Platelet-based coagulation: different populations, different functions

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REVIEW ARTICLE

Platelet-based coagulation: different populations, different functions

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Summary. Platelets in a thrombus interact with (anti)coagulation factors and support blood coagulation. In the concept of cell-based control of coagulation, three different roles of platelets can be distinguished: control of thrombin generation, support of fibrin formation, and regulation of fibrin clot retraction. Here, we postulate that different populations of platelets with distinct surface properties are involved in these coagulant functions. Platelets with elevated Ca^{2+} and exposed phosphatidylserine control thrombin and fibrin generation, while platelets with activated $\alpha_{IIb}\beta_3$ regulate clot retraction. We review how coagulation factor binding depends on the platelet activation state. Furthermore, we discuss the ligands, platelet receptors and downstream intracellular signaling pathways implicated in these coagulant functions. These insights lead to an adapted model of platelet-based coagulation.

Keywords: coagulation factors, clot retraction, fibrin formation, platelet receptors, procoagulant activity, thrombin generation.

Introduction

Since the discovery of platelets as essential blood constituents of an arterial thrombus, it has rapidly become clear that platelets also contribute to fibrin clot formation. On the other hand, coagulation is often still regarded as a merely plasmatic process, characterized by initiation, propagation and termination phases of thrombin generation [1]. In the conventional scheme, the extrinsic coagulation cascade starts with tissue factor binding to factor VIIa, whether or not in complex with FXa, resulting in the cleavage of traces of prothrombin into thrombin. Thrombin amplifies its own generation by proteolytically activating other coagulation factors. In a next phase, these active factors are inactivated by anticoagulation factors, such as tissue factor pathway inhibitor (TFPI), activated protein C, antithrombin, and C1 inhibitor.

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It is widely accepted that phospholipid membranes containing the negatively charged lipid phosphatidylserine (PS) are required to propagate and enhance the coagulation reactions. This membrane dependency implies ultimate cellular control of the coagulation process as a whole. In the blood system, activated platelets with surface-exposed PS cleave high amounts of FX and prothrombin into the proteolytically active forms, FXa and thrombin [2,3]. However, platelets also have other roles in coagulation. They provide a scaffold for the formation of fibrin fibers [3], and, once fibrin clots are formed, they regulate the process of clot retraction [4].

The interwoven nature of platelet activation and the coagulation system has been observed in numerous studies. For instance, in the most common *in vivo* thrombosis models, i.e. ferric chloride-induced damage of arteries or arterioles, the thrombotic process is similarly sensitive to defects in platelet activation and to inhibition of coagulation [5]. This *in vivo* work has collectively shown that: thrombus formation is triggered by collagen, as well as by tissue factor and thrombin [6–9]; both platelets and coagulation contribute to arterial and venous thrombus formation [10,11]; and fibrin formation already occurs at initial stages of thrombus formation [12].

On the basis of literature evidence, we postulate that different platelet populations may have different roles in the coagulation process, depending on their activation state and surface properties. In the following, we will discuss this from the perspectives of: (i) interaction of platelets with (anti)coagulation factors; (ii) heterogeneity of platelet populations with respect to control of thrombin generation, fibrin formation, and clot retraction; and (iii) receptors and signaling processes leading to these different platelet populations.

Platelet interactions with (anti)coagulation factors

For coagulation control, platelets need to be able to interact with coagulation factors. The reported evidence for such interactions is summarized below.

Fibrinogen and fibrin

The fibrinogen receptor, integrin $\alpha_{IIb}\beta_3$, is the most abundantly expressed platelet glycoprotein (GP). Platelet–fibrinogen-binding

requires activation of $\alpha_{IIb}\beta_3$ via conformational changes, a response that is induced by most platelet agonists [13]. This integrin is also supposed to bind fibrin. However, whether integrin-bound fibrinogen is directly converted into fibrin is unclear. Specific fibrin receptors have not been reported, although a role for platelet GPIb in fibrin binding has been proposed [14].

Prothrombin and other vitamin K-dependent factors

Phospholipid membranes with negatively charged phospholipids, i.e. PS and, to a lesser extent, phosphatidylethanolamine, greatly facilitate the binding of coagulation factors and the generation of FXa and thrombin [2,3]. Platelet activation by strong agonists, e.g. thrombin and collagen, is required for PS exposure. As shown by flow cytometry, the PS-exposing platelets characteristically display high-affinity binding sites for vitamin K-dependent (anti)coagulation factors, i.e. prothrombin, FVII, FIX, FX, and proteins C, S, and Z [3,15]. Gamma-carboxyglutamate (Gla) domains, which are present in all vitamin K-dependent factors, mediate Ca^{2+}-dependent factor binding to the PS surface. Hence, PS-exposing platelets serve as assembly sites for components of the tenase complex (FVIIIa, FIXa, and FX) and the prothrombinase complex (FVa, FXa, and prothrombin).

Investigations with fluorescence microscopy have shown that labeled FX and prothrombin only bind to PS-exposing platelets in a thrombus [16,17]. However, once cleaved into thrombin, the prothrombin label appears to redirect to the sites of fibrin fibers [17], which is in agreement with indications that fibrin acts as a site for thrombin. So far, specific protein receptors on platelets for vitamin K-dependent factors such as FIX or FX have not been identified [2,3]. A recent proposal, however, is that these factors may stay bound to the platelet surface after the formation of cysteine bridges, catalyzed by protein disulfide isomerase isoforms [18]. Unlike some other cell types, platelets lack the signaling FXa receptor, protease-activated receptor (PAR)2 [19]. An unexpected finding was that FXa, similarly to ADAM-10/17, influences platelet activation by cleaving the collagen receptor GPVI [20,21].

Thrombin

Thrombin is the only vitamin K-dependent factor known to bind to specific receptor proteins on platelets. Thrombin interacts with high specificity with PARs, i.e. the PAR1 and PAR4 isoforms in human platelets, and the PAR3 and PAR4 isoforms in mouse platelets. Of these, only PAR1 and PAR4 are cleavable by thrombin. In addition, high-affinity thrombin-binding sites are present on GPIb, whereas GPV serves as a thrombin cleavage substrate [22].

Tissue factor

Similarly to megakaryocytes, platelets express limited amounts of tissue factor, e.g. upon sepsis [23,24]. It is, however, doubted whether the levels of tissue factor present in platelets are physiologically relevant [25]. Furthermore, activated platelets release considerable amounts of TFPI, which restricts thrombus growth [26]. Hence, platelet-derived TFPI is expected to rapidly inactivate any tissue factor present at the platelet surface.

FV

Platelet α-granules contain ~ 20% of the blood content of FV. Once released, platelet FV becomes activated by traces of thrombin. It has been reported by one group that platelet-derived FVa is more resistant to protease inactivation than the FVa in plasma [27], but this finding needs confirmation. FVa binds to PS-exposing (platelet) membranes via its C2 domain. Bound to PS-exposing platelets, it interacts with FXa to form local prothrombinase complexes [17,28]. With flow cytometry, a subpopulation of (thrombin-activated) platelets has been detected with intermediate FVa binding [29]. This is in agreement with the observation that FVa is incorporated into the membranes of coated platelets [30], and this may point to gradual accumulation of FVa at the platelet surface.

FVII

In platelets stimulated by collagen and thrombin, binding of recombinant FVIIa to GPIbα, is described, subsequently leading to FX activation [31]. The proposed mechanism is that FVIIa interaction with the GPIb–V–IX complex facilitates the Gla domain-dependent binding to PS-exposing membranes. Platelet-bound FVIIa can restore thrombus formation of platelets lacking $\alpha_{IIb}\beta_3$ by enhancing PS exposure [32]. This may explain why recombinant FVIIa can be an effective prohemostatic drug in patients with Glanzmann's thrombasthenia.

FVIII and FIX

Although it is not a vitamin K-dependent factor, FVIIIa is able to bind to PS-exposing membranes via its C2 domain, probably in a similar manner as FVa [33]. This binding of FVIIIa enhances FIXa binding, and thus stimulates tenase complex formation and FXa generation on PS-exposing platelets. As FVIII in plasma is carried by von Willebrand factor (VWF), FVIII can also interact with platelets via VWF and GPIb–V–IX. However, in kinetic studies of fibrin formation, a role for VWF-bound FVIII in platelet coagulant activity could not be detected [34]. The binding of FIXa appears to be confined to PS-exposing platelets, as determined by annexin A5-labeling studies, and relies on prior elevation of cytosolic Ca^{2+} [35,36].

FXI and FXII

Decades ago, it was proposed that platelets promote fibrin formation in an FXII-dependent manner [37]. Recent observations point to marked roles of FXI and FXII in arterial thrombus formation in mice, suggesting that platelets, in some

way, stimulate the intrinsic pathway of coagulation [38]. One explanation for the thrombus-stimulating effect of FXI and FXII is the binding of FXII to collagen, and its subsequent activation in the presence of prekallikrein and high molecular weight kininogen [39]. There is, however, also some evidence that FXI and FXII can bind to the platelet surface via GPIbα [40]. However, an early suggestion that platelet-bound FXIa is active and cleaves FIX could not be confirmed. Recent studies with human and mouse platelets point to binding of FXI to the platelet apolipoprotein E receptor 2 (LRP8) [41]. It is still not quite clear whether FXII can be activated on the surfaces of platelets. Some studies suggest that polyphosphates released from platelets may fulfil such a role [42], but recent data indicate that the size of platelet-derived polyphosphates (60–100 phosphate residues) is too small for this action [43]. On the other hand, platelet-size polyphosphates do accelerate the activation of FXI by thrombin [44].

FXIII

The coagulation factor FXIIIa acts as a transglutaminase in the cross-linking of fibrin fibers. Experiments with immobilized FXIIIa have shown that platelets bind to this protein via $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ [45]. FXIIIa has also been implicated in the chemical cross-linking of secretory proteins from the α-granules at the surfaces of so-called coated platelets (see below) [46]. However, other tissue-type transglutaminases may also contribute to this process [47].

Anticoagulation factors

Platelets secrete significant amounts of the anticoagulant proteins TFPI and protease nexin-1 (PN-1, gene *SERPINE2*). Binding of TFPI has been detected at the surfaces of coated platelets [48]. PN-1 inactivates thrombin, tissue-type plasminogen activator, and plasmin, thereby acting as a platelet-dependent, negative regulator of coagulation and fibrinolysis [49]. The anticoagulant vitamin K-dependent factors protein S and protein C, both of which bind to PS membranes, can suppress thrombin generation at the platelet surface. It has been reported that activated protein C binds to the platelet LRP8 receptor [50], but the importance of this interaction for the regulation of anticoagulation is unknown. The anticoagulant protein Z acts as an FXa inhibitor [51,52]; whether it binds to platelets is unclear. Similarly, binding sites are not known for anticoagulant proteins such as antithrombin and C1 inhibitor, which block the active sites of coagulation factors.

As summarized in Table 1, most coagulation factors seem to interact preferentially with PS-exposing platelets. These highly activated platelets provide the assembly sites for coagulation factor complex formation, causing greatly enhanced generation of FXa and thrombin. How the anticoagulation factors operate at the platelet surface is not clear. Another unresolved issue is whether all (anti)coagulation factors bind to a PS-exposing platelet at the same time, or whether, perhaps, fibrin or anticoagulation binding to platelets displaces other coagulation factors.

Different platelet populations controlling distinct coagulation steps

Research on the role(s) of platelets in coagulation is complicated by the formation of distinct populations of activated platelets, e.g. with or without PS exposure. Below, we discuss evidence from the literature on how this heterogeneity can determine the different coagulant roles of platelets.

Table 1 Reported interactions of (anti)coagulation factors with platelets; for explanation and references, see text

(Anti)coagulation factor	Binding site on platelets and result	Platelet population
Fibrin(ogen)	Fibrinogen: binding to $\alpha_{IIb}\beta_3$. Precursor for fibrin formation. Fibrin receptor unknown	Aggregating and clot-retracting platelets
Factor II	FII (prothrombin): binding to PS (via Gla domain)	FII: PS-exposing platelets
	FIIa (thrombin): binding to PAR1, PAR3, PAR4, and GPIb–V–IX	FIIa: all platelets
Tissue factor	Weakly expressed. Physiologic role unclear	NA
Factor V	Released by platelets. Binding to PS (via C2 domain); other receptors unknown. Activation of prothrombin	PS-exposing and coated platelets
Factor VII	Binding to PS (via Gla domain) and GPIb–V–IX Activation of FIX and FX	PS-exposing platelets
Factor VIII	Binding to PS (via C2 domain), indirectly via GPIb–V–IX. Activation of FX	PS-exposing platelets
Factor IX	Binding to PS (via Gla domain). Activation of FX	PS-exposing platelets
Factor X	Binding to PS (via Gla domain); other receptors unknown. Activation of prothrombin	PS-exposing and coated platelets
Factor XI	Binding to LRP8 and GPIb–V–IX	Not described
Factor XII	Binding site unclear	Not described
Factor XIII	Binding to $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$	PS-exposing and coated platelets
TFPI	Released by platelets; binding site unclear	Coated platelets
Protease nexin-1	Released by platelets; binding site unclear	NA
Protein C	Binding to PS (via Gla domain) and LRP8	PS-exposing platelets
Protein S	Binding to PS (via Gla domain)	PS-exposing platelets

GP, glycoprotein; NA, not applicable; PAR, protease-activated receptor; PS, phosphatidylserine; TFPI, tissue factor pathway inhibitor.

Procoagulant platelets (agonist-induced PS exposure)

Within a thrombus, formed in a damaged vessel *in vivo* or on a collagen matrix in vitro, platelets with different surface properties are clearly distinguishable [53,54]. Aggregated platelets in the inner thrombus core, close to activating components of the vessel wall, have formed pseudopods, undergone secretion, and bind fibrinogen (see below). Discoid platelets at the outer part of growing thrombi may easily detach and lack these activation markers. A distinct population of highly activated platelets is present close to collagen fibers and as patches around a thrombus. These platelets are characterized by surface-exposed PS (detected with the probes annexin A5 and lactadherin), prolonged cytosolic Ca^{2+} rises, a rounded structure, and the ability to bind coagulation factors such as FV and FX [16,17]. They also produce procoagulant microparticles. Particularly under coagulant conditions, large patches of PS-exposing platelets are formed, implying a role of thrombin as a platelet agonist. The population of PS-exposing platelets has also been described as procoagulant platelets and platelets with a sustained calcium-induced platelet morphology [55,56]. Several studies have shown that *in vivo* injection of annexin A5 or lactadherin (chelating exposed PS) significantly impairs thrombus formation in mouse vessels [10,57], indicating the functional importance of this platelet population.

Studies with isolated coagulation factors have shown that PS-exposing platelets enhance the activities of the tenase and prothrombinase complexes by almost 1000-fold [2,58]. Recent ex vivo flow studies have indicated that platelets with prolonged high Ca^{2+} and PS exposure also catalyze fibrin network formation, although fibrin formation does not appear to be limited to these sites [34]. Jointly, these findings indicate that PS exposure is a prerequisite for platelet-dependent thrombin generation, with fibrin formation not being directly linked to this response.

Coated platelets

Dale *et al*. [30,59] have identified a population of platelets, formed after stimulation with collagen plus thrombin, that have been termed coated platelets. A characteristic of these platelets is their ability to irreversibly bind several α-granular proteins, including FV, thrombospondin, fibrinogen, fibronectin, and VWF [60]. It is considered that these proteins cluster together at the platelet surface and form an immobilized coat. The first reports proposed that FXIII activity is required for chemical cross-linking of these granular proteins to serotonin residues on the platelet membrane [46]. However, in studies with mice deficient in FXIII, it was shown that at least fibrinogen and VWF can bind to coated platelets in the absence of FXIIIa, suggesting (additional) involvement of a tissue-type transglutaminase expressed by platelets [47]. Considering that coated platelets also expose PS [30], the question arises of whether these two populations are identical. This is suggested by the findings that coated and PS-exposing platelets are both formed in response to strong agonists (e.g. collagen and thrombin), through the same cellular processes (e.g. mitochondrial permeability transition pore [MPTP] formation), and that both are accompanied by shedding of microparticles [59,61–64]. Partial overlap of these two populations has indeed been observed in studies using dual labeling of thrombi with annexin A5 and a serotonin probe [16]. On the other hand, stimulation of washed platelets with the Ca^{2+} ionophore A23187 resulted in the formation of PS-exposing platelets without a coat of FVa [30] or high fibrin(ogen) [47]. Together, these findings suggest that coat formation occurs secondary to PS exposure, most likely as a consequence of the assembly of platelet secretion products