are known to be active in coagulation, are localized almost exclusively at the platelet membrane interior. Intact platelets therefore hardly stimulate clotting reactions whereas disrupted platelets do. The effect of increased platelet disruption by sonication on the ability of platelets to support prothrombin and factor X activation in our assay systems is shown in fig.1. The release of lactate dehydrogenase indicating platelet disruption, runs parallel with the activity of the platelet sonicates in both activation assays. Apparently, sonication has changed the platelet membrane, such that the negatively charged lipids become exposed and can participate in the two activation reactions.

An interesting non-physiological platelet activator is the divalent cation ionophore A23187 which is a potent trigger of various platelet reactions (19-21). The activity of ionophore stimulated platelets in our reactions was tested by incubation of washed platelets with 1 μM A23187 or with Me_2SO , the

Fig.1. Effect of sonication time on platelet release of lactate dehydrogenase and on the activities in factor X- and prothrombin activation. Washed platelets (3×10^6 /ml) were sonicated for varying time periods in the MSE sonicator set at the lowest power output. Samples (0.475 ml) from the sonicate were taken to determine the amount of lactate dehydrogenase present. Samples of 0.312 ml were taken to determine the activity of the sonicates in the reaction systems for prothrombin- and for factor X activation. The three assays were carried out exactly as described for activated platelets in Materials and Methods. (○-○), lactate dehydrogenase activity; (●-●), activity in prothrombin activation; (▲-▲), activity in factor X activation.

solvent for A23187, as control. After 5 min stirring at 37°C and with 3 mM CaCl_2 present, the proteins for activation of either prothrombin- or factor X were added and the rates of thrombin- and factor Xa formation determined. The results are presented in table 2. Unstimulated platelets have a low activity in both systems. Incubation with A23187 resulted in a 50-60 fold increase of platelet activity in both systems, equal to 71% and 62% of the activity of sonicated platelets in activation of prothrombin and factor X respectively. The ionophore effect is not due however to platelet lysis, because the platelet lactate dehydrogenase discharged from the platelets was 3% of that total present. Addition of A23187 to sonicated platelets or artificial phospholipid vesicles did not influence the rates of thrombin or factor Xa formation. The ionophore and the solvent Me_2SO therefore do not influence membrane structure or the activity of the clotting factors under our reaction conditions. When the ionophore stimulated platelets were centrifuged (10 min, $7000 \times g$), 17% of the platelet activity both in intrinsic factor X activation and in prothrombin activation remained in the supernatant. This indicates that 83% of the activity is platelet bound and is not released.

Table 2 also gives the rates of prothrombin- and factor X activation found with vesicles composed of purified lipid components as present in the human platelet. The data shown are for $1 \mu\text{M}$ of total phospholipid present in the

Table 2. Effect of platelets, erythrocytes, and vesicles of platelet lipid composition on the rate of thrombin- and factor Xa formation. The effect on the rates of thrombin- and factor Xa formation by platelets, erythrocytes and synthetic membranes was measured in our reaction system for prothrombin and factor X activation as described in Materials and methods. The final concentration of platelets and of erythrocytes in the reaction mixture was 2.5×10^6 /ml. Therefore the concentration of phospholipids in the reaction mixtures as provided by erythrocytes, platelets and synthetic vesicles was $1 \mu\text{M}$ (the platelet phospholipid content was measured to be $0.4 \mu\text{Moles}/10^9$ platelets). The synthetic vesicles were composed of (in mole percentage): phosphatidylserine 11%, phosphatidylinositol 4%, phosphatidylethanolamine 28%, sphingomyeline 19%, phosphatidylcholine 38% and cholesterol in a cholesterol to phospholipid ratio of 1:1. Preparation of platelets, erythrocytes and synthetic vesicles is described in Materials and Methods.

	Rate of thrombin formation (nM IIa.min ⁻¹)	Rate of factor Xa formation (nM Xa.min ⁻¹)
Washed platelets, not stimulated	17	0.3
Platelets stimulated by $1 \mu\text{M}$ A23187 during 5 min	843	19.4
Platelets disrupted by sonication	1188	31.5
Vesicles with a phospholipid composition found in platelets	1214	34.2
Human erythrocytes	7	0.0
Human erythrocytes incubated with $1 \mu\text{M}$ A23187 during 5 min	19	0.0
Human erythrocytes disrupted by sonication	1325	28.9

reaction mixture, which allows direct comparison with the results for the platelet experiments that were carried out at 2.5×10^6 platelets/ml. (10^{11} platelets contain $40 \mu\text{moles}$ of phospholipid; P. Comfurius, unpublished). It can be seen that vesicles of platelet lipid composition have the same activity in prothrombin- and factor X activation as platelets disrupted by sonication. Table 2 also shows results of experiments with erythrocytes, which have a phospholipid composition and membrane asymmetry similar to platelets (16,27). Washed erythrocytes are not active in our two assays, but as with platelets, activity appears after sonication (38). In contrast to platelets, incubation

Fig.2. Dose-response curves showing the relationship between the activity of platelets in the reaction systems for prothrombin- and factor X activation after stimulation with various concentrations of ionophore A23187.

Platelet suspensions containing 3×10^6 platelets/ml were adjusted to 3 mM CaCl_2 , and stirred at 350 rev/min. To the platelets was added 5 μl of an appropriate A23187 dilution in dimethyl sulfoxide. After 10 min at 37 °C, either release of lactate dehydrogenase was measured (not shown) or the components of our reaction systems for prothrombin- or factor X activation were added to determine rates of thrombin and factor Xa formation as described in Materials and Methods. (●) Activity in prothrombin activation; (▲) activity in factor X activation.

of the red blood cells with 1 μM A23187 fails to enhance the activity of erythrocytes in prothrombin- and factor X activation. The effect of A23187 on platelet activation was investigated further by varying the ionophore concentration, the activation time by ionophore, and the platelet concentration at optimal and suboptimal ionophore concentration.

Fig.2 indicates that the major effects of ionophore are obtained in the range between 0.2 and 0.5 μM A23187, and that the activity of platelets in prothrombin activation is seen at slightly lower A23187 concentrations than the activity in factor X activation.

The appearance of activity in time is shown in fig.3A,B. In our assays, thrombin- and factor Xa formation are determined over time periods of 30 and 90 seconds respectively, which precludes measurement of platelet activation times shorter than 1 min. With optimal A23187 (1 μM), maximal activation is already seen after 1 min. With suboptimal A23187 (0.4 μM) the activity in prothrombin activation appears rapid and in factor X activation somewhat slower. Platelet activities after activation remained stable for at least 15 min. A small decline in factor X- activating activity is seen. Prolonged incubation at suboptimal A23187 concentration, did not increase the level of

Fig.3. Time course of appearance of platelet activity in factor II- and factor X activation after triggering of the platelets at two A23187 concentrations. Platelets $3 \times 10^6/\text{ml}$ at 3 mM CaCl_2 (320 μl) were incubated at 37°C while stirring and 5 μl of ionophore A23187 was added to a concentration (in 325 μl) of either 1 μM (closed circles) or 0.4 μM A23187 (open circles). After the time intervals indicated in the figures, the components for either the prothrombin- or the factor X activation system were added and the rates of prothrombin- and factor X activation were determined as described in Materials and Methods. (A) Activity of platelets in prothrombin activation; (B) activity in factor X activation.

activity.

Fig.4 shows the rates of thrombin- (fig.4A) and factor Xa formation (fig.4B) when varying amounts of platelets are stimulated for 5 min with either 3 μM or with 0.35 μM A23187, or with none (control Me_2SO).

For comparison, we also determined the activities after platelet disruption by sonication. The rate of prothrombin activation is proportional to the amount of sonicated platelets up to 10×10^6 platelets/ml; at higher platelet concentration, the rate levels off, because of prothrombin depletion. The reaction system for factor X activation begins to deviate from linearity at lower platelet sonicate concentrations (5×10^6 platelets/ml), possibly because the factor VIIIa concentration becomes limiting. When platelets are stimulated with 3 μM A23187, the rates of prothrombin- and factor X activation increase in parallel with the rates found for sonicated platelets. We conclude that

Fig.4. Relationship between the rates of prothrombin- and factor X activation and the concentration of either sonicated platelets or ionophore-stimulated platelets.

Platelets were sonicated or stimulated with either 3 μM or 0.35 μM A23187 or with Me_2SO at platelet concentrations as indicated in the figure. After 5 min, the platelet suspensions (0.325 ml) were supplemented to a final volume of 0.5 ml, with the components for activation of prothrombin- and factor X and the rates of thrombin- and factor Xa formation were determined as in Materials and Methods.(A) Activity in the reaction system for prothrombin activation;(B) activity in the reaction system for factor Xa activation. (○) Washed platelets, (▲) 0.35 μM A23187, (■) 3 μM A23187, (●) after sonication.

stimulation at 3 μM A23187 results in optimal activation over the whole range of platelet concentrations shown in fig.4A,B. With suboptimal A23187 (0.35 μM) proportionality, particularly in factor X activation, is only seen in the lower concentration ranges. It appears that at higher platelet concentrations, the effective ionophore concentration is lowered, such that a constant intermediate level of activation is not achieved. In agreement with fig.2 is the observation that platelets activated with 0.35 μM A23187 are relatively more active in prothrombin- than in factor X activation.

Phospholipase A_2 treatment of A23187-activated platelets. If A23187 stimulation results in appearance of phosphatidylserine at the platelet surface, it

Fig.5. Effect of phospholipase A_2 incubation on the activity of ionophore A23187 stimulated platelets in the reaction systems for prothrombin- and factor X activation.

Platelets were stimulated by A23187 while stirring at 37°C , in a volume of 0.325 ml containing 3 mM Ca^{2+} , $1\text{ }\mu\text{M}$ A23187 and 10^7 platelets/ml. After 1 min at 37°C , $5\text{ }\mu\text{l}$ of phospholipase A_2 (20 IU/ml) was added and after the time-intervals indicated in the figure the components for prothrombin- and factor X activation were added, and the rates of factor IIa- and Xa formation determined as described in Materials and Methods. (●) Rate of thrombin formation; (▼) rate of factor Xa formation. Rates measured 1 min after platelet stimulation were chosen as 100% activities.

was of interest to test whether elimination of phosphatidylserine would lead to loss of activity in the two activation reactions.

Therefore A23187-stimulated platelets were treated with phospholipase A_2 from *Naja naja*. This particular lipase was found to abolish activity of platelets in prothrombin activation and was also able to hydrolyse phosphatidylserine (13,39). A time course of the effect of phospholipase A_2 incubation on the capacity of A23187 stimulated platelets to support prothrombin- and factor X activation is shown in fig.5. We find that the activity of A23187 stimulated platelets in both assays can be completely abolished by incubation with phospholipase A_2 . However, the activity of platelets in factor X activation decreases faster than the activity in prothrombin activation. Phospholipase treatment was not accompanied by platelet lysis, since lactate dehydrogenase release was less than 5% of that found after sonication. The phospholipase A_2 preparation was highly purified and did not inactivate any of the protein components of the reaction systems for prothrombin- and factor X activation.

Effect of ionophore A23187 on platelet aggregation and release. Soon after characterization of A23187 (40) its effect on platelets was studied (19-21). A23187 induces release of serotonin and ATP; mobilizes intracellular Ca^{2+} stores, and causes aggregation. In view of the activity of A23187- stimulated

Fig.6. Aggregation of washed platelet suspensions with ionophore A23187. Washed platelets (3×10^7 /ml) were stirred at 37°C in an aggregometer with 3 mM CaCl_2 . A23187 was added to a final concentration of $0.75 \mu\text{M}$, $0.35 \mu\text{M}$ and $0.15 \mu\text{M}$. The figure shows the recorder tracings observed at these concentrations. The rate of aggregation was taken as the slope of the dotted line. The bar represents 30 seconds.

Fig.7. Dose response curves indicating the relationship between platelet aggregation and ATP release and the concentration of ionophore A23187.

Platelets (3×10^7 /ml) were aggregated with varying A23187 concentrations in the aggregometer. The rates of aggregation were calculated as described in fig.6 and presented as percentage of the maximal aggregation rate found at $1 \mu\text{M}$ A23187. The release of ATP was determined in the supernatants of platelets (3×10^7 /ml) which were incubated for 5 min at 37°C at the A23187 concentration shown in the figure. ATP determination was as described in Materials and Methods. Release activity was presented as percentage of maximal released ATP found at $0.75 \mu\text{M}$ A23187 (●) ATP release, (▲) aggregation.

platelets in prothrombin- and factor X activation described here it was of interest to investigate the relationship of these activities with release and aggregation. The aggregation reactions were done by stirring washed platelets (3.0×10^7 /ml) at 37°C in presence of 3 mM CaCl_2 , in the aggregometer. The recorder tracings obtained at three different A23187 concentrations are shown in fig.6. In the absence of added Ca^{2+} very little aggregation can be induced by A23187 under our conditions, but upon addition of Ca^{2+} rapid aggregation is observed (not shown). Slopes were determined, as indicated by the dotted line in fig.6, and considered a measure for the rate of aggregation. A plot of aggregation rates versus A23187 concentration is shown in fig.7. The curve with closed circles in fig.7 represents ATP release in the platelet super-

Fig.8. Dose response curve indicating the dependence of factor V release from platelets on the concentration A23187.

Washed platelets ($1 \times 10^7/\text{ml}$) were stirred at 37°C and stimulated for 2 min at the A23187 concentrations indicated in the figure. Factor Va release was determined by addition of phospholipid vesicles, factor Xa and prothrombin and measurement of the rate of thrombin formation as described in Materials and Methods. Maximal released activity was 89.6% of the factor Va activity found after the platelets were lysed with 0.025% Triton X-100, with 10 mM CaCl_2 added to maintain Va activity.

nant in response to A23187 activation. ATP release is seen within a narrow range of A23187 concentrations and excess ionophore inhibits ATP release.

