

Review Article

Platelet Activation and Blood Coagulation

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Summary

Platelet activation and blood coagulation are complementary, mutually dependent processes in haemostasis and thrombosis. Platelets interact with several coagulation factors, while the coagulation product thrombin is a potent platelet-activating agonist. Activated platelets come in a procoagulant state after a prolonged elevation in cytosolic $[Ca^{2+}]_i$. Such platelets, e. g. when adhering to collagen via glycoprotein VI, expose phosphatidylserine (PS) at their outer surface and produce (PS-exposing) membrane blebs and microvesicles. Inhibition of aminophospholipid translocase and activation of phospholipid scramblase mediate the exposure of PS, whereas calpain-mediated protein cleavage leads to membrane blebbing and vesiculation. Surface-exposed PS strongly propagates the coagulation process by facilitating the assembly and activation of tenase and prothrombinase complexes. Factor IXa and platelet-bound factor Va support these activities. In addition, platelets can support the initiation phase of coagulation by providing binding sites for prothrombin and factor XI. They thereby take over the initiating role of tissue factor and factor VIIa in coagulation activation.

Introduction

There is no doubt that platelet activation and blood coagulation are mutually dependent, interactive processes. Fibrin, formed upon coagulation, stabilises the platelet plug during the haemostatic response. Also, in thrombosis, aggregated platelets and fibrin form the main constituents of intra-arterial thrombi. The physical interactions between platelets and coagulation factors are being revealed in these days. Many if not all coagulation factors appear to bind to platelets either via their glycoprotein receptors or via phospholipids that become exposed following platelet activation. In this paper, we review these interactions as well as the activation processes in platelets that lead to changes in these interactions. We will first describe the activation conditions and signalling pathways that result in the surface exposure of procoagulant phospholipids, and then evaluate the enzymatic mechanisms of control of transmembrane phospholipid asymmetry. Finally, we focus on the interactions between platelets and coagulation factors that are involved in the initiation and the propagation of blood coagulation. The role of platelets in the termination of blood coagulation, e. g. inactivation of factors Va and VIIIa by activated protein C, is not discussed.

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Bleb Formation and Exposure of Procoagulant Phospholipids

Originally, the contribution of platelets to the coagulation process was ascribed to platelet factor 3. In search for the identity of platelet factor 3, it became clear that platelets which most actively propagate the coagulation reactions are characterised by the presence of phosphatidylserine (PS) at their membrane surface. These platelets have also formed membrane blebs, which are shed into the circulation as procoagulant platelet-derived microvesicles.

Platelet Receptors Involved in Bleb Formation and Phosphatidylserine Exposure

Several investigators have demonstrated that stimulation of platelets in suspension with collagen and thrombin causes surface-exposure of procoagulant PS. Calcium ionophores (A23187 or ionomycin) are even more active in evoking this reaction, suggesting that increased intracellular Ca^{2+}, $[Ca^{2+}]_i$ a key element in this process (1, 2). Typically, during the process of PS exposure (described in detail below), platelets dramatically change in shape. They loose most of their cytoskeletal structure, round off to balloon-like structures, and form membrane blebs [reviewed in (3, 4)]. Nowadays, it is clear that all these responses are evoked in platelets that interact with fibrillar collagen, even in the absence of thrombin (5, 6), or in platelets that bind to fibrin in coagulating plasma (7, 8). The procoagulant transformation of platelets thus occurs precisely at sites where coagulation is desired (collagen in vessel wall) or is present (fibrin clot).

Adhesion and activation of platelets through the collagen receptor glycoprotein VI is a potent trigger for inducing bleb formation and PS exposure (9, 10), as apparent from studies with the glycoprotein VI ligands, Gly-Pro-Hyp collagen-related peptide (11) and convulxin (12). Binding via glycoprotein VI primes for several platelet-activation events, but also for additional platelet-collagen interactions, e. g. via adhesive receptors like integrin $\alpha_2\beta$ (13). Glycoprotein VI activation is a potent trigger of bleb formation and PS exposure. Platelet adhesion *per se* potentiates the procoagulant effect mediated by glycoprotein VI

(10), possibly by triggering additional activation steps and/or ensuring optimal glycoprotein VI-ligand contact.

Bleb formation and surface exposure of PS are also effectively triggered in platelets in coagulating plasma (7). Again, adhesive receptors seem to be important, because antibodies against integrin $\alpha_{IIb}\beta_3$ (receptor of fibrinogen and fibrin) and glycoprotein Ib-V-IX (vWF receptor) reduce platelet-dependent thrombin formation in plasma to a similar degree as platelet-activation inhibitors like prostacyclin (7, 14). Integrin $\alpha_{IIb}\beta_3$ antagonists reduce PS exposure and factor V binding to platelets, and they augment the anticoagulant effect of heparins (15). Platelet-dependent thrombin formation is also suppressed by antibodies against vWF (7). For reasons that are unclear, antibody-based antagonists of integrin $\alpha_{IIb}\beta_3$ are much better inhibitors of thrombin generation than peptide antagonists (16). Accordingly, the successful clinical use of anti-$\alpha_{IIb}\beta_3$ drugs (17) may in part rely on their anticoagulant effect next to their anti-aggregatory effect. One should however realise that these compounds may also have anticoagulant effects that are not related to PS exposure. As indicated below, integrin $\alpha_{IIb}\beta_3$ and glycoprotein Ib-V-IX bind coagulation factors (prothrombin and factor XI, respectively), implying that antibodies against these glycoproteins can also act by displacement of the bound coagulation factors.

Various authors have described the platelet agonists and receptors involved in "coagulation-mediated" PS exposure. Thrombin, although being a potent agonist for aggregation and release reactions, causes only little PS exposure when added to washed platelets in suspension or to platelets adhering to fibrinogen (18, 19). The rather weak procoagulant effect of thrombin resembles that of agonists of the protease-activated receptor 1 (PAR1), whereas agonists of the second thrombin receptor PAR4 are ineffective (20). This suggests that thrombin cleavage of PAR1 rather than of PAR4 triggers the procoagulant response. In addition, thrombin binds and activates glycoprotein Ib, which enhances the active state of platelets particularly for interaction with fibrin (21). It has been proposed that glycoprotein Ib-bound thrombin is responsible for PS exposure in (gel-filtered) platelets that are stimulated with thrombin in a way requiring both PAR1 and integrin $\alpha_{IIb}\beta_3$ activation (22). Because thrombin forms fibrin and platelet-fibrin interactions significantly contribute to the platelet-dependent coagulation in plasma (7, 23), it is well conceivable that platelet binding via integrins to (trace amounts of) fibrin stimulates the procoagulant activity of thrombin-stimulated platelets.

Since the interaction of platelet glycoprotein Ib-V-IX with vWF highly depends on the local wall shear rate (24), this shear dependency may also exist for the thrombin/vWF-dependent procoagulant response. High shear stress indeed induces a vWF- and Ca^{2+}/calpain-dependent shedding of microvesicles from platelets (25). The shear-dependent microvesiculation was found to be increased by PAR1 activation (26), and inhibited by integrin $\alpha_{IIb}\beta_3$ antagonists (27). Others have described that high shear and vWF potentiate the PS exposure of fibrin-adherent, thrombin-stimulated platelets (28).

Signaling Pathways towards Bleb Formation and Phosphatidylserine Exposure

Most if not all platelet-activating agents causing bleb formation (microvesiculation) and PS exposure evoke a potent rise in $[Ca^{2+}]_i$ (see Fig. 1). From single-cell imaging studies, it has become evident that platelet binding to fibrillar collagen or other glycoprotein VI ligands (collagen-related peptide or convulxin) induces a prolonged, non-spiking increase in $[Ca^{2+}]_i$, which precedes both membrane blebbing and PS exposure (6, 10). Activated glycoprotein VI triggers a complex cascade of tyrosine and serine protein kinases. As a result, phospholipase C-γ2 becomes active, which generates inositol 1,4,5-trisphosphate required for Ca^{2+} mobilisation from intracellular Ca^{2+} stores (29). Calcium mobilisation induces the process of store-regulated Ca^{2+} entry from the extracellular medium, which in platelets leads to substantial amplification of the Ca^{2+} signal (30, 31).

It has been described that glycoprotein VI-mediated bleb formation and PS exposure are both diminished by interventions reducing the Ca^{2+} signal, i. e. by antagonists of protein tyrosine kinases, elevation in cyclic AMP and chelation of intracellular Ca^{2+} (6). When induced by Ca^{2+} ionophores, these events are again blocked by Ca^{2+} chelation, but not by protein kinase inhibition (6, 32). This suggests that the action of Ca^{2+} in the response is not mediated by protein kinases. With glycoprotein VI agonists, influx of extracellular Ca^{2+} appears to be essential to raise $[Ca^{2+}]_i$ to sufficiently high levels to trigger PS exposure and microvesiculation (6, 9). Others have reported that Ca^{2+} influx, and thus PS exposure, is dependent on the presence of 3-phosphorylated phosphoinositides and activation of Bruton's tyrosine kinase (33).

Platelet agonists such as ADP, thromboxane and PAR1 peptide SLFFRN, which activate G_q-coupled receptors, all stimulate phospholipase C-β isoforms (34). This results in a relatively short production of inositol 1,4,5-trisphosphate and a transient, spiking Ca^{2+} signal (35, 36). Thrombin, which acts through phospholipase C-β and -γ isoforms (37), induces a sustained, non-spiking Ca^{2+} signal only at high dose (38). The short Ca^{2+} response may explain why the phospholipase C-β-stimulating agents promote no more than little PS exposure. Inhibitors of sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) such as thapsigargin cause a moderate $[Ca^{2+}]_i$ elevation, consisting of both Ca^{2+} mobilisation and influx. The SERCA inhibitors strongly potentiate and prolong the Ca^{2+} response evoked by thrombin, and significantly increase the weak effect of thrombin on PS exposure (39, 40). Also with

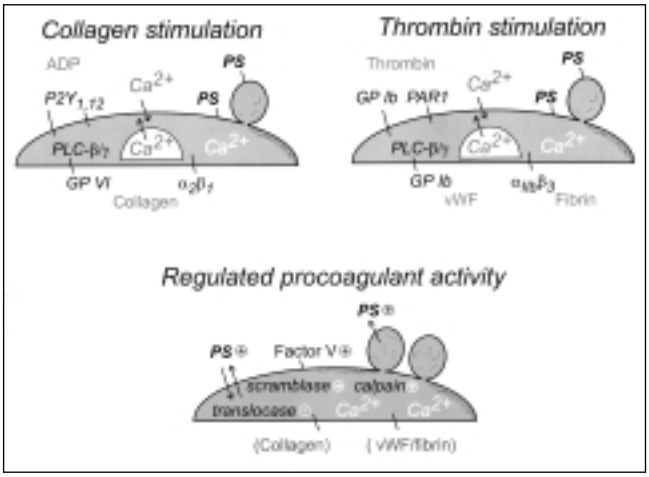

Fig. 1 Function of cytosolic Ca^{2+} in collagen- and thrombin-stimulated bleb formation, PS exposure and procoagulant activity. Phospholipase C (PLC) activation, mediated by glycoprotein VI or PAR1, causes Ca^{2+} mobilisation from intracellular stores and subsequent Ca^{2+} influx from the extracellular medium. Increased $[Ca^{2+}]_i$ is a prerequisite for protrusion of membrane blebs and exposure of procoagulant PS. Platelet adhesion via integrin $\alpha_2\beta_1$ (collagen), glycoprotein Ib (vWF) and integrin $\alpha_{IIb}\beta_3$ (fibrin) potentiates these responses, as indicated. Released ADP, acting via the $P2Y_1$ and $P2Y_{12}$ receptors, potentiates the Ca^{2+} signal and the adhesion, respectively. This is indicated for collagen stimulation, but is also true for thrombin. Elevated $[Ca^{2+}]_i$ stimulates phospholipid scramblase (PS exposure), calpain (blebbing) and factor V secretion (prothrombinase); it inhibits aminophospholipid translocase avoiding PS to be pumped back

these agents, influx of external Ca^{2+} and consequent high levels of $[Ca^{2+}]_i$ are needed for bleb formation and PS exposure (6, 41). Taken together, the findings indicate that, regardless of the agonist, a high elevation in $[Ca^{2+}]_i$ (μM range) persisting during a certain time (minute range) is required for evoking membrane blebbing and PS exposure. The store-regulated Ca^{2+} influx, probably via Trp channels, typically increases both the degree and the time of elevation (6, 38).

Effects of Elevated Free Cytosolic Ca^{2+}

The processes of bleb formation and PS exposure differ from other known Ca^{2+}-dependent platelet activation events, e.g. shape change and secretion, in being insensitive towards Ca^{2+}-calmodulin kinase inhibitors. Bleb formation and subsequent microvesiculation, but not PS exposure, are known to be mediated by Ca^{2+}-dependent autolysis and activation of the protease μ-calpain (39, 41-43). Caspase proteases are unlikely to be involved (44). As calpain degrades several cytoskeletal proteins, it is appreciated that this degradation results in dissociation of the membrane skeleton from the plasma membrane, which facilitates protrusion of membrane patches that are no longer interacting with the cytoskeleton. Entry of Ca^{2+} appears to be required for optimal calpain activation (39, 43). Inhibitor studies showed that the p38 mitogen-activated protein kinase pathway is involved in the Ca^{2+}/calpain-dependent bleb formation, perhaps by mediating phosphorylation of proteins modulating actin polymerisation (10). Others have proposed that, along with calpain activation, (Ca^{2+}-dependent) protein tyrosine dephosphorylation mediates bleb/microvesicle formation (45). Elevated $[Ca^{2+}]_i$ may thus have more targets than only calpain.

The Ca^{2+}-dependent step or process leading to PS exposure most probably regards activation of a phospholipid scramblase (see below). At present, there is no evidence for involvement of Ca^{2+}-dependent protein kinases. Platelets from humans (46) and mice (33) exhibiting increased $[Ca^{2+}]_i$ levels also show enhanced exposure of PS and increased microparticle release. Conversely, in platelets from patients with storage pool deficiency, where the thrombin-induced Ca^{2+} signal is shortened due to lack of ADP release, also the platelet- (PS-)dependent prothrombinase activity is reduced (47). This is consistent with the observation that blocking of the ADP receptors leads to a reduced platelet procoagulant activity and formation of microvesicles (48, 49). Thus, autocrine activating effects of released ADP through the purinergic $P2Y_1$ and $P2Y_{12}$ receptors can potentiate the procoagulant effects of other agonists.

In summary, both bleb formation and PS exposure require a prolonged rise in $[Ca^{2+}]_i$, which under physiological conditions is most likely triggered by phospholipase C activation and subsequent Ca^{2+} influx (Fig. 1). Agonists mediating such high Ca^{2+} responses are collagen (via glycoprotein VI) and high doses of thrombin/vWF (via PAR1 and glycoprotein Ib), with ADP being stimulatory (via purinergic receptors). Adhesion-mediated events though potentiate the procoagulant responses both in case of activation with collagen (integrin $\alpha_2\beta_1$) and thrombin/vWF (integrin $\alpha_{IIb}\beta_3$).

Control of Exposure of Procoagulant Phospholipids

Procoagulant phospholipid membranes, such as offered by activated platelets and platelet-derived microvesicles, amplify the process of blood coagulation by several orders of magnitude. Although surface-exposed PS is the major procoagulant phospholipid, other plasma membrane phospholipids modulate its coagulant effect. Therefore, a tight regulation of the transmembrane distribution of the phospholipids is essential to control the haemostatic process.

Specific Roles of Membrane Phospholipids in the Coagulation Process

Phosphatidylserine-containing membranes strongly accelerate two important reactions of the coagulation process, the tenase and prothrombinase reactions. Electrostatic and hydrophobic interactions are involved in the binding of vitamin K-dependent coagulation factors (enzymatic factors IXa and Xa and non-enzymatic cofactors Va and VIIIa) to such membranes (50). The lipid-dependent interaction has various effects. It leads to increased local concentration of coagulation factors, allows conformational changes required for optimal function of the coagulation proteins, facilitates transfer of substrate and product between the coagulation complexes, and it restricts the activity of the coagulation process to areas of injury (51-53).

Optimal activity for the tenase and prothrombinase complexes is observed at phospholipid surfaces containing 10-15 mol% PS, with a higher PS content resulting in decreased catalytic efficiency (50). Both tenase and prothrombinase interact with the PS-containing membrane in a stereo-selective manner, preferring the naturally occurring PS isomer phosphatidyl *L*-serine. Other phospholipids modulate the procoagulant activity of PS-containing membranes. In particular phosphatidylethanolamine (PE) enhances the catalytic properties of membranes with a low PS content, mainly because it increases the membrane affinity of the hydrophobic factors Va and VIIIa (54, 55). In contrast, sphingomyelin (SM) profoundly reduces the catalytic ability of such membranes. The latter effect is explained by a more tight packing of acyl chains caused by the highly saturated SM, which lowers the hydrophobic penetration of coagulation factors and thus their optimal functioning. Fluidifying cholesterol moderately improves the procoagulant activity of SM-containing membranes (50).

Regulation of Membrane Phospholipid Asymmetry

Slightly more than half of the phospholipids in the plasma membrane of platelets and other blood cells consists of the choline-containing phospholipids PC and SM, while the remaining is mostly comprised of the aminophospholipids PS and PE. Phosphatidylinositol polyphosphates and phosphatidic acid comprise <5% of the membrane phospholipids. In platelets, the content of procoagulant PS amounts to approximately 10%. In resting platelets, as in other cells, the outer leaflet of the plasma membrane comprises the majority of the choline-containing phospholipids (PC and SM), whereas the inner leaflet facing the cytosol sequesters the aminophospholipids (PS and PE) (56). This asymmetric lipid organisation is maintained by various membrane enzymes acting cooperatively and synchronously.

First indications for involvement of a protein that shuttles phospholipids from one membrane leaflet to another date from 1984, when a rapid inward migration of spin-labelled analogues of PS and PE was measured ($t^1/_2$ ~10 min) in human erythrocytes (57). Later studies showed that the transporting protein only uses aminophospholipids – with a slight preference for PS over PE –, and thus could be termed aminophospholipid translocase (56). Its transport activity is blocked by sulfhydryl-reactive agents and increased $[Ca^{2+}]_i$, but also by *o*-vanadate and fluoride, consistent with the notion that one molecule of ATP is needed for the transport of every phospholipid molecule. It is emphasised that mere inhibition of the translocase activity does not result in loss of transmembrane phospholipid asymmetry. Recently, a novel

P-type ATPase has been cloned and proposed as a candidate protein of the aminophospholipid translocase (58). However, its involvement in lipid transport across the plasma membrane has been questioned by others [discussed in (50)].

In addition to the inward transport of aminophospholipids, the plasma membrane exhibits a slow, continuous movement of phospholipids from the inner to the outer leaflet ($t^1/_2$ ~1.5 h). This movement also requires ATP and concerns choline- and aminophospholipids (59, 60). The protein responsible for this slow activity has been identified in erythrocytes as multidrug resistance protein-1 (MRP1), a member of the family of ATP-binding cassette (ABC) proteins (61, 62). It can be inferred that the dynamic asymmetric transbilayer distribution of phospholipids in the plasma membrane of platelets, like in erythrocytes, results from the concerted action of aminophospholipid translocase and MRP1.

After collapse of the membrane phospholipid asymmetry, the procoagulant activity of platelets is increased. The most prominent feature of phospholipid scrambling is exposure of PS at the cell surface. Yet, the scrambling process is bi-directional and involves all plasma membrane phospholipids, including PE and SM. Bearing in mind that PE amplifies and SM attenuates the procoagulant activity of PS, the outward and inward movements of these respective phospholipids during scrambling will enforce the procoagulant activity of platelets. As mentioned above, phospholipid scrambling requires a persistent rise in $[Ca^{2+}]_i$ in platelets. Consequently, reducing $[Ca^{2+}]_i$ will arrest the scrambling process, and eventually switch on the aminophospholipid translocase to restore lipid asymmetry and lower the procoagulant activity. The low PS exposure induced by thrombin alone may thus be explained by the transient (spiking) elevation in $[Ca^{2+}]_i$ with this agonist, which limits the scrambling activity. In addition, thrombin stimulation evokes a 2-3 fold enhancement of translocase activity, which may help in pumping back the exposed PS to the inner membrane leaflet.

Although phospholipid scrambling is not directly coupled to ATP hydrolysis, prolonged ATP depletion results in gradual loss of scrambling activity, at least in red cells. Scrambling can be restored upon ATP repletion, suggesting that one or more phosphorylated components contribute to this process (56). Various non-enzymatic mechanisms have been proposed to explain the phospholipid scrambling, including calpain-mediated loss of membrane-cytoskeletal interactions, complex formation of Ca^{2+} and phosphatidylinositol 4,5-bisphosphate, and transient disturbances of the membrane bilayer (hexagonal phase) upon formation of microvesicles, but each of these proposals has been questioned or disproved (56).

Two research groups successfully reconstituted a protein fraction derived from platelet or erythrocyte membranes into proteoliposomes with a functional, Ca^{2+}-inducible scrambling activity (63, 64). Sims and co-workers succeeded in cloning a protein with this activity from human erythrocytes, which they termed human phospholipid scramblase, hPLSCR1. Later on, three homologues of this protein were found (65). The function of hPLSCR1 as a major plasma membrane phospholipid scramblase is however disputable for a number of reasons. Transcriptional up-regulation of the cloned protein by treating cells with interferon was not accompanied by increased scrambling activity, and levels of PLSCR1 did not correlate with PS exposure in cells undergoing apoptosis (65). Furthermore, the rate of scrambling activity of the reconstituted recombinant protein was more than two orders of magnitude slower than that observed in blood cells. Finally, lipid scrambling was not impaired in mice where the PLSCR1 gene was deleted, although the scrambling activity might have been rescued here by related proteins (hPLSCR2-4) (PJ Sims, personal communication).

Impaired Regulation of Phospholipid Asymmetry

Altered regulation of platelet phospholipid asymmetry is incidentally seen in patients. In the Scott syndrome, impairment of phospholipid scramblase is accompanied by moderately severe bleeding. In the two well-documented cases of the Scott syndrome, the bleeding phenotype appears to transmit as an autosomal recessive trait affecting various hematopoietic cell lineages (66, 67). The most straightforward laboratory diagnosis for the syndrome is complete absence of a developing procoagulant surface on platelets and erythrocytes upon treatment with Ca^{2+}-ionophores due to lack of PS exposure. However, the combined action of collagen and thrombin results – at least in platelets from the propositus – in PS exposure that is reduced by only 70% in comparison to platelets from healthy individuals. This is consistent with a decreased rate and extent of tenase and prothrombinase activity in the activated patient's platelets (68). In addition to a diminished procoagulant activity, microvesicle formation is also impaired in patients with Scott syndrome (2, 69). Both defects seem not to be related to an abnormal calcium response or impaired calpain activation. Some studies with Scott platelets indicate reduced tyrosine phosphorylation (69) and decreased pseudopod formation (70). Although this is suggestive for an altered signal transduction, the implications of these observations are still unclear (71). Interestingly, EBV-transformed lymphoblasts from the Scott patient appear to have normal levels of hPLSCR1 mRNA, with a deduced amino acid sequence identical to that of control cells. This finding is another argument against the function of hPLSCR1 as a scramblase, but it can also indicate that the defect in the Scott syndrome concerns a regulatory component. This would be consistent with the observation that Scott lymphoblasts are unable to expose PS when challenged with Ca^{2+}-ionophore, but exhibit normal PS exposure during apoptosis (72).

Opposite to the Scott syndrome, enhanced PS exposure and microvesicle formation has been detected in particular patients with thrombotic complications (73). Patients with Wiskott-Aldrich syndrome, attributed to mutations in the WASP gene, have platelets with enhanced exposure of PS and increased microparticle release (46). These altered responses could be related to increased levels of $[Ca^{2+}]_i$ (46).

Interactions of Platelet Membrane Proteins and Coagulation Factors

Evidence is accumulating that besides PS and other phospholipids also protein components of the platelet membrane play important roles in the coagulation process. Various coagulation factors interact with surface glycoproteins of non-stimulated and stimulated platelets. The binding contributes to the thrombogenic potential of platelets, especially during initiation of the coagulation.

Platelet-dependent Activation of Factor IX

Factor IX is activated via two pathways, namely by the complex of tissue factor and factor VIIa at the surface of tissue factor-bearing cells, and by platelet-bound factor XIa (Fig. 2). The existence of two factor IX-activating pathways has puzzled investigators for a long time, until the discovery of tissue-factor pathway inhibitor (TFPI). Then, it was realised that the factor XIa pathway is critical for producing sufficient amounts of factor IXa to stop severe bleeding, once the tissue factor-factor VII route is terminated by TFPI (74). Factor IX is considered to interact with platelets through one chain of dimeric factor XIa, with the other chain binding directly to the platelet surface (75).

Platelet-dependent Activation of Factor XI

How factor XI functions in coagulation, particularly under conditions where tissue-factor pathway inhibitor (TFPI) suppresses the extrinsic route, has only recently been discovered. Because factor XI deficiency leads to bleeding mainly upon severe trauma or surgery, it was postulated that, once TFPI is active, factor XI is needed to ensure sufficient factor IXa generation for sustained factor Xa production (76). Walsh and co-workers identified the key elements in factor XIa-dependent factor IXa generation. They found that thrombin feedbacks factor XI activation, and that this process is stimulated 5,000-10,000-fold by platelets activated with the PAR1 agonist SFLLRN (77, 78). Procoagulant phospholipids were unable to mimic the effect of platelets. The binding site for factor XI on activated platelets is identified as the glycoprotein Iba chain of the glycoprotein Ib-V-IX complex (79). Since thrombin also binds to this complex (see above), factor XI-glycoprotein Ib interaction can facilitate the cleavage of factor XI by thrombin. This could explain why Bernard-Soulier platelets (lacking glycoprotein Ib) have a reduced (factor XI-dependent) thrombin formation. But, because glycoprotein Ib is readily available on normal unstimulated platelets, it remains unclear why platelets need to be stimulated (e.g. with SFLLRN or thrombin) to exert their factor XI-dependent procoagulant activity.

Assembly of Intrinsic Factor X-Activating Complex on Platelets

The serine protease factor IXa cleaves factor X into its active form, factor Xa. Appreciable factor X activation requires a PS-containing membrane surface for its assembly with the factors VIIIa and IXa (the tenase complex) (80). Next to this, binding and competition experiments have led to the postulation of a protein receptor model for tenase assembly. Herein, it is assumed that thrombin-stimulated platelets expose non-lipid binding sites for the factors VIIIa, IXa and X (81, 82). An argument for the involvement of protein components besides PS was found in the observation that annexin V only partly inhibited the activation of factor X on activated platelets (83). However, studies with mutated recombinant proteins provide little reason to assume factor IXa binding sites other than anionic phospholipids. For instance: *i)* specific amino acid residues within the EGF2 domain of factor IXa equally mediate its binding to PS-containing vesicles and to activated platelets (84); *ii)* modified factor IXa proteins with reduced phospholipid-binding properties fail also to bind to activated platelets (85); *iii)* factor IXa proteins which show high kinetic activity in the presence of phospholipid vesicles retain this activity when bound to platelets (84, 86); and *iv)* point mutations in the Gla-containing region of factor IX inhibit its binding not only to PS-containing vesicles but also to activated platelets (87). At present, it is thus questionable whether protein-type receptors significantly contribute to factor X activation.

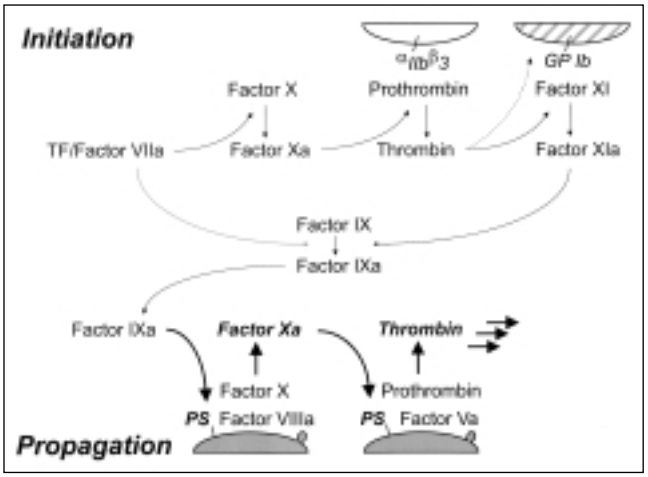

Fig. 2 Procoagulant effects of platelets in the initiation and propagation phases of blood coagulation. Prothrombin-integrin $\alpha_{IIb}\beta_3$ interaction and factor XI-glycoprotein Ib interaction ensure factor IX activation in the initiation phase, triggered by tissue factor (TF)-factor VIIa. During the propagation phase of coagu-lation, factor IXa stimulate the tenase reaction on PS-exposing, activated platelets, after which factor Xa stimulates prothrombinase. The generated thrombin feeds backs to stimulate platelets and to generate activated cofactors

Assembly of Prothrombin-Activating Complex on Platelets

The prothrombinase complex consists of the non-enzymatic cofactor Va, the serine protease factor Xa and the substrate prothrombin. Procoagulant phospholipids (PS) stimulate prothrombinase assembly and thrombin formation by several orders of magnitude (see above). Yet, non-lipid membrane components have been identified on (activated) platelets that support the binding of prothrombinase factors. Plow and co-workers have demonstrated that prothrombin binds to purified integrin $\alpha_{IIb}\beta_3$ independently of phospholipids (88). This interaction is specific, as it is inhibited by the $\alpha_{IIb}\beta_3$ antagonist abciximab and by RGD peptides. In case of intact platelets, prothrombin binding does not require activation of $\alpha_{IIb}\beta_3$, such in contrast to the RGD-dependent binding of fibrinogen to this integrin. It thus follows that platelet activation (i.e. $\alpha_{IIb}\beta_3$ activation) is needed for fibrinogen molecules to compete with prothrombin for integrin receptor sites. Integrin activation already occurs at low concentrations of thrombin, which suggests that integrin-prothrombin interactions are most relevant at the start of thrombin formation.

Interestingly, blockade of integrin $\alpha_{IIb}\beta_3$ markedly delays the prothrombin activation by factor Xa, but does not reduce the amount of thrombin generated (89). This also implies that integrin $\alpha_{IIb}\beta_3$-mediated thrombin generation only controls the initiation phase of the coagulation process, i.e. when platelets start to become activated. Once coagulation is started and platelets are fully activated, both enhanced prothrombin binding and increased thrombin generation have been observed (90). These findings are explained by assuming that anionic phospholipids on activated platelets provide the majority of the sites where the prothrombin-activating complexes are assembled.

The existence of non-lipid receptors for factor Xa on platelets has been debated. Miletich et al. reported in 1977 that neither factor IX, factor X nor prothrombin could compete with factor Xa for its binding to the surface of activated platelets (91). The investigators then provided evidence that part of the factor Xa receptor on platelets is (platelet) factor Va (92). Recently, Tracy and co-workers questioned whether PS exposure alone is sufficient for assembly of an efficient prothrombin-converting complex. These investigators proposed that the platelet receptor for factor Xa consists of anionic phospholipids, membrane-bound factor Va and effector cell protease receptor-1 (EPR1, a 65-kDa protein) (93). Support for involvement of EPR1 came from studies that compared the binding characteristics of factor Xa onto phospholipid vesicles and thrombin-activated platelets (94, 95). Others, however, were unable to demonstrate involvement of an EPR1-like protein in prothrombinase assembly at the surface of activated platelets (19).

Recent work has further developed the early idea that platelet factor V plays an important role in thrombin formation. Subpopulations of

platelets that are stimulated with collagen and thrombin (COAT) express high levels of surface-bound, α-granule-derived factor V. The expression of this so-called COAT-factor V parallels the surface exposure of PS. Only platelets expressing COAT factor V may be able to bind factor Xa (96). The COAT platelets are also characterised by their capacity of binding several serotonin-conjugated adhesive and procoagulant proteins (97). That platelet-derived factor V has a significant role in thrombin formation also appears from the recognition that in some patients bleeding disorders could be related to quantitative or qualitative abnormalities in this factor, such as in platelet factor V Quebec (98) and New York (99).

Roles of Platelets in Initial and Sustained Coagulation

It is well appreciated that the tissue factor-driven thrombin generation is divided into an initiation and a propagation phase. Either phase is usually assigned to different cell types: initiation on tissue factor-bearing cells (vessel wall and leukocytes) and propagation on PS-exposing cells (platelets or microvesicles). However, the newly discovered functions of glycoprotein Ib and integrin $\alpha_{IIb}\beta_3$ in supporting thrombin generation urge to refine this scheme of blood coagulation. This is summarised in Fig. 2.

During the initiation phase, the tissue factor-factor VIIa complex on the vessel wall or leukocytes generates small amounts of factor IXa and Xa (extrinsic pathway), which generates only low amounts of thrombin. It appears that trace amounts of factor Xa can activate prothrombin which is bound to integrin $\alpha_{IIb}\beta_3$ on unstimulated platelets, generating surface-localised thrombin. These thrombin molecules activate platelets (via PAR1, PAR4 and glycoprotein Ib-V-IX) and provide binding sites for factor XI on the glycoprotein Ib complex. Subsequent activation of bound factor XI by thrombin then triggers a pathway allowing generation of factor IXa, Xa and thrombin, independently of tissue factor.

After the initiation phase of coagulation, thrombin formation needs to be propagated to ensure normal haemostasis. This process is greatly supported by collagen-bound, glycoprotein VI-activated platelets and by fibrin-bound platelets in the developing clot. These platelets expose PS and factor V and thus provide a procoagulant surface, at which the tenase and prothrombinase complexes are efficiently assembled, resulting in a vast acceleration of thrombin generation.

Platelets thus play various – initial and sustained – roles in optimising coagulation. The many-fold interrelationships that are being unravelled between (activated) platelets and coagulation proteins make it understandable why established anti-platelet drugs do not only have anti-aggregatory effects, but also exhibit anti-coagulant activity. Because of this dual role of platelets, it is evident that dosage of these drugs should not be based on platelet activation measurements *per se*.

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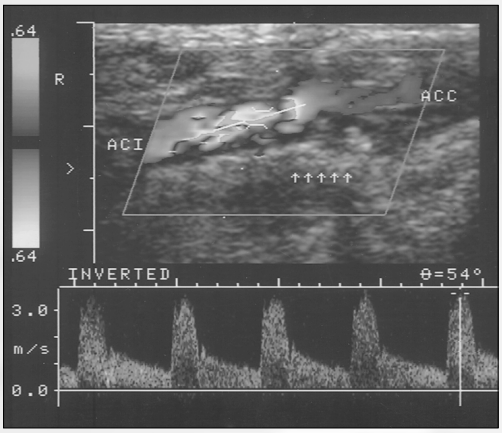

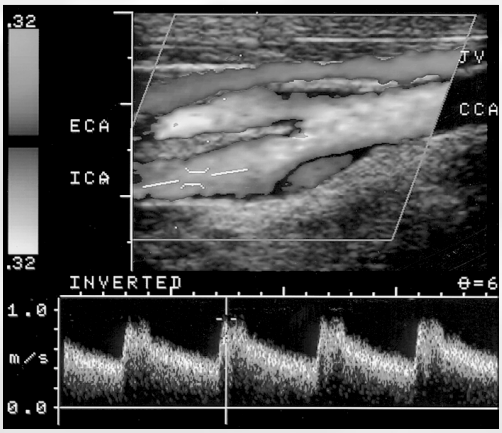

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