Since ATP, like ADP, Ca^{2+} and serotonin are situated in the platelet dense bodies, it was of interest to study the release of factor V, one of the proteins stored in the alpha-granules, a different platelet storage organel (41). Factor V release was studied with a new assay. In the assay factor Xa, prothrombin, CaCl_2 and phospholipid are added to the platelets. Excess phospholipid is present to minimize the platelet lipid contribution. Reaction conditions were chosen such that the rate of prothrombin activation is proportional to the concentration of factor Va present. An advantage of the assay is that no centrifugation step is involved, thus avoiding the possibility that released but platelet bound factor V is not measured leading to an underestimation of factor V release. The rate of thrombin formation in the assay is linear in time, indicating that under our reaction conditions factor V is present as factor Va. The results obtained when 1×10^7 platelets/ml are stimulated with varying A23187 concentrations are shown in fig.8. As found for platelet activities in prothrombin- and factor X activation and in ATP release, the release of factor V was complete within 1 min, even at suboptimal A23187 concentration at which not all factor V is released. Maximal factor V release induced by A23187 represented 90% of the factor V(a) activity found in platelets lysed with 0.025% Triton X-100. Table 3 summarizes the A23187 concentrations to obtain half-maximal response of the various platelet functions. Half maximal activities of ATP release, factor V release, activity in factor II- and X activation and aggregation are found between 0.30 and $0.41 \mu\text{M}$ A23187. The concentration differences are not large enough to conclude that

Table 3. Ionophore concentration required to obtain half-maximal effect on platelet function.

The A23187 concentrations at which 50% of optimal activity is obtained were compiled from the results shown in figs.2,7 and 8.

Platelet function	concentration A23187 (μ M)
release of ATP	0.30
prothrombin activation	0.33
release of factor V(Va) activity	0.35
aggregation	0.40
intrinsic factor X activation	0.41

certain processes are mutually unrelated but do not rule out a relationship between release of α -granules and appearance of procoagulant activity in factor II- and X activation.

DISCUSSION

The main result presented in this paper is, that human platelets after a short incubation with the divalent cation ionophore A23187 are stimulated 50-60 fold in their capacity to support the prothrombin- and the intrinsic factor X activation reactions. The effect is not due to platelet lysis, and is specific in the sense that erythrocytes cannot be stimulated. The activity is platelet- bound, since 83% of the activity can be sedimented with the platelets. The increased activity of platelets after A23187 stimulation in prothrombin-and factor X activation, can be attributed to increased exposure at the platelet surface of negatively charged procoagulant phospholipid, most likely phosphatidylserine. This conclusion was obtained using two lipid dependent measuring systems, in which rates of thrombin- and factor Xa formation are directly proportional to the lipid concentration. Proportionality was observed for phospholipid vesicles, for platelet sonicates and for A23187 triggered platelets, which allowed direct comparison of activity of these preparations.

Phospholipid vesicles of platelet lipid composition, in a concentration as present in platelets gave the same rates of activation as a platelet sonicate. Therefore the platelet membrane proteins, that are present in the sonicate do not influence the activity of the phospholipid present in the sonicate in factor X- and prothrombin activation. Moreover the ionophore triggered platelets show activities in prothrombin- and factor X activation well below those

of vesicles of platelet phospholipid composition (69% and 57% respectively). Thus, sufficient procoagulant phospholipid is present in the platelet membrane, to account for the activities of A23187-activated platelets in prothrombin and factor X activation. There appears no compelling reason to suppose a role for platelet membrane proteins in prothrombin- and factor X activation, nor to postulate the existence of non-lipid structures that bind clotting factors.

The negatively charged procoagulant phospholipids phosphatidylserine and phosphatidylinositol comprise 11% and 4% respectively of the total platelet phospholipid. In the unstimulated platelet most of these are located at the inner leaflet of the platelet membrane. Bevers et al. reported that after A23187 stimulation 50% of the phosphatidylserine and 90% of the phosphatidylethanolamine was exposed at the platelet surface, no significant amount of phosphatidylinositol was found (22). By itself, phosphatidylethanolamine is not procoagulant, therefore phosphatidylserine exposure most likely explains the procoagulant activity of platelets after A23187 stimulation. The experiments with vesicles of variable phosphatidylserine content indicate a different phosphatidylserine requirement for the factor X- and prothrombin activation reactions. The quotient of the rate of prothrombin- and the rate of factor X activation is high at 2.5% phosphatidylserine and much lower for vesicles of 10-15% phosphatidylserine. The quotient of the rates of prothrombin- and factor X activation found for platelets after sonication or after A23187 stimulation corresponds best with the quotient of the rates found for vesicles containing 10-15% phosphatidylserine. This suggests that the phosphatidylserine content of platelets after sonication or after stimulation by A23187 is between 10-15%. This indicates that sonication and platelet stimulation by A23187 result in rearrangement of membrane lipids such that phosphatidylserine is nearly equally distributed over the two membrane leaflets.

When stimulated at suboptimal A23187 concentration, the platelets are relatively more active in prothrombin- than in factor X activation. For example, the concentrations A23187 required to induce half- maximal activity are 0.33 μM and 0.41 μM A23187 respectively. Again, the comparison with vesicles of varying phosphatidylserine content provides an explanation. Vesicles containing low mole percentages of phosphatidylserine, were relatively more active in prothrombin- than in factor X activation. This suggests that with suboptimal ionophore, incomplete phosphatidylserine exposure at the platelet surface is obtained. Thus, platelets stimulated at increasing A23187 concentrations are most likely increasingly enriched in phosphatidylserine

content in the platelet outer membrane leaflet.

The digestion experiments with phospholipase A₂ provide additional evidence for the presence of negatively charged phospholipids like phosphatidylserine at the platelet outer surface after triggering by A23187. The progressive decline and abolishment of activity of A23187-stimulated platelets in the reactions activating factor II and X upon phospholipase treatment can be explained by progressive degradation of phosphatidylserine molecules at the platelet outer surface. The more rapid fall of platelet activity in factor X activation compared to prothrombin activation, can be explained by the different phosphatidylserine requirements of the two reaction systems. If the phosphatidylserine content after A23187 stimulation is between 10 and 15%, then a decline of this percentage would decrease the rate of factor X activation more strongly than it would affect the rate of prothrombin activation, which still proceeds rapidly at 2.5% phosphatidylserine.

An important step forward in the understanding of the role of platelets in clotting reactions was Miletich's finding that platelets that have undergone the release reaction possess 200-300 high affinity binding sites (receptors) for factor Xa (6). Factor Xa binding was dependent on platelet release induced by thrombin or by A23187. Later, it was found that thrombin merely served to release platelet factor Va and that released factor Va functioned as a receptor for factor Xa, by strongly enhancing factor Xa binding to platelets (7). When factor Va was added exogenously to non-stimulated platelets, the same number of factor Xa binding sites were found (8,12). The effect of A23187 on factor Xa binding, as the effect of thrombin, must be ascribed to factor Va release by the ionophore. However, the effect of exogenously added factor Va, on factor Xa binding to ionophore stimulated platelets has not been investigated. From the results published by the group of Majerus (6-8), by Dahlbäck and Stenflo (9,10), and by the group of Mann (10,12), it can be concluded that irrespective of thrombin stimulation human platelets possess 200-400 sites, whereas 900 binding sites for the factor Xa-Va complex are present on the bovine platelet. Until now no detailed reports have appeared on the binding of factor IXa, and how it is influenced by presence of factor VIIIa, to phospholipid vesicles or to activated platelets. The similarities between the factor II- and X activating systems (4,5) and our own unpublished data suggest that in the presence of negatively charged phospholipids factor VIIIa enhances the binding of factor IXa in the same way as the binding of factor Xa is increased by the presence of factor Va (30,42). Thus, if in the presence of platelets, factor Va is a receptor for factor Xa, one may consider platelet

Table 4. Calculation of binding sites for the prothrombin activating complex at untriggered and at ionophore A23187-stimulated human platelets. Binding sites were calculated from kinetic data shown in table 2. The activation rates were divided by the V_{max} for prothrombin activation (4). The maximally possible concentration is 15 nM factor Xa-Va, given enzyme and cofactor concentrations present in our reaction mixtures. Multiplication with Avogadro's number and division by the platelet concentration (2.5×10^8 platelets/ml) results in the number of enzyme complexes bound at the platelet surface.

	Prothrombin activation no trigger	A23187
Rates of thrombin formation from table 2 ($\text{nM IIa} \cdot \text{min}^{-1}$)	17	843
Platelet bound concentration of factor Xa-Va (nM)	8.5×10^{-3}	421×10^{-3}
Number of factor Xa-Va complexes bound per platelet	2040	101200

bound factor VIIIa a receptor for factor IXa. From the kinetic data presented in this paper, binding data can be inferred if it is assumed that the observed activation rates for prothrombin, were produced by platelet bound factor Xa-Va enzyme complexes. Further assumptions are that all the platelet binding sites are filled with factor Xa-Va, that sufficient substrate is present to achieve V_{max} and that the V_{max} with platelets is the same as determined with phospholipid vesicles. These assumptions are met for the prothrombin activating system, considering the various dissociation constants and kinetic parameters reported in the literature (4,30,42,43). For the intrinsic factor X activation system a similar calculation will be possible as soon as information is available on the binding of factors IXa and VIIIa to platelets.

The binding data calculated from our kinetic data are presented in table 4. Unstimulated washed platelets contain 2040 binding sites per platelet for the prothrombin activating complex, comparable with the 200-900 binding sites per platelet reported in the literature that were identified by direct binding experiments. After stimulation by ionophore, the 50 fold enhancement of the rate of thrombin and factor Xa formation corresponds to an increase in sites from 2040 to about 100000 sites per platelet for the prothrombinase complex.

Erythrocytes have a similar phospholipid and membrane asymmetry as platelets (16,27), but cannot be activated by A23187. Sickled erythrocytes will accelerate clotting in vitro (44), due to exposure of phosphatidylserine at the sickle-cell surface. The altered membrane asymmetry may have resulted from

transbilayer phospholipid movement or flip-flop across the membrane, a mechanism also proposed for platelets (38). Both cell types may therefore possess the postulated flip-flop mechanism. Why A23187 only activates the process in platelets is unknown.

Ionophore A23187 induces several platelet responses, including rapid shape change, release, aggregation, liberation of arachidonic acid from endogenous phospholipids (19-21,45-47), and as shown here increases activity in prothrombin- and factor X activation. The mechanism of A23187 action is thought to involve the liberation of Ca^{2+} from endogenous platelet stores into the cytoplasm. The various responses might be triggered by increases in the level of cytoplasmic Ca^{2+} . It may be speculated that erythrocytes cannot be activated by A23187 because in these cells the Ca-stores are absent. For a discussion of the role of Ca^{2+} , see: Gerrard et al (48). The appearance of procoagulant activity is not directly related to arachidonic acid liberation. Both thrombin and A23187 release arachidonic acid, but thrombin alone is an inefficient inducer of procoagulant activity (13).

The effect of A23187 on the procoagulant activity of platelets described in this paper may be an important tool in further elucidation of the mechanism by which a platelet procoagulant surface is generated under physiological conditions.

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CHAPTER V

ASSEMBLY OF THE INTRINSIC FACTOR X ACTIVATING COMPLEX; INTERACTIONS BETWEEN FACTOR IXa, FACTOR VIIla, AND PHOSPHOLIPID

SUMMARY

The activation of blood coagulation factor X by factor IXa is strongly stimulated by the non-enzymatic cofactors phospholipid, Ca^{2+} and activated factor VIII. In this paper we derived an expression, which enabled the determination of binding parameters of factor IXa for phospholipids (either in the absence or presence of factor VIIla) from kinetic measurements of factor X activation. It is shown that rates of factor X activation in the presence of phospholipids could be saturated with an excess factor VIIla at limiting amounts of factor IXa and vice versa. These data indicate that the enzymatic unit in the intrinsic factor X activator is a 1:1 stoichiometrical complex of factor IXa and factor VIIla bound to phospholipid. Titrations with factor IXa at fixed concentrations of phospholipid and factor X show that the apparent dissociation constant of factor IXa for phospholipid is lowered from 10^{-6} M to 10^{-8} M by the presence of factor VIIla. We conclude that in analogy to the factor Xa-factor Va interaction in the prothrombinase complex, factor VIIla functions as a high-affinity binding site ("receptor") for factor IXa in intrinsic factor X activation. Therefore, factor VIIla increases the V_{max} of factor X activation by 1) enhancing the k_{cat} of the reaction and 2) increasing the amount of phospholipid-bound factor IXa that participates in factor X activation.

INTRODUCTION

During blood coagulation the zymogen factor X is converted into factor Xa by the serine protease factor IXa (For a review see ref. 1). The rate of factor Xa formation is strongly increased by the presence of negatively charged phospholipids, Ca^{2+} ions and activated factor VIII (For a review on factor VIII see ref. 2). It is thought that during factor X activation, factor IXa and VIIla are associated in a complex adsorbed at the phospholipid surface, that is termed the intrinsic factor X activator or the intrinsic factor X activating complex (3-7). A detailed understanding of the mode of action of this complex ultimately requires quantification of the interactions between the components. Binding parameters i.e. dissociation constants and number of binding sites have been determined for interaction of factors IXa and X with phospholipid vesicles of varying composition (8,9). Until now no data are available, however, on interactions that involve factor VIII.

The organisation of the components of the factor X activating complex as postulated above strongly resembles the structure of the prothrombinase complex (3-10). Data on the interactions between the components of the latter complex have been obtained by several methods. One technique was to infer binding parameters from the kinetics of prothrombin activation (11,12).

The binding data presented in this paper, derived from kinetic studies of factor X activation, provide evidence that a factor IXa-factor VIIIa-phospholipid complex is the catalytic unit in factor X activation and further indicate that the incorporation of factor IXa in the enzymatic complex is strongly promoted by activated factor VIII.

MATERIALS AND METHODS

Materials. S2337 (Bz-Ile-Glu (Piperidyl)-Gly-Arg-p-nitroanilide) was purchased from Kabi AB, Sweden; Sephacryl-S300 was from Pharmacia Sweden. Phosphatidylcholine (18:1_{cis}/18:1_{cis}-phosphatidylcholine) was from Sigma Chemicals St. Louis USA; Sphingomyelin was from Koch-Light, England. All reagents were of highest grade available supplied by Merck, Darmstadt, Germany and by Sigma Chemicals, St. Louis USA.

Phospholipids and phospholipid vesicles. Phosphatidylethanolamine and phosphatidylserine (both 18:1_{cis}/18:1_{cis}) were prepared by enzymatic synthesis (13). Vesicles were prepared by sonication in a buffer containing 50 mM Tris-HCl and 175 mM NaCl pH 7.9 as described before (10). The composition of the vesicles was: 25% phosphatidylserine, 28% phosphatidylethanolamine, 19% sphingomyelin, 28% phosphatidylcholine and cholesterol in a 1:1 molar ratio with respect to the total phospholipid concentration.

Proteins. Bovine factors X₂, IXa and thrombin were prepared as described previously (6). Factor VIII:C was purified from bovine blood using the method of Vehar and Davie (14), modified as follows. After chromatography on sulfate-sepharose we omitted the factor X-sepharose step because factor VIII failed to bind to this column. The final purification step for factor VIII consisted of gelfiltration on Sephacryl-S300. Factor VIII eluted as a broad peak corresponding to the elution position of bovine catalase (M_r 232 000). Analysis of the column fractions by SDS-polyacrylamide gelelectrophoresis followed by silver staining showed two predominant bands at 250 000 and 74 000. Factor VIII stock solutions were dialyzed against a buffer containing 10 mM morpholinoethane

sulfonic acid, 175 mM NaCl, 10 mM CaCl₂, 10% glycerol, adjusted to pH 6.5 with 1 M Tris. Factor VIII was diluted in the same buffer containing 10 mg/ml human albumin.

Activation of factor VIII. Factor VIII was activated by thrombin. Optimal conditions were determined as described before by direct measurement of the effect of factor VIIla on the rate of factor X activation (6). In a typical experiment factor VIII was activated for 1 min at 37 °C and pH 7.9 at a thrombin concentration of 3.65 nM (130 ng/ml, 0.36 NIH-units/ml).

Factor X activation. Rates of factor Xa formation were determined with the specific chromogenic substrate S2337 as described before (6).

Quantification of factor VIII. The concentration of factor VIII was determined by a one-stage clotting assay using factor VIII deficient plasma and was expressed in units/ml, assuming the presence of 1 unit/ml of factor VIII in plasma pooled from 10 cows. The purified factor VIII preparation had a specific activity of 1600-3000 units/mg in a clotting assay, assuming a $A_{280}^{1\%} = 10.0$. It is uncertain which of the bands in the factor VIII:C preparation as seen on SDS-gels are associated with procoagulant activity. Therefore the concentration of active factor VIII:C cannot be determined from absorbance at 280nm. The results in this paper provide evidence for factor IXa-factor VIIla complex formation at the phospholipid surface. When it is assumed that factor IXa and factor VIIla are present in a 1:1 molar stoichiometrical complex, it appeared possible to determine the molar concentration of factor VIII. The method involves saturation of a limiting known amount of factor IXa with factor VIIla, which provides the activity of the factor IXa-VIIla complex; followed by saturation of a limiting unknown amount of factor VIIla with factor IXa. With $V(VIIla)$ and $V(IXa)$ being the rates of factor X activation determined for a known concentration of factor IXa saturated with factor VIIla and for an unknown amount of factor VIIla saturated with factor IXa, respectively, the molar factor VIIla concentration can be calculated from the equation:

$$\frac{V(IXa)}{[VIIla]} = \frac{V(VIIla)}{[IXa]}$$

RESULTS

Derivation of binding parameters from kinetic measurements. In previous studies (6) we have demonstrated that the activation of factor X by factor IXa is increased 3 orders of magnitude by negatively charged phospholipids and that the presence of the non-enzymatic protein cofactor VIIIa causes an additional 200 000-fold rate enhancement. Kinetic analysis (6) revealed that factor VIIIa increases the $V_{\max}$ of factor X activation whereas the presence of phospholipid bilayers decreases the K_m for factor X. These effects of accessory components on the kinetic parameters of factor X activation do explain the above mentioned rate enhancements. The drop of the K_m in the presence of phospholipids is explained in a model in which phospholipids promote the formation of the enzyme-substrate complex by binding both factor IXa and factor X. The factor X and factor IXa concentrations in the presence of phospholipids can be chosen such that only factor IXa molecules which are incorporated in either factor IXa-phospholipid- or factor IXa-factor VIIIa-phospholipid complexes (factor IXa_{bound}) contribute to the rate of factor X activation (V). In that case

$$V = c \cdot [IXa]_{\text{bound}} \quad (I)$$

where c is a proportionality factor determined by the kinetic parameters of factor X activation. The dissociation constant K_d for dissociation of factor IXa from kinetically functional binding sites is given by

$$K_d = \frac{[IXa]_{\text{free}} \cdot ([\text{sites}]_{\text{total}} - [IXa]_{\text{bound}})}{[IXa]_{\text{bound}}} \quad (II)$$

where $[IXa]_{\text{free}}$ is the concentration factor IXa free in solution and $[\text{sites}]_{\text{total}}$ is the total concentration of factor IXa binding sites (phospholipid or factor VIIIa-phospholipid).

Rearrangement of equation II yields:

$$\frac{1}{[IXa]_{\text{bound}}} = \frac{K_d}{[IXa]_{\text{free}} \cdot [\text{sites}]_{\text{total}}} + \frac{1}{[\text{sites}]_{\text{total}}} \quad (III)$$

Substitution of equation I in III results in:

$$\frac{1}{V} = \frac{1}{c} \cdot \frac{K_d}{[IXa]_{\text{free}} \cdot [\text{sites}]_{\text{total}}} + \frac{1}{[\text{sites}]_{\text{total}}} \quad (IV)$$

A plot of V^{-1} vs. $[IXa]_{\text{free}}^{-1}$ is linear, with the intercept at the ordinate being $c^{-1}[\text{sites}]_{\text{total}}^{-1}$ and the intercept at the abscissa equal to $-K_d^{-1}$. The K_d is the

FIG.1. Effect of factor IXa on the rate of factor X activation in the absence (A) and presence (B) of factor VIIIa. Factor IXa and phospholipid vesicles were prewarmed at 37 °C in 0.3 ml of a buffer containing 50 mM Tris-HCl, 175 mM NaCl, 10 mM CaCl₂, pH 7.9. Factor X activation was started by addition of 0.2 ml prewarmed factor X (panel A) or 0.1 ml of activated factor VIII plus 0.1 ml of factor X (panel B). After 60s and 120s (A) or 45s and 90s (B) samples were taken and assayed for factor Xa as described in Experimental Procedures. The final composition of the reaction mixtures was: factor IXa as indicated, 25 μM phospholipid, 0.5 μM factor X, 50 mM Tris-HCl, 175 mM NaCl, pH 7.9, 6 mM CaCl₂, 0.5 mg/ml human albumin. The composition of the reaction mixtures of the experiments shown in panel B were identical to panel A, except for the presence in B of thrombin-activated factor VIII in a final concentration of 0.012 nM (0.011 units/ml). The insets depict double-reciprocal plots of the titration curves.

concentration $[IXa]_{\text{free}}$ required to occupy 50% of the sites_{total}, which will result in half-maximal factor X activation rates. When $[IXa]_{\text{bound}} \ll [IXa]_{\text{free}}$ (a condition met in the experiments presented in this paper), the free factor IXa concentration equals the total concentration of factor IXa, which allows the estimation of the binding parameters of factor IXa for phospholipid from intercepts in plots of V^{-1} vs $[IXa]_{\text{total}}^{-1}$.

Assembly of the intrinsic factor X activating complex, studied by varying the factor IXa concentration. The relationship between the rate of factor X activation and the concentration of factor IXa, in the presence of phospholipids, was examined to determine whether binding data for factor IXa could be derived from kinetic experiments. In the two experiments shown in fig.1, initial rates

Table 1. Assembly of the intrinsic factor X activating complex in absence and presence of factor VIIa at varying phospholipid and factor X concentration. The reaction conditions were as described in figure 1. The concentration of factor VIIa, when present, was 0.017 nM. $V(IXa)$ is the rate of factor X activation at infinitely high concentration of factor IXa. Kd^{app} is the factor IXa concentration required to obtain 0.5 $V(IXa)$.

Components in reaction mixture		Absence of factor VIIa		Presence of factor VIIa	
Factor X (μM)	Phospholipid (μM)	Kd^{app} factor IXa (nM)	$V(IXa)$ (nM Xa/min)	Kd^{app} factor IXa (nM)	$V(IXa)$ (nM Xa/min)
0.5	10	370	2.8	6.7	9.1
0.5	25	770	10.4	10.0	11.4
0.5	50	1110	23.2	16.1	12.3
0.2	50	830	14.3	5.1	7.2
0.05	50	480	4.2	3.0	4.1

of factor X activation were determined as a function of the concentration factor IXa at fixed concentrations of phospholipid vesicles (25 μM) and factor X (0.5 μM). In panel A no factor VIIa was added and in panel B thrombin activated factor VIII at a concentration of 0.012 nM (0.011 units/ml) was present. In both experiments the rate of factor X activation reached a plateau value and could, therefore, be saturated with respect to factor IXa. However the factor IXa concentration required for saturation was considerably lower in the presence of factor VIIa (B) than in its absence (A). Double-reciprocal plots of the data of fig.1 show a linear relationship between the plotted variables. Straight lines in double-reciprocal plots are in accordance with equation IV. This expression was derived for titration of single identical binding sites with factor IXa, which upon occupation, contribute to the rate of factor X activation. By applying equation IV to the kinetic data, apparent Kd values for the dissociation of factor IXa from factor IXa-phospholipid and factor IXa-factor VIIa-phospholipid complexes could be calculated. The Kd^{app} found in the absence of factor VIIa was 770 nM and in the presence of factor VIIa 10 nM.

Additional factor IXa titration experiments in the absence and presence of factor VIIa were carried out at varying phospholipid and factor X concentrations. All double-reciprocal plots were linear, and binding and kinetic data calculated from the plots are summarized in table 1. It is shown that all Kd^{app} values for factor IXa are 50-100 fold lowered by the presence of factor VIIa, indicating that factor IXa has a higher affinity for factor VIIa-phospholipid complexes than for phospholipid alone. The small effects of

Fig.2. Double reciprocal plots of the effect of factor IXa on the rate of factor X activation at varying concentration of activated factor VIII.

Factor IXa titration curves were made at four different factor VIIIa concentrations as described in the legend of fig.1. The final composition of the reaction mixtures (0.5 ml) was: 50 μ M phospholipid, 0.5 μ M factor X, 3-50 nM factor IXa, 50 mM Tris-HCl, 175 mM NaCl, pH 7.9, 6 mM CaCl_2 , 0.5 mg/ml human albumin and?

(○—○), 0.0125 nM factor VIIIa;
(▲—▲), 0.033 nM factor VIIIa;
(■—■), 0.0375 nM factor VIIIa
(●—●), 0.1 nM factor VIIIa.

varying phospholipid and factor X concentrations on K_d^{app} will be further considered in the discussion.

In the absence of factor VIIIa, the $V(\text{IXa})$ values (rates of factor X activation obtained by extrapolation to infinitely high concentration of factor IXa) increased at increasing phospholipid concentrations. The proportional increase of $V(\text{IXa})$ with the phospholipid concentration is consistent with an increased number of sites for factor IXa available at the phospholipid surface. In the presence of factor VIIIa, $V(\text{IXa})$ was hardly affected by the phospholipid concentration, suggesting that under these conditions factor VIIIa rather than phospholipid limits the total amount of factor IXa-factor VIIIa-phospholipid complexes which can be formed. Both in the absence and presence of factor VIIIa, decrease of the factor X concentration resulted in a decrease of $V(\text{IXa})$, which is due to the fact that rates of factor X activation were measured at a factor X concentration around the K_m . The K_m for factor X at 50 μ M phospholipid was 0.15 μ M in the absence of factor VIIIa and was 0.053 μ M with factor VIIIa (data not shown).

To test the suggestion that, at the phospholipid concentrations given in table 1 and fig.1, factor VIIIa is the limiting component for the functional binding

Table 2. Factor IXa titrations at four different factor VIIla concentrations. The K_d^{app} and $V(IXa)$ values were calculated from the double reciprocal plots shown in fig.2.

Factor VIIla	K_d^{app}	$V(IXa)$	$V(IXa)/[VIIla]$
(nM)	(nM)	(nM Xa/min)	(nM Xa/min per nM VIIla)
0.0125	16.7	12.3	984
0.033	17.8	28.2	855
0.0375	14.0	35.2	939
0.1	16.0	92.6	926

sites for factor IXa, titration experiments with factor IXa were carried out at four different factor VIIla concentrations. The double-reciprocal plots obtained are shown in fig.2 and the binding and kinetic parameters derived from these plots are summarized in table 2. The $V(IXa)$ increases proportionally with the amount of factor VIIla present. The K_d^{app} is not influenced by the factor VIIla concentration indicating that the affinity of factor IXa for functional binding sites is independent of the amount of factor VIIla present. These results and the observation that in the presence of factor VIIla variation of the phospholipid concentration does not affect the $V(IXa)$, strongly suggest that in these experiments most of the factor VIIla molecules are bound to the phospholipid bilayers where they form the kinetically functional binding sites for factor IXa.

Assembly of the factor X activating complex at increasing factor VIIla concentrations. Having shown that the rate of factor Xa formation at limiting factor VIIla concentrations is saturable with respect to factor IXa, it was of interest to examine whether with limiting amounts of factor IXa, the rate of factor Xa formation could also be saturated with factor VIIla. Varying amounts of activated factor VIII were added to reaction mixtures containing factor IXa, phospholipid, Ca^{2+} and factor X. The factor IXa concentration determined by active-site titration (15) was 0.017 nM. As shown in fig.3, the rate of factor X activation was also saturable with factor VIIla. The double-reciprocal plot is linear and from the intercept at the ordinate the value for $V(VIIla)$ is calculated, which represents the rate of factor Xa formation when all factor IXa molecules are complexed with factor VIIla. With $V(VIIla)$ and $V(IXa)$ determined under the same experimental conditions the molar concentration of factor VIIla can be calculated as outlined in materials and methods. This enabled the plotting of factor VIIla in molar terms (fig.3). The K_d^{app} thus found for the binding of factor VIIla to factor IXa in the presence of

Fig.3. Effect of varying amounts of activated factor VIII on the rate of factor X activation in the presence of a limiting amount of factor IXa.

Factor X activation rates were measured as in fig.1B. Factor VIII activation was at constant concentration of 70 units/ml of factor VIII and 3.65 nM of thrombin. The final composition of the reaction mixture for factor X activation was: 50 mM Tris-HCl, 175 mM NaCl, 6 mM $CaCl_2$, pH 7.9, 2.5 mg/ml human albumin, factor VIIa as indicated in the figure, 0.9 nM thrombin, 0.017 nM factor IXa 25 μ M phospholipid vesicles and 0.5 μ M factor X_2 .

phospholipid is 6.7 nM. This value is in the same range as the K_d^{app} values for factor IXa binding to factor VIIa in the presence of phospholipids (table 1).

DISCUSSION

In this paper we present evidence for the hypothesis that during intrinsic factor X activation, factor IXa and factor VIIa are complexed together at the phospholipid surface. Evidence for factor IXa-factor VIIa interaction is provided by the observation that the rate of factor X activation is saturable with excess factor IXa in the presence of limiting amounts of factor VIIa and vice versa. The amount of complex formed appeared proportional to the amount of limiting component present. The saturation curves were hyperbolic suggesting that single identical sites were titrated in each case. By titration of a limited amount of factor VIIa with factor IXa in the presence of phospholipid a K_d^{app} for factor IXa between 3-16 nM was found. Titration of a limited amount of factor IXa with factor VIIa in the presence of phospholipid re-

sulted in a K_d^{app} of 6.7 nM for factor VIIIA. We further carried out factor X activation experiments in the absence of factor VIIIA. The K_d^{app} for factor IXa binding to phospholipids obtained from kinetic experiments varied between 0.4-1.1 μ M. These values are comparable to binding parameters determined by Nelsestuen et al. (8) in direct binding studies. At a suboptimal Ca^{2+} concentration they observed a K_d of 2 μ M for dissociation of factor IX or for RVV-activated factor IX from phospholipid vesicles. Comparison of factor X activation experiments in the absence and presence of factor VIIIA indicates that the apparent K_d for factor IXa binding is lowered 50-100 fold in the presence of factor VIIIA. We conclude that factor VIIIA functions as a high affinity binding site for factor IXa at the phospholipid surface. It is likely that the combination of factor IXa-phospholipid and factor IXa-factor VIIIA interactions promote the incorporation of factor IXa into the factor X activating complex. In that respect factor VIIIA has a similar function as factor Va in prothrombin activation. Nesheim et al. (11) and Lindhout et al. (12) reported that factor Va promotes the binding of factor Xa to negatively charged phospholipid surfaces.

The dependence of K_d^{app} from the phospholipid and the factor X concentration is not completely understood. The following factors may contribute to the observed dependence: a) Up to 15% of added factor IXa is bound, which at the higher phospholipid concentration results in somewhat higher K_d^{app} values b) Factor X binds to phospholipids with a K_d of 50 nM (9) and may compete with factor IXa for binding to the phospholipid surface. To appreciate the interaction of factor VIIIA with factor IXa and phospholipids it would be important to study factor VIIIA binding using a direct binding assay. Presently, however, the instability of activated factor VIII preparations does not allow such an approach.

In our previous experiments on the intrinsic factor X activator we used factor VIII complex consisting of factor VIII:C and von Willebrand factor (6). In the present work we used a factor VIII:C preparation (14) that contains little or no von Willebrand factor. Using factor VIII complex, a V_{max} of 500 moles of factor Xa min^{-1} per mol of factor IXa was reported, whereas in this study the V_{max} value is 900. This result suggests that the von Willebrand factor does not strongly influence the V_{max} of factor Xa formation.

The physiological implications of the high-affinity binding sites for factor IXa, formed by factor VIIIA at a lipid- or activated platelet surface appear important. The concentration of factor VIII in human plasma is 0.25 nM (unpublished observations). The concentration of factor IX, which is at least

200-fold higher, is approximately 60 nM (16). During the clotting process only small amounts of factor IX are converted into factor IXa and the concentrations of factors VIIla and IXa will remain in the nanomolar range. The 50-100 fold lowering of the Kd for factor IXa binding (to the nM range) by factor VIIla therefore allows more efficient factor IXa-factor VIIla complex formation. Previously we found that factor VIIla caused a 200 000 fold enhancement of the Vmax of factor Xa formation (6). It now appears that the overall enhancement is explained by two major effects of factor VIIla: a) greatly enhanced catalytic power of factor IXa-VIIla-phospholipid, compared to factor IXa-phospholipid; b) enhanced factor IXa affinity for the lipid surface in the presence of factor VIIla, which increases the amount of phospholipid-bound factor IXa that participates in factor X activation.

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CHAPTER VI

KINETIC STUDIES OF PROTHROMBIN ACTIVATION

The effect of factor Va and phospholipids on the formation of the enzyme-substrate complex.

SUMMARY

The kinetic parameters of bovine prothrombin activation by factor Xa were determined in the absence and presence of factor Va as a function of the phospholipid concentration and composition.

In the absence of factor Va the K_m for prothrombin increases proportional with the phospholipid concentration and correlates well with the affinity of prothrombin for the different membranes. Phospholipid vesicles with a high affinity for prothrombin yield low K_m values compared to membranes with less favourable binding parameters. At limited phospholipid concentrations the V_{max} of prothrombin activation correlates with the binding affinity of factor Xa for the various phospholipid vesicles. Membranes with a high affinity for factor Xa have high V_{max} values, while for membranes with a low affinity a low V_{max} is observed. Extrapolation of double reciprocal plots of $1/V_{max}$ versus $1/[\text{phospholipid}]$ to infinite phospholipid concentrations, a condition at which all factor Xa would participate in prothrombin activation, yields a k_{cat} of $2-4 \text{ min}^{-1}$ independent of the type and amount of acidic phospholipid present in the vesicles. Also in the presence of factor Va the K_m for prothrombin varies proportional with the phospholipid concentration. There is, however, no correlation between the binding parameters and the K_m . Factor Va drastically lowers the K_m for prothrombin for vesicles which have low affinity for prothrombin. Vesicles composed of 20 mole% phosphatidylglycerol and 80 mole% phosphatidylcholine have a K_m of $0.04 \mu\text{M}$ when factor Va is present, compared to $2.2 \mu\text{M}$ determined in the absence of factor Va. Vesicles that contain low amounts of phosphatidylserine also have considerably lower K_m values for prothrombin in the presence of factor Va than in its absence. When factor Va is present the V_{max} of prothrombin activation is virtually independent of the affinity of factor Xa for phospholipid. Over a wide range of phospholipid concentrations and compositions a k_{cat} of 4500 min^{-1} is observed. Our data establish that prothrombin activation by factor Xa in the presence of factor Va and phospholipid is independent of the prothrombin density at the phospholipid surface. A model in which the enzymatic unit of prothrombinase consists of a three component complex (factor Xa-factor Va-phospholipid) with an increased affinity for soluble prothrombin can explain the observed effects of factor Va on the K_m for prothrombin. Therefore, factor Va accelerates prothrombin activation by: 1) increasing the k_{cat} of prothrombin activation; 2) promoting the binding of factor Xa; 3) promoting the assembly of the enzyme-substrate complex.

INTRODUCTION

The activation of the blood coagulation factor prothrombin is catalyzed by prothrombinase, an enzymatic complex composed of the serine protease factor Xa and the non-enzymatic cofactors factor Va, phospholipid and Ca^{2+} (1). Previous kinetic studies revealed that prothrombin activation by factor Xa alone is an inefficient process with a low V_{max} of thrombin formation and a high K_{m} for prothrombin (2). Negatively charged phospholipids plus Ca^{2+} stimulate prothrombin activation by lowering the K_{m} for prothrombin, while factor Va greatly enhances prothrombin activation by increasing the V_{max} (2,3). All protein components of the prothrombinase complex bind to phospholipid. This led to the proposal that phospholipid membranes act as a surface at which the proteins involved in prothrombin activation interact with each other. The increased prothrombin concentration at the phospholipid-water interface explains the observed decrease of the K_{m} for prothrombin in the presence of phospholipid. This model, in which the K_{m} is determined by the local density of prothrombin at the membrane surface (2,4) also explains the increase of the K_{m} observed at increasing phospholipid concentration.

In an alternative model postulated by Nelsetuen (5), the prothrombinase complex is viewed as a dissociable three-component enzyme (Factor Xa-factor Va-phospholipid) that acts on soluble prothrombin. The increased affinity for prothrombin is the result of additive free energies of prothrombin-factor Va and prothrombin-phospholipid interactions occurring at the active site of prothrombinase. Recently, Pusey and Nelsetuen (6) indeed demonstrated that the K_{m} for prothrombin is independent of the prothrombin density at the membrane surface when factor Va is present. However, previous kinetic studies on prothrombin activation in the absence of factor Va showed that the observed K_{m} did correlate with the prothrombin density at the phospholipid surface (2). These observations suggest that factor Va may alter the mechanism by which prothrombin enters the catalytic site of the prothrombin activating complex. The present report concerns a detailed kinetic analysis of the effects of factor Va on the kinetic parameters of prothrombinase. Phospholipid vesicles that contain various amounts of different acidic phospholipids were used in order to vary the affinity of the membranes for prothrombin and to test whether a correlation exists between the prothrombin density at the phospholipid surface and the observed K_{m} for prothrombin. It is shown that in the absence of factor Va the K_{m} correlates with the affinity of prothrombin for phospholipid, while in the presence of factor Va such a correlation does not

exist. These findings suggest that the mode of action of phospholipids in prothrombin activation with and without factor Va cannot be described in one unique model.

MATERIALS AND METHODS

Reagents. S2238 was purchased from AB Kabi Diagnostica, p-NPGB was from Nutritional Biochemicals. Ovalbumin, human serum albumin (fatty acid free), soy bean trypsin inhibitor, RVV-X, Echis Carinatus venom and 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC) were obtained from Sigma. DEAE-Sephadex A-50, QAE Sephadex A-50, SP-Sephadex C-50, Sephadex G-100 and CNBr activated Sepharose-4B were purchased from Pharmacia. Soybean trypsin inhibitor was coupled to CNBr-activated Sepharose 4B according to the method of Cuatrecasas (7). All reagents used were of the highest grade commercially available.

Proteins. Bovine prothrombin and prethrombin-1 were purified according the method of Owen et al. (8). Thrombin was purified as described earlier (2). Bovine Factor X was purified as described by Fujikawa et al. (9). RVV-X was purified from the crude venom by the method of Schiffman et al. (10). Factor Xa was prepared from factor X activated with RVV-X (11). Factor V and Factor Va were obtained as described by Lindhout et al. (12). Factor Xa, prothrombin and thrombin were stored at -80°C in 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml human serum albumin (pH 7.9). Factor Va was stored in the same buffer containing 5 mM CaCl_2 .

Protein concentrations. The molar concentration of thrombin was determined by active site titration with p-NPGB according to Chase and Shaw (13). Prothrombin and prethrombin-1 concentrations were determined by the same method after complete activation with Echis Carinatus venom. Factor Xa concentrations were determined by active site titration according to Smith (14). The concentration of factor Va was determined by kinetic analysis as described by Lindhout et al. (12).

Phospholipid and phospholipid vesicle preparations. 1,2-dioleoyl-sn-glycero-3-phosphoserine (PS), 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (PG) and 1,2-dioleoyl-sn-glycero-3-phosphate (PA) were prepared from 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC) by enzymatic synthesis as described by Comfurius and Zwaal (15). Lipid analysis was carried out as described previously (16). Single bilayer phospholipid vesicles were prepared according to the method of De Kruijff et al. (17). Phospholipid concentrations were determined by phosphate analysis as described by Böttcher et al. (18).

Measurement of rates of prothrombin activation. Factor Xa and phospholipids with or without factor Va were incubated for 5 min at 37 °C in a buffer containing 50 mM Tris, 175 mM NaCl, 5 mM CaCl₂ and 0.5 mg/ml human serum albumin at pH 7.9. Prothrombin activation was started by the addition of prewarmed prothrombin in the same buffer. The final concentration of factor Xa in experiments in which factor Va was absent, was 1 nM. In the presence of factor Va (5 nM) prothrombin activation was carried out at 1 pM factor Xa. After different time intervals samples were taken from the prothrombin activating mixture and transferred to cuvettes containing the thrombin specific chromogenic substrate S2238. The cuvettes (thermostated at 37 °C) contained 1 ml of 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 20 mM EDTA, 0.5 mg/ml ovalbumin and 235 µM S2238. The thrombin concentration was determined by measuring the rates of absorbance change recorded at 405 minus 500 nm on an Aminco DW-2A spectrophotometer in the dual-wavelength mode. The amount of thrombin was calculated from a calibration curve made with known amounts of active site-titrated thrombin.

Kinetic analysis. Michaelis-Menten kinetics was applied to analyse the kinetic data of prothrombin activation. The kinetic parameters were obtained from initial rate measurements. Thrombin formation rates were linear in time and care was taken that small amounts of substrate were converted during the time course of the experiments. Rates of thrombin formation were calculated and expressed as thrombin formed per min per factor Xa. Km and Vmax, were determined by statistical analysis of the Lineweaver-Burk plots as described by Eisenthal and Cornish-Bowden (19). The Lineweaver-Burk plots were straight lines over a wide range of prothrombin concentrations. No deviation from linearity could be detected with prothrombin concentrations that were more than 5-fold above or below the Km for prothrombin.

RESULTS AND DISCUSSION

The effect of phospholipid vesicles containing different polar headgroups on the kinetic parameters of prothrombin activation.

Previous kinetic studies revealed that negatively-charged phospholipids promote factor Xa-catalyzed prothrombin activation by lowering the K_m for prothrombin and that the observed K_m increased proportional with the amount of phospholipid present (2). Two models have been proposed to explain the effects of phospholipid on the K_m for prothrombin:

1. In the so-called "bound substrate model" (2,4) it is suggested that the overall prothrombin density at the phospholipid surface determines the rate of prothrombin activation by phospholipid-bound factor Xa. When phospholipids are present, the prothrombin concentration in a shell surrounding the phospholipid surface greatly exceeds that in free solution, which explains the drastic drop of the K_m for prothrombin. Increasing the phospholipid concentration lowers the prothrombin density at the phospholipid surface with a consequential increase of the observed K_m .
2. In the so-called "free substrate model" (5) the soluble prothrombin concentration directly determines the rate of prothrombin activation by phospholipid-bound factor Xa. The enzymatic unit of the prothrombin activating complex is thought to consist of a dissociable two component (factor Xa-phospholipid) or three component (factor Xa-factor Va-phospholipid) complex, that binds prothrombin from solution with increased affinity. The affinity for soluble prothrombin is determined by the additional free energies of prothrombin binding to the individual components of this complex. Increasing the phospholipid concentration lowers the free prothrombin concentration since prothrombin binds to phospholipid outside the enzymatic domain; hence more prothrombin has to be added to obtain the free prothrombin concentration required for half saturation of the enzymatic unit. Therefore, also in the free substrate model the K_m for prothrombin increases proportional with the phospholipid concentration.

The fundamental difference between the two models is that in the bound substrate model lateral diffusion of prothrombin across the phospholipid surface is an essential step in the formation of the enzyme-substrate complex, while in the free substrate model the enzyme-substrate complex is formed by direct interaction of prothrombin with the enzymatic unit.

The bound substrate model predicts that the V_{max} and K_m are directly

Table I. The effect of factor Va on the kinetic parameters of prothrombin activation measured in the presence of vesicles containing various acidic phospholipid. Varying amounts of prothrombin were activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM CaCl_2 , 0.5 mg/ml human serum albumin, 50 μM phospholipid and 1 nM factor Xa and no factor Va or 1 pM factor Xa and 5 nM factor Va. Thrombin formation rates and kinetic parameters were determined as described in Materials and Methods.

Vesicle phospholipid composition	K_m (μM)	V_{max} ($\text{IIa min}^{-1}\text{Xa}^{-1}$)
<u>- factor Va</u>		
PS/PC (25/75; M/M)	0.11	2.56
PA/PC (25/75; M/M)	0.10	2.78
PG/PC (25/75; M/M)	1.81	0.17
<u>+ factor Va</u>		
PS/PC (25/75; M/M)	0.14	4050
PA/PC (25/75; M/M)	0.11	4184
PG/PC (25/75; M/M)	0.04	3345

related to the binding parameters of, respectively, factor Xa and prothrombin for phospholipid. Vesicles with increased affinity for factor Xa bind more factor Xa, hence more factor Xa will participate in prothrombin activation and higher V_{max} values will be observed. Vesicles with an increased affinity for prothrombin will yield lower K_m values since less prothrombin is required to obtain the prothrombin density at the phospholipid surface at which half of the phospholipid-bound factor Xa is saturated. In order to test these predictions we determined kinetic parameters of prothrombin activation for vesicles that contain negatively charged phospholipids with different polar headgroups (PS, PA, PG), which are known to have different binding parameters for factor Xa and prothrombin (20). Table I shows, that in the absence of factor Va, both the V_{max} and the K_m depend on the type of acidic phospholipid present in the phospholipid vesicles. PS and PA, which at the experimental pH have 2 negative charges in their headgroup (PS also contains 1 positive charge), give considerably higher V_{max} and lower K_m values than PG, which contains only one negative charge. The observed kinetic parameters relate very well to previously reported binding parameters. Membranes, that contain PS and PA have a higher affinity for prothrombin and factor Xa, than those containing PG. Table I also reveals that the kinetic parameters, determined in the presence of factor Va, do not reflect the differences in binding parameters for factor Xa and prothrombin. When factor Va is part of the prothrombin activating complex the V_{max} is hardly affected by the type of acidic phospholipid present in the

Fig. 1. The effect of the mole percentage PG in PG-PC vesicles on the kinetic parameters of prothrombin activation. Various amounts of prothrombin were activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM CaCl₂, 0.5 mg/ml human serum albumin, 50 μM phospholipid and 1 nM factor Xa and no factor Va (○-○) or 1 pM factor Xa and 5 nM factor Va (●-●). The mole percentage PG present in the phospholipid vesicles is indicated in the figure. Thrombin formation rates, Vmax (figure 1A) and Km (figure 1B) were determined as described in Materials and Methods.

membrane bilayer. This can be attributed to the fact that factor Va promotes the binding of all added factor Xa to phospholipid vesicles, since rates of prothrombin activation in our experiments were optimized with respect to the amount of factor Va present. Moreover, the Km does not relate to the binding parameters of prothrombin for the phospholipid vesicles per se. Vesicles containing PG, which have the lowest affinity for prothrombin, have the most favourable Km for prothrombin. Therefore, we have to conclude that in the presence of PG containing vesicles and factor Va, the Km for prothrombin is not determined by the prothrombin density at the membrane surface.

Kinetic parameters of prothrombin activation for vesicles containing various mole percentages of acidic phospholipid.

The question arises whether the effects of factor Va on the kinetic parameters of prothrombin activation (especially on the Km) are a unique feature of PG containing vesicles or that they must be attributed to the low affinity of these vesicles for prothrombin and factor Xa. To this end we determined the Km and Vmax for vesicles that contain various mole percentages of the acidic phospholipids PG or PS. Lowering the mole percentage of acidic phospholipid in these vesicles results in a weaker binding of factor Xa and prothrombin (20). Fig 1A shows the Vmax values determined for vesicles that contain various mole percentages PG. Without factor Va there is a considerable increase of the Vmax

Fig. 2. The effect of the mole percentage PS in PS-PC vesicles on the kinetic parameters of prothrombin activation. The experimental conditions are described in the legend of figure 1. (A) represents the effect of mole percentage PS on the Vmax and (B) the effect of mole percentage PS on the Km (○-○ no factor Va present; ●-● 5 nM factor Va present).

when the mole percentage PG in the vesicles is increased. In the presence of factor Va, the Vmax is much less dependent on the PG content of the vesicles. At more than 40 mole% PG a maximal value is observed, which suggests that factor Va is able to promote the binding of all added factor Xa to the phospholipid vesicles.

The Km values obtained for vesicles with different mole percentages PG are presented in Figure 1B. Without factor Va, the Km increases when the mole percentage PG is lowered. The increase of the Km for prothrombin runs parallel with the increase of the dissociation constant of the prothrombin-phospholipid complex. Two striking differences are observed, however, when factor Va is present: 1) In the presence of factor Va the Km for prothrombin is 10-40 fold lower than in its absence and 2) in the presence of factor Va the Km is independent of the binding affinity of prothrombin for the phospholipid vesicles between 20 and 100 mole% PG.

Essentially the same effects on the kinetic parameters are found when PS is utilized as acidic phospholipid. Unfortunately the mole percentage PS is limited to 40% since at higher percentages the vesicles tend to aggregate due to the presence of CaCl₂. Fig 2A shows that in the absence of factor Va there is a continuous increase of the Vmax when the mole percentage PS in the phospholipid vesicles is increased from 2.5% to 40%. With factor Va the Vmax is independent of the mole fraction PS, which again indicates that under these conditions all factor Xa molecules are bound to phospholipid and participate

Table II. Kinetic parameters of prethrombin-1 activation. Various amounts of prethrombin-1 were activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM CaCl₂, 0.5 mg/ml human serum albumin, 50 μ M phospholipid, 1 pM factor Xa and 5 nM factor Va. Measurement of thrombin formation rates and determination of kinetic parameters were carried out as described in Materials and Methods.

Vesicle phospholipid composition	Km (μ M)	Vmax(Ila min ⁻¹ Xa ⁻¹)
PS/PC (25/75; M/M)	11.3	1711
PA/PC (25/75; M/M)	12.1	1563
PG/PC (25/75; M/M)	8.1	1352

in prothrombin activation. Fig. 2B reveals that in the absence of factor Va the Km for prothrombin increases when the binding affinity of prothrombin for the vesicles is decreased. Factor Va has minor effects on the Km of prothrombin for vesicles containing between 20 and 40 mole% PS. At amounts of PS below 20 mole% factor Va seems to compensate for the reduced affinity of prothrombin for phospholipid; much lower Km values are observed in the presence of factor Va than in its absence.

It is even questionable whether in the presence of factor Va prothrombin-phospholipid interactions still contribute to the formation of the enzyme-substrate complex. Since the phospholipid binding capacity of prothrombin resides in its fragment-1 region, it is interesting to compare prothrombin and prethrombin-1 (which lacks fragment-1) as substrates for prothrombinase.

Table II gives the kinetic parameters for prethrombin-1 activation, determined in the presence of factor Va and vesicles containing either PS, PA or PG as acidic phospholipid. For all vesicles the Km values for prethrombin-1 are 100-200 fold higher than for prothrombin (cf. Table I). These experiments actually exclude a variant of the free substrate model in which prothrombin-phospholipid interactions do not contribute to the formation of the enzyme-substrate complex. In such a model the Km for prothrombin would only depend on its affinities for factor Xa and factor Va. The large difference between the Km for prothrombin and prethrombin-1 strongly suggests that prothrombin-phospholipid interactions are essential for the binding of prothrombin to the prothrombinase complex. We have as yet no explanation for the fact that the Vmax of prethrombin-1 activation is 2-3 fold lower than the Vmax for prothrombin activation. The important information contained in the experiments reported in this section is that the kinetic parameters of prothrombin activation in the presence of factor Va and phospholipid have no relation to the prothrombin density at the phospholipid membrane.

The effect of the phospholipid concentration on the K_m for prothrombin.

Previous kinetic studies of prothrombin activation carried out in our laboratory revealed that the K_m for prothrombin has to be regarded as an apparent K_m (K_m^{app}) since it increases at increasing phospholipid concentrations (2). For both the free substrate and the bound substrate model an equation can be derived, that relates the observed K_m with the phospholipid concentration and the binding parameters of prothrombin for the phospholipid vesicles present in the kinetic experiment. The total prothrombin concentration required to attain the observed K_m equals the sum of the concentrations of free and phospholipid-bound prothrombin (eq 1).

$$K_m^{app} = [PT_{free}] + [PT_{bound}] \quad (1)$$

Since the concentration of factor Xa present in our experiments is far below the concentration of binding sites (S_{total}) for vitamin K-dependent coagulation factors provided by the phospholipid vesicles

$$K_d = \frac{[PT_{free}] \cdot ([S_{total}] - [PT_{bound}])}{[PT_{bound}]} \quad (2)$$

where K_d is the dissociation constant of the prothrombin-phospholipid complex. Defining q as the maximal amount of prothrombin, that can bind to phospholipid (Mole/Mole) gives

$$[S_{total}] = q \cdot [\text{phospholipid}] \quad (3)$$

Substitution of eq 3 into eq 2, followed by rearrangement of eq 2 yields

$$[PT_{bound}] = \frac{q \cdot [\text{phospholipid}]}{K_d \cdot [PT_{free}]^{-1} + 1} \quad (4)$$

Substituting eq 4 into eq 1 gives the relation between K_m and the phospholipid concentration (eq 5).

$$K_m^{app} = [PT_{free}] + \frac{q \cdot [\text{phospholipid}]}{K_d \cdot [PT_{free}]^{-1} + 1} \quad (5)$$

This equation is applicable both to the free and the bound substrate model. In the free substrate model the free prothrombin concentration ($[PT_{free}]$) at the observed K_m^{app} is constant and independent of the amount of phospholipid present. Since also K_d and q are constants for a particular kind of phospho-

lipid the K_m^{app} will be linearly dependent on the phospholipid concentration. In the bound substrate model the K_m^{app} is attained at a particular prothrombin density at the phospholipid surface. The latter model is defined in such a way that this prothrombin density is independent of the phospholipid concentration and composition. Since the required prothrombin density for a certain kind of phospholipid is always reached at the same free prothrombin concentration, $[PT_{free}]$ at K_m^{app} is constant and a plot of K_m^{app} versus the concentration phospholipid is also a straight line in the bound substrate model. Differences exist between the intercept at the ordinate and the slope of the line when eq 5 is applied to the free or the bound substrate model. The intercept at the ordinate is actually the K_m that would be measured at infinitely low phospholipid concentration. We will call this value the intrinsic K_m of the prothrombinase complex. In the free substrate model the intrinsic K_m is the real K_m for prothrombin since no excess of phospholipid is present outside the enzymatic domain that can bind prothrombin and lower the free prothrombin concentration that interacts with the enzymatic complex. In the bound substrate model, $[PT_{free}]$, that is the intrinsic K_m , is the free prothrombin concentration required to obtain the prothrombin density at the phospholipid surface at which half of the phospholipid-bound factor Xa is involved in prothrombin activation. In this model the intrinsic K_m directly relates to the binding parameters of prothrombin for the kind of phospholipid vesicles used in the kinetic experiment. Membranes with less favourable binding parameters for prothrombin will have higher intrinsic K_m values, since more free prothrombin has to be present to obtain the required prothrombin density at the phospholipid surface.

The slopes of the K_m^{app} versus [phospholipid] plots contain important information.

The slope , $\frac{q}{K_d.[PT_{free}]^{-1} + 1}$ equals $[PT_{bound}]/[\text{phospholipid}]$ (eq 4),

which is actually the prothrombin density at the phospholipid surface required at K_m^{app} . In the bound substrate model this prothrombin density is defined to be a constant, which is independent of the phospholipid concentration and composition. When this model is applicable to prothrombin activation plots of K_m^{app} versus [phospholipid] will yield a set of parallel lines for phospholipid vesicles with different binding parameters for prothrombin. In the free substrate model there are no restraints on the prothrombin density at the membrane surface at K_m^{app} . The slope of the K_m^{app} versus [phospholipid] plots

Fig. 3. The effect of the phospholipid concentration on the K_m of prothrombin for phospholipid vesicles containing various mole percentages PS. Various amounts of prothrombin were activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM CaCl_2 , 0.5 mg/ml human serum albumin, amounts of phospholipid indicated in the figure and 1 nM factor Xa and no factor Va (A) or 1 pM factor Xa and 5 nM factor Va (B). The mole percentage PS present in the vesicles was: 2.5% ($\Delta-\Delta$), 5% ($\blacktriangle-\blacktriangle$), 10% ($\square-\square$), 20% ($\blacksquare-\blacksquare$), 30% ($\circ-\circ$) or 40% ($\bullet-\bullet$). Thrombin formation rates and the kinetic parameters were determined as described in the Materials and Methods.

is therefore in this model a function of the binding parameters of prothrombin and the intrinsic K_m ($[\text{PT}_{\text{free}}]$). Phospholipid vesicles with favourable binding parameters for prothrombin (high q and low K_d) will produce steep lines in the K_m versus [phospholipid] plot.

Figure 3 shows the effect of increasing phospholipid concentrations on the K_m for prothrombin measured in the absence and presence of factor Va. In this experiment phospholipid vesicles containing different mole percentages PS were used. The plots obtained in the absence and presence of factor Va are distinctly different. The slopes of the lines vary in the absence of factor Va 2-3 fold and in the presence of factor Va 70-fold for vesicles with mole percentages PS between 2.5 and 40%. Table III summarizes the intrinsic K_m values and the slopes calculated from the data presented in figure 3. In order to determine accurate intrinsic K_m values for 20, 30 and 40 mole% PS a separate experiment was carried out at phospholipid concentrations between 1 and 25 μM (data not shown). In the absence of factor Va the intrinsic K_m shows a wide variation for vesicles that contain different amounts of PS. At 2.5 mole% PS the intrinsic K_m is 3.35 μM , while at 40 mole% PS a value below 0.01 μM is calculated (the intrinsic K_m was actually too low to be accurately measured).

Table III. The effect of the mole percentage PS on the intrinsic Km for prothrombin and the slope of plots of K_m^{app} versus [phospholipid]. Intrinsic Km values and the slope of plots of K_m^{app} versus [phospholipid] for vesicles containing different mole percentages PS were calculated from the data presented in Figure 3. The intrinsic Km for vesicles containing, 20, 30 and 40 mole% PS was calculated from Km versus [phospholipid] plots using a phospholipid concentration between 1 and 25 μ M (data not shown).

PS (Mole%)	-factor Va		+factor Va	
	slope (M/M)	Intrinsic Km (μ M)	slope (M/M)	Intrinsic Km (μ M)
2.5	7.32×10^{-3}	3.35	- ^a	0.145
5	5.50×10^{-3}	1.30	0.07×10^{-3}	0.072
10	4.55×10^{-3}	0.325	0.25×10^{-3}	0.055
20	2.47×10^{-3}	0.023	1.35×10^{-3}	0.034
30	2.46×10^{-3}	<0.01 ^b	4.18×10^{-3}	0.027
40	2.67×10^{-3}	<0.01 ^b	5.28×10^{-3}	0.030

^a It was not possible to determine an accurate value for the slope.

^b To determine accurate values for the intrinsic Km, phospholipid concentrations below 1 μ M were necessary, which gave prothrombin activation rates that were too low to be measured.

When factor Va is present there is much less variation of the intrinsic Km. For vesicles with amounts of PS between 10 and 40 mole% the intrinsic Km is almost constant (0.055-0.030 μ M). From the different effects of PS on the slope of the K_m^{app} versus phospholipid plot and on the intrinsic Km it is obvious that the mode of action of phospholipids on prothrombin activation in the absence and presence of factor Va cannot be described in one unique model. The wide variation of slopes of K_m^{app} versus [phospholipid] plots determined in the presence of factor Va (fig. 3B) indicates that only the free substrate model can adequately describe the observed phenomena. In that case the slopes of the lines should correlate with known prothrombin binding parameters for the phospholipid membranes used in this experiment. Parameter fitting of the lines according to eq 5 indeed yields values for Kd and prothrombin binding sites per phospholipid (M/M), that fulfill the requirements of slope and intercept and which are in close agreement with prothrombin binding parameters for these membranes reported by Nelsestuen and Broderius (20). Table IV summarizes the binding parameters for prothrombin obtained by parameter fitting and those reported in the literature. The variation of the intrinsic Km (real

Table IV. Prothrombin binding parameters derived from plots of the K_m versus the phospholipid concentration for prothrombin activation in the presence of factor Va.

PS ^a (Mole%)	K _d (μ M)	sites/ phospholipid (M/M)	K _d ^b (μ M)	sites/ phospholipid ^b (M/M)
5	5.1	0.005	5.5	0.004
10	1.5	0.007	1.6	0.007
20	0.3	0.012	0.3	0.011
30	0.1	0.013	0.1	0.012
40	0.05	0.013	0.1	0.013

a) 2.5 Mole% PS is omitted since no direct binding data were available.

b) from Nelstuen and Broderius (1977)

K_m for prothrombinase) with the mole percentage PS observed in the presence of factor Va is also adequately explained in the free substrate model, since there is no correlation between the intrinsic K_m and the binding parameters of prothrombin for vesicles with varying mole percentage PS. In the free substrate model the intrinsic K_m is not only determined by the free energy of prothrombin-phospholipid interaction but also by the free energies of prothrombin-factor Va and prothrombin-factor Xa interactions. Since information about the quantitative contribution of each of these interactions is lacking no prediction of the actual value of the intrinsic K_m can be made.

The data obtained in the absence of factor Va are qualitatively better explained in the bound substrate model. The intrinsic K_m varies parallel with the dissociation constants reported for the prothrombin-phospholipid complex (20) and the slopes of the plots of K_m^{app} versus [phospholipid] (although they should be parallel in the bound substrate model) vary considerably less (2-3 fold) in the absence of factor Va than in its presence. It is impossible, however, to obtain by parameter fitting binding constants for prothrombin that are comparable to those determined in direct binding studies and at the same time meet the requirements of slope and intercept of the experimentally observed K_m^{app} versus [phospholipid] plot. This is caused by the low value of the intrinsic K_m , determined at the higher mole percentages PS. Intrinsic K_m 's below 0.01 μ M (Table III) cannot be reconciled with either of the two models and known parameters of prothrombin binding to phospholipid.

The effect of the phospholipid concentration on the V_{max} of prothrombin activation. In both the free substrate and the bound substrate model prothrombin activation is catalyzed by phospholipid-bound factor Xa. Figure 4A shows that without factor Va, the V_{max} is dependent on both the phospholipid concentra-

Fig. 4. The effect of the phospholipid concentration on the $V_{\max}$ of prothrombin activation for phospholipid vesicles containing various mole percentages PS. The experimental conditions are described in the legend of figure 3. (A) no factor Va present and (B) plus 5 nM factor Va. The mole percentage PS present in the vesicles was: 2.5% ($\triangle-\triangle$), 5% ($\blacktriangle-\blacktriangle$), 10% ($\square-\square$), 20% ($\blacksquare-\blacksquare$), 30% ($\circ-\circ$), or 40% ($\bullet-\bullet$).

tion and the amount of PS present in the membranes. Increasing the phospholipid concentration or the mole percentage PS results in an increase of the $V_{\max}$ of prothrombin activation. This is explained by the fact that more factor Xa is bound to the phospholipid vesicles and hence more factor Xa participates in prothrombin activation. Double reciprocal plots of $1/V_{\max}$ versus $1/[\text{phospholipid}]$ are straight lines (figure 5), which upon extrapolation to infinite phospholipid concentration yield $V_{\max}$ values between 2-4 thrombin formed/min/factor Xa independent of the amount of PS in the membranes. This shows that the catalytic activity of phospholipid-bound factor Xa is hardly affected by the phospholipid composition of the vesicles. In view of the fact that all added factor Xa participates in prothrombin activation, the $V_{\max}$ at infinite phospholipid concentration is actually the k_{cat} of prothrombin activation. Figure 4B shows that there is no correlation between the $V_{\max}$ and the affinity of factor Xa for phospholipid when factor Va is present. Only minor changes of $V_{\max}$ are observed for membranes containing between 2.5 and 40 mole% PS in a phospholipid concentration range of 10 to 300 μM . Apparently factor Va promotes the affinity of factor Xa for phospholipid to such an extent that almost all factor Xa molecules are bound to phospholipid and participate in prothrombin activation. Factor Va increases the k_{cat} of prothrombin activation three orders of magnitude. In the absence of factor Va the k_{cat} of prothrombin

Fig. 5. Double reciprocal plots of $1/V_{\max}$ against $1/[\text{phospholipid}]$ for vesicles containing various mole percentages PS. The experimental conditions for prothrombin activation in the absence of factor Va are described in the legend of figure 3. The mole percentage PS present in the vesicles was: 2.5% ($\triangle-\triangle$), 5% ($\blacktriangle-\blacktriangle$), 10% ($\square-\square$), 20% ($\blacksquare-\blacksquare$), 30% ($\circ-\circ$) or 40% ($\bullet-\bullet$).

activation is $2-4 \text{ min}^{-1}$ while in the presence of factor Va values of 4500 min^{-1} are observed.

CONCLUDING REMARKS

In this paper we presented an extensive study of the effects of different phospholipids on the kinetic parameters of prothrombin activation in order to discriminate between the two models (i.e. the bound substrate model and the free substrate model) that have been proposed to explain the mode of action of phospholipids in prothrombin activation.

The effects of phospholipids on the K_m for prothrombin determined in the absence of factor Va cannot be satisfactorily explained in either of the two proposed models. The K_m for prothrombin measured in the absence of factor Va varies parallel with the binding affinity of prothrombin for phospholipid (membranes with a low affinity have a high K_m , while for membranes with a high affinity a low K_m is measured). Neither in the bound nor in the free substrate model it is possible, however, to correlate the observed K_m on a quantitative basis with known binding parameters of prothrombin for phospholipid.

The mode of action of phospholipid in prothrombin activation in the pre-

sence of factor Va can be adequately explained in the free substrate model for the following reasons:

- 1) Factor Va causes a considerable decrease of the K_m for prothrombin when phospholipid vesicles with a low affinity for prothrombin are part of prothrombinase.
- 2) There is no relation between the observed K_m and the prothrombin density at the membrane surface when prothrombin is activated in the presence of factor Va and phospholipid vesicles with a low affinity for prothrombin.
- 3) The slopes of K_m^{app} versus [phospholipid] plots vary considerably for membranes with different affinities for prothrombin, which is one of the predictions of the free substrate model
- 4) The experimentally obtained K_m^{app} versus [phospholipid] plots can be theoretically fitted with binding parameters for prothrombin reported in the literature.

Our experimental data for prothrombin activation in the presence of factor Va support the proposal of Nelsestuen (5) that prothrombinase is a dissociable three component enzyme (factor Xa-factor Va-phospholipid) that converts soluble prothrombin; the observed increase of affinity for prothrombin, measured as a decrease of the K_m , is the result of prothrombin-factor Va and prothrombin-phospholipid interactions that occur near the active site of prothrombinase. From its effect on the K_m it is obvious that factor Va promotes the binding of prothrombin to the prothrombinase complex. This can be accomplished by 1) a direct interaction of prothrombin with factor Va, which provides additional free energy for the binding of prothrombin to the enzymatic complex and/or 2) a factor Va induced rearrangement of phospholipid molecules in the enzymatic domain that results in the creation of a better phospholipid surface for prothrombin binding. The first possibility is supported by direct binding studies (21) in which it was shown that factor Va promotes the binding of prothrombin to negatively charged phospholipids. The formation of a prothrombin-factor Va complex with a 1:1 stoichiometry is indicative for direct prothrombin-factor Va interaction. Insufficient quantitative data prevent, however, direct correlation of the effect of factor Va on prothrombin binding to phospholipids with its effect on K_m^{app} . With respect to the second possibility Mayer and Nelsestuen (22) reported that factor Va is able to cause lateral phase separation (clustering) of acidic phospholipids in membranes and suggested that this lateral phase separation may be an important process in the formation of the prothrombinase complex. Whether this clustering of acidic phospholipid molecules by factor Va also promotes prothrombin binding to the

membrane is, however, unknown. At present, we cannot draw conclusions with regard to the two possibilities mentioned above.

It is to be expected that the intrinsic K_m should continuously decrease when the mole percentage acidic phospholipid in membranes is increased since this would result in an increased free energy of prothrombin-phospholipid interaction. However, at mole percentages PS exceeding 5% the intrinsic K_m is virtually constant (Table III), although direct binding experiments (20) revealed that the affinity of the membranes for prothrombin still increases. The intrinsic K_m for prothrombinase in the presence of factor Va and membranes containing a high mole percentage PS is even higher than the intrinsic K_m measured in the absence of factor Va, which seems to contradict the proposal that factor Va promotes the affinity of the prothrombinase complex for prothrombin. These phenomena can be explained when the rate constants of prothrombinase are considered. From the intrinsic K_m and the k_{cat} a k_{cat}/K_m of $2.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ is calculated for a prothrombinase complex consisting of factors Xa and Va and phospholipids that contain 5 or more mole% PS. This indicates that prothrombinase under these conditions is a diffusion controlled process in which a further increase of the binding affinity of phospholipids does not further change the kinetic parameters because of diffusion limitation. Hence the intrinsic K_m will become constant for vesicles that contain 5 or more mole% PS. When factor Va is absent k_{cat}/K_m is three orders of magnitude lower, which indicates that prothrombin activation under these conditions is not diffusion limited. This can explain why the intrinsic K_m values decrease below those observed in the presence of factor Va. The rate constant of prothrombinase of $2.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ exceeds the rate constant for association of prothrombin with a single binding site on a PS containing membrane ($k_1 = 3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) reported by Wei et al. (23). This rate constant, which was determined in the absence of factor Va, is not compatible with the rate constant of prothrombinase with factor Va. To account for the rate constant of prothrombin activation factor Va has to increase the association rate constant of prothrombin binding to the complex.

In conclusion factor Va has three important effects in prothrombinase: 1) it accelerates prothrombin activation by increasing the k_{cat} more than 1000-fold, 2) it promotes the binding of factor Xa to phospholipids and therefore increases the amount of factor Xa that participates in prothrombin activation 3) it promotes the affinity of prothrombinase for its substrate prothrombin on membranes which have a low binding affinity for prothrombin. The latter effects presumably have physiological importance. It has been proposed that

activated blood platelets provide the phospholipid surface at which prothrombin activation takes place. Activated platelets expose relatively small amounts of acidic phospholipids (16). On platelet membrane areas with low PS density factor Va will have an important role in the binding of prothrombin and factor Xa and the subsequent formation of the enzyme-substrate complex.

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CHAPTER VII

CONCLUSIONS AND SUMMARY

Prothrombin activation and intrinsic factor X activation are strongly stimulated by the presence of membranes containing negatively charged phospholipids and by non-enzymatic protein cofactors (factor Va for prothrombin- and factor VIIIa for factor X activation). The primary object of the studies presented in this thesis was to investigate the possibility that, during hemostasis, blood platelets provide the membrane surface required for prothrombin- and factor X activation. In chapter II we demonstrated that platelets are most active in promoting prothrombin activation, when they are stimulated by the combined action of collagen and thrombin. Unstimulated platelets or platelets stimulated with either collagen or thrombin alone were much less active in this respect. The phospholipid composition of the outer platelet surface was studied by treatment of activated and control platelets with phospholipase A_2 . Phospholipids from unstimulated platelets and from platelets stimulated by either collagen or thrombin were hardly susceptible to phospholipase A_2 . In contrast, when platelets were activated by collagen plus thrombin, up to 20% of the total amount of platelet phospholipids could be degraded by phospholipase A_2 , without significant cell lysis. The hydrolysis of glycerophospholipids comprises 25% of the phosphatidylserine-, 25% of the phosphatidylcholine- and 30% of the phosphatidylethanolamine content of the platelets stimulated with collagen plus thrombin. Using different probes to determine the phospholipid distribution over the inner- and outer leaflet of the platelet's plasma membrane, other authors reported that phosphatidylserine is almost exclusively located in the interior of unstimulated and thrombin stimulated platelets (1-4). Structural changes in the plasma membrane of platelets after stimulation with collagen plus thrombin may cause both the increased susceptibility of phospholipids to phospholipase A_2 and the exposure of phosphatidylserine required for efficient prothrombin activation.

It was expected that exposure of phosphatidylserine at the surface of platelets triggered with collagen plus thrombin would evoke the platelet procoagulant surface not only for prothrombin activation but also for intrinsic factor X activation. In chapter III we studied the role of stimulated platelets in prothrombin- and factor X activation with purified coagulation

factors and chromogenic substrates to measure rates of thrombin- and factor Xa formation. Using amounts of enzymes, substrates and protein cofactors that were saturating with respect to rates of substrate activation, the rates of prothrombin- and factor X activation found with platelets stimulated with collagen plus thrombin were increased 10 and 20 fold, respectively, as compared with unstimulated platelets. Stimulation of platelets with either collagen or thrombin alone resulted in a complete aggregation and serotonin release but induced less platelet procoagulant activity in prothrombin- and factor X activation than stimulation with collagen plus thrombin. The platelet procoagulant activity induced by collagen plus thrombin could be sedimented with the platelets upon centrifugation. Treatment of the stimulated platelets with phospholipase A₂ almost completely abolished the platelet activity in prothrombin- and factor X activation. This indicates that phospholipids in the outer leaflet of the platelet membrane are essential for the interaction of platelets with the clotting factors of the prothrombin- and factor X activating complexes.

In the assay systems used for prothrombin- and intrinsic factor X activation, the rates of thrombin- and factor Xa formation were dependent on the presence of phosphatidylserine in artificial phospholipid membranes. When low concentrations of enzymes and protein cofactors were employed for prothrombin- and factor X activation, the rates of thrombin- and factor Xa formation increased upon increasing the amount of phosphatidylserine in phospholipid vesicles. In contrast, the prothrombin- and factor X activation determined at saturating concentrations of enzymes and protein cofactors, were optimal with vesicles containing relatively low amounts of phosphatidylserine. This made the assay systems for prothrombin- and factor X activation suitable probes for the detection of various mole percentages phosphatidylserine in phospholipid bilayers. When the generation of a procoagulant surface on platelets stimulated with collagen plus thrombin was probed with the assay systems for prothrombin- and factor X activation using sub-saturating concentrations of enzymes and protein cofactors, half maximal platelet procoagulant activity was found within 2½ minutes after the onset of platelet stimulation. With saturating concentrations factor Xa and factor Va for the activation of prothrombin, higher rates of thrombin formation were observed in a later stage of platelet activation; half-maximal procoagulant activity was found 9 minutes after the addition of collagen plus thrombin to the platelets. We propose that the phosphatidylserine exposed at the surface of platelets triggered with collagen plus thrombin is not randomly distributed in the outer monolayer of the plate-

let membrane. Domains with a high phosphatidylserine density are generated shortly after platelet stimulation and are detected with the systems for prothrombin- and factor X activation using saturating and sub-saturating concentrations of clotting factors. Domains with a low phosphatidylserine density, which are formed later after triggering are probed exclusively with systems containing saturating amounts of clotting factors.

In chapter IV it was shown that with platelets, which were stimulated by the calcium ionophore A23187, 50-60 fold higher rates of prothrombin- and factor X activation were found than with non-stimulated platelets. The concentration of ionophore required for the induction of platelet procoagulant activity was similar to the concentrations required for platelet aggregation and release of ATP and factor V. The procoagulant activity, which was not due to platelet lysis, could be sedimented with the platelets by centrifugation and was completely abolished by treatment of the stimulated platelets with phospholipase A₂. Incubation of A23187 stimulated platelets with phospholipase A₂ has been shown to degrade at least half of the amount of phosphatidylserine present in platelets (3,5). We suggest that phosphatidylserine at the surface of platelets stimulated by A23187 is essential for the platelet procoagulant activity in prothrombin- and factor X activation.

In chapter V and VI studies are presented which describe the role of phospholipid bilayers and protein cofactors in the enzymatic complexes for prothrombin- and factor X activation. In previous studies (6,7) it was demonstrated that in prothrombin- and factor X activation phospholipids decrease the K_m for the respective substrates, whereas the V_{max} of the reactions is increased by the presence of their protein cofactors. As shown in chapters V and VI, the protein cofactors increase the V_{max} of prothrombin- and factor X activation not only by an increase of the k_{cat} of the reactions but also by promoting the incorporation of enzymes in the enzyme-protein cofactor-phospholipid complexes. In chapter V, the binding parameters of factor IXa for artificial bilayers consisting of phospholipids found in platelets were inferred from kinetic measurements of factor X activation in the absence and presence of factor VIIIa. Rates of factor X activation were measured at varying concentrations of factor IXa in reaction mixtures containing Ca²⁺, factor X and phospholipid vesicles. Double reciprocal plots of rates vs. factor IXa concentrations were straight lines. Maximal rates of factor X activation inferred from these plots were proportional to the phospholipid concentration, indicating that phospholipids provide kinetically functional binding sites for factor IXa. The factor IXa concentration required for half-maximal rates of

factor Xa formation was considered to be the factor IXa concentration required for the occupation of half the total amount of functional binding sites. The apparent dissociation constant so obtained varied between 0.4-1.1 μM which was not far from the reported dissociation constant obtained by direct binding studies for the binding of factor IXa to phospholipid. (8). Also when factor VIIIa was present in the factor X activating reaction mixture the rates of factor Xa formation could be saturated with factor IXa. Double reciprocal plotting of rates and factor IXa concentrations revealed straight lines, indicating that single functional binding sites for factor IXa were involved. Maximal rates of factor Xa formation were proportional to the factor VIIIa concentrations and independent of the phospholipid concentration present. These findings suggest that all factor VIIIa was bound to phospholipid, phospholipid-bound factor VIIIa being the kinetically functional binding site for factor IXa. The apparent dissociation constant determined for factor IXa binding to factor VIIIa-phospholipid complexes is 50-100 fold lower than found for the apparent dissociation constant of factor IXa in the absence of factor VIIIa. We conclude that factor VIIIa bound at (platelet) phospholipid surfaces functions as a high affinity receptor for factor IXa. Factor VIIIa increases the V_{max} of factor X activation by enhancing the k_{cat} of the reaction and by increasing the amount of phospholipid-bound factor IXa that participates in factor X activation.

Studies on the effect of factor Va and phospholipids on the formation of the enzyme-substrate complex for prothrombin activation are presented in chapter VI. Kinetic parameters of prothrombin activation were determined as a function of phospholipid composition and concentration. In the absence of factor Va and at limited amounts of phospholipid vesicles the V_{max} correlates with the binding affinity of factor Xa for the different membranes. Membranes with a high affinity for factor Xa have high V_{max} values, whereas for membranes with a low affinity a low V_{max} is found. The k_{cat} , which is defined as the V_{max} at saturating phospholipid concentration, that is when all factor Xa is bound and enzymatically active, was 2-4 min^{-1} independent of the phospholipid composition. In the presence of factor Va the k_{cat} was independent of the type and amount of acidic phospholipid in the vesicles; the value observed was 4500 min^{-1} . To achieve a V_{max} value of $\frac{1}{2} k_{\text{cat}}$, considerably less phospholipid was required in the presence of factor Va than in its absence. Apparently, in the presence of factor Va more factor Xa participates in prothrombin activation than in its absence, which is attributed to the property of factor Va to promote the binding of factor Xa to phospholipid bilayers, a phenomenon also

reported by other authors (9,10).

In the absence and presence of factor Va, the Km for prothrombin increases proportional with the phospholipid concentration. In the absence of factor Va the Km correlates with the affinity of prothrombin for the various phospholipid vesicles. Phospholipid membranes with a high affinity for prothrombin yield low Km values compared to membranes with low affinity for prothrombin, a correlation that does not exist when factor Va is present. Vesicles containing low amounts of acidic phospholipids have considerably lower Km values for prothrombin in the presence of factor Va than in its absence. The effects of factor Va on the Km for prothrombin can be explained by a model in which soluble prothrombin is the substrate for the enzymatic unit of prothrombinase that consists of a three component complex (factor Xa - factor Va - phospholipid) and in which factor Va promotes the enzyme-substrate complex formation.

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De prothrombine- en intrinsieke factor X- activeringsreacties worden sterk versneld door de aanwezigheid van membranen met negatief geladen fosfolipiden en door niet-enzymatische eiwit cofactoren (factor Va voor prothrombine- en factor VIIIa voor factor X activering). De studies die in dit proefschrift zijn weergegeven, hadden tot doel de mogelijkheid te onderzoeken dat tijdens de hemostase, bloedplaatjes de membraan-oppervlakken verschaffen die nodig zijn voor prothrombine- en factor X activering.

In hoofdstuk II toonden we aan dat plaatjes de prothrombine activering het best bevorderen, als zij gestimuleerd worden door de gecombineerde werking van collageen en thrombine. Niet- gestimuleerde plaatjes of plaatjes die waren gestimuleerd met alleen collageen of thrombine waren hierin veel minder actief. De fosfolipide samenstelling van het plaatjes buiten-oppervlak werd geanalyseerd door geactiveerde- en controle plaatjes te behandelen met fosfolipase A_2 . Fosfolipiden van niet-gestimuleerde plaatjes en van plaatjes gestimuleerd door alleen collageen of thrombine waren nauwelijks gevoelig voor fosfolipase A_2 . Als daarentegen, plaatjes werden geactiveerd door collageen plus thrombine, kon 20% van de totale hoeveelheid plaatjesfosfolipiden worden afgebroken door fosfolipase A_2 , zonder significante cel lyse. De hydrolyse van glycerofosfolipiden omvat 25% van het fosfatidylserine-, 25% van het fosfatidylcholine- en 30% van het fosfatidylethanolamine gehalte van de met collageen plus thrombine gestimuleerde plaatjes. Andere auteurs (1-4) vonden, met behulp van verschillende methoden ter bepaling van de fosfolipide verdeling over de binnen- en buiten-monolaag van het plasma-membraan, dat fosfatidylserine bijna uitsluitend aanwezig is in het inwendige van het niet-gestimuleerde en thrombine- gestimuleerde plaatje. Structurele veranderingen in het plasma-membraan van plaatjes na stimulering met collageen plus thrombine zouden zowel de verhoogde gevoeligheid van fosfolipiden voor fosfolipase A_2 als het beschikbaar komen van fosfatidylserine, nodig voor een efficiënte prothrombine activering, kunnen veroorzaken.

Verwacht werd, dat door het beschikbaar komen van fosfatidylserine in het oppervlak van met collageen plus thrombine gestimuleerde plaatjes, een procoagulant oppervlak zou ontstaan dat niet alleen geschikt is voor prothrombine activering maar ook voor intrinsieke factor X activering. In hoofdstuk

III bestudeerden wij de rol van gestimuleerde plaatjes in de prothrombine- en factor X activering met behulp van gezuiverde stoffactoren en chromogene substraten voor het meten van de snelheden van thrombine- en factor Xa vorming. Met hoeveelheden enzymen, substraten en eiwit cofactoren die verzadigend waren met betrekking tot de snelheid van substraat activering, waren de snelheden van prothrombine- en factor X activering in aanwezigheid van collageen plus thrombine gestimuleerde plaatjes 10- resp. 20 maal hoger dan werd gevonden met niet-gestimuleerde plaatjes. Stimulering van plaatjes met alleen collageen of thrombine resulteerde in een volledige aggregatie en release van serotonine maar induceerde minder procoagulante activiteit in prothrombine- en factor X activering dan stimulering met collageen plus thrombine. De procoagulante activiteit van plaatjes geïnduceerd door collageen plus thrombine, kon door centrifugering, samen met de plaatjes, worden gesedimenteerd. De activiteit van plaatjes in de prothrombine- en factor X activering verdween na behandeling van de gestimuleerde plaatjes met fosfolipase A₂. Dit geeft aan dat fosfolipiden in de buiten-monolaag van het plasma-membraan essentieel zijn voor de interactie van plaatjes met de stoffactoren van de prothrombine- en factor X activerende complexen.

In de meetsystemen, die werden gebruikt voor prothrombine- en factor X activering, waren de snelheden van vorming van thrombine en factor Xa afhankelijk van de aanwezigheid van fosfatidylserine in artificieel samengestelde fosfolipide membranen. Wanneer lage concentraties enzymen en eiwit cofactoren werden gebruikt voor de activering van prothrombine en factor X, namen de snelheden van thrombine- en factor Xa vorming toe als de hoeveelheid fosfatidylserine in fosfolipide vesicles werd verhoogd. Als daarentegen verzadigende concentraties enzymen en cofactoren werden gebruikt, verliep de activering van prothrombine en factor X optimaal met vesicles die relatief lage hoeveelheden fosfatidylserine bevatten.

Dit maakte de meetsystemen voor prothrombine- en factor X activering geschikt voor het detecteren van verschillende mol-percentages fosfatidylserine in fosfolipide bilagen. Wanneer het genereren van een procoagulant oppervlak op collageen plus thrombine gestimuleerde plaatjes werd gevolgd met meetsystemen voor prothrombine- en factor X activering met niet-verzadigende concentraties enzymen en eiwit cofactoren, werd half-maximale procoagulante activiteit gevonden binnen 2½ minuut na stimulering van het plaatje. Met verzadigende concentraties factor Xa en factor Va voor de activering van prothrombine werden hogere snelheden van thrombinevorming waargenomen, echter in een later stadium van plaatjes activering; half-maximale procoagulante activiteit werd

gevonden, 9 minuten na de toevoeging van collageen plus thrombine aan de plaatjes. Een verklaring die hiervoor kan worden gegeven is, dat het fosfatidylserine, dat na stimulering beschikbaar komt op het oppervlak van plaatjes, ongelijkmatig is verdeeld in de buiten-monolaag van het plaatjesmembraan. Gebieden met een hoge fosfatidylserine dichtheid worden gevormd kort na het stimuleren van de plaatjes en worden gedetecteerd met systemen voor prothrombine- en factor X activering met verzadigende- en niet-verzadigende concentraties stoffactoren. Gebieden met een lage fosfatidylserine dichtheid, die later na de stimulering worden gevormd, kunnen uitsluitend worden aangetoond met systemen die verzadigende hoeveelheden stoffactoren bevatten. In hoofdstuk IV werd aangetoond dat met plaatjes, die waren gestimuleerd door calcium-ionofoor A23187, snelheden werden gevonden voor prothrombine- en factor X activering die 50-60 maal hoger waren dan met niet-gestimuleerde plaatjes. De ionofoor concentratie die nodig was voor het induceren van plaatjes procoagulante activiteit kwam overeen met de concentratie die nodig was voor plaatjes aggregatie en release van ATP en factor V. De procoagulante activiteit, die niet kon worden toegeschreven aan cel lyse, kon samen met de plaatjes worden gesedimenteerd door centrifugatie en verdween na behandeling van de gestimuleerde plaatjes met fosfolipase A_2 . Eerder was aangetoond dat het incuberen van A23187-gestimuleerde plaatjes met fosfolipase A_2 resulteerde in de afbraak van tenminste de helft van de hoeveelheid fosfatidylserine, die in plaatjes aanwezig is (3,5). Wij stellen voor dat fosfatidylserine op het oppervlak van met A23187 gestimuleerde plaatjes, essentieel is voor de plaatjes procoagulante activiteit in de activering van prothrombine en factor X.

In hoofdstuk V en VI zijn studies gepresenteerd naar de rol van fosfolipide bilagen en eiwit cofactoren in de enzymatische complexen voor prothrombine- en factor X activering. In eerdere experimenten (6,7) werd aangetoond dat, in de prothrombine- en factor X activering, de K_m voor de respectievelijke substraten wordt verlaagd door fosfolipiden, terwijl de V_{max} van de reacties wordt verhoogd door de aanwezigheid van hun eiwit cofactoren. In de hoofdstukken V en VI wordt aangetoond dat de eiwit cofactoren de V_{max} van prothrombine- en factor X activering niet alleen verhogen door een verhoging van de k_{cat} van de reacties, maar tevens door het bevorderen van de inbouw van enzymen in enzym - eiwit cofactor - fosfolipide complexen. In hoofdstuk V werden de bindingsparameters van factor IXa voor bilagen bestaande uit fosfolipiden die ook in het plaatje aanwezig zijn, afgeleid van kinetische metingen van de activering van factor X in aan- en afwezigheid van factor VIIIa. De snelheden van factor Xa vorming werden gemeten bij verschillende concentraties factor

IXa, in reactie mengsels die Ca^{2+} , factor X en fosfolipiden bevatten. Rechte lijnen werden gevonden wanneer de reciproke snelheden werden uitgezet tegen de reciproke factor IXa concentraties. De maximale snelheden van factor X activering die met behulp van deze plots werden bepaald, waren evenredig met de fosfolipide concentratie, hetgeen erop wijst dat fosfolipiden de kinetisch functionele bindingsplaatsen leveren voor factor IXa. De concentratie factor IXa die nodig is om half-maximale snelheden van factor Xa vorming te verkrijgen, werd beschouwd als de factor IXa concentratie die nodig was voor de bezetting van de helft van alle functionele bindingsplaatsen. De zo verkregen, kinetisch afgeleide, dissociatie constante varieerde tussen 0.4-1.1 μM en was vergelijkbaar met de in de literatuur gerapporteerde dissociatie constante, die werd gevonden met directe bindingsstudies (8).

Ook als factor VIIa aanwezig was in het reactie mengsel voor factor X activering, was het mogelijk de snelheden van factor Xa vorming te verzadigen met factor IXa. Het dubbel reciprook uitzetten van snelheden en factor IXa concentraties leverde rechte lijnen op, hetgeen wijst op de betrokkenheid van één soort functionele bindingsplaats voor factor IXa. De maximale snelheden van factor Xa vorming waren evenredig met de factor VIIa concentraties en onafhankelijk van de fosfolipide concentratie.

Deze waarnemingen suggereren dat alle factor VIIa gebonden was aan fosfolipiden en dat de fosfolipide-gebonden factor VIIa de kinetisch functionele bindingsplaats is voor factor IXa. De kinetisch bepaalde dissociatie constante, die werd gevonden voor de binding van factor IXa aan factor VIIa-fosfolipide complexen, is 50-100 maal lager dan die werd gevonden voor factor IXa in afwezigheid van factor VIIa. Wij concluderen dat factor VIIa, dat gebonden is aan (plaatjes)fosfolipide oppervlakken, functioneert als een receptor met hoge affiniteit voor factor IXa. Factor VIIa verhoogt de V_{max} van factor X activering door het verhogen van de k_{cat} van de reactie en door het vergroten van de hoeveelheid factor IXa dat aan fosfolipiden gebonden is en dat deelneemt aan de activering van factor X.

Studies naar het effect van factor Va en fosfolipiden op de vorming van het enzym-substraat complex voor de activering van prothrombine zijn gepresenteerd in hoofdstuk VI. De kinetische parameters van prothrombine activering werden bepaald als functie van de fosfolipide concentratie en fosfolipide samenstelling. In afwezigheid van factor Va en bij beperkte hoeveelheid fosfolipide vesicles, correleert de V_{max} met de bindings affiniteit van factor Xa voor de verschillende membranen. Membranen met een hoge affiniteit voor factor Xa geven hoge V_{max} waarden, terwijl voor membranen met een lage affiniteit een

lage V_{max} wordt gevonden. De k_{cat} , die is gedefinieerd als de V_{max} bij verzadigende fosfolipide concentratie, waarbij alle factor Xa gebonden is en enzymatisch actief, was $2-4 \text{ min}^{-1}$ onafhankelijk van de fosfolipide samenstelling. In aanwezigheid van factor Va was de k_{cat} onafhankelijk van het soort en de hoeveelheid zure fosfolipiden in de vesicles; de gevonden waarde was 4500 min^{-1} . Om een V_{max} waarde te vinden gelijk aan $\frac{1}{2} k_{cat}$, was aanzienlijk minder fosfolipide nodig in aanwezigheid van factor Va dan in afwezigheid hiervan, hetgeen wordt toegeschreven aan de eigenschap van factor Va om de binding van factor Xa aan fosfolipide bilagen te bevorderen; een eigenschap die ook is gerapporteerd door andere auteurs (9,10).

Zowel in aanwezigheid als in afwezigheid van factor Va, stijgt de K_m voor prothrombine proportioneel met de fosfolipide concentratie. In afwezigheid van factor Va correleert de K_m met de affiniteit van prothrombine voor de verschillende fosfolipide vesicles. Fosfolipide membranen met een hoge affiniteit voor prothrombine geven lage K_m waarden vergeleken met membranen met een lage affiniteit voor prothrombine, een correlatie die niet bestaat als factor Va aanwezig is. Vesicles met lage percentages zure fosfolipiden hebben aanzienlijk lagere K_m waarden voor prothrombine in aanwezigheid van factor Va dan in afwezigheid hiervan.

De effecten van factor Va op de K_m voor prothrombine kunnen worden verklaard met een model waarin prothrombine in de oplossing het substraat is voor de enzymatische unit van prothrombinase, die bestaat uit drie componenten (factor Xa - factor Va - fosfolipide) en waarin factor Va de enzym-substraat complexvorming bevordert.

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren te Haarlem op 9 december 1952. Hij behaalde het diploma HBS-B in 1970 aan het Triniteits Lyceum te Haarlem. Vanaf 1970 studeerde hij scheikunde aan de Universiteit van Amsterdam. In april 1974 werd het kandidaatsexamen afgelegd, gevolgd door het doctoraal examen in november 1978. Zijn hoofdvak, bijvak en speciale richting waren respectievelijk biochemie, klinische chemie en kristallografie.

Van mei 1979 tot oktober 1984 is hij als wetenschappelijk assistent in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O./FUNGO) en werkzaam bij de capaciteitsgroep Biochemie van de Rijksuniversiteit Limburg te Maastricht. Gedurende deze periode werd onder leiding van Prof.Dr. R.F.A. Zwaal en Dr. J. Rosing het onderzoek verricht dat heeft geleid tot deze dissertatie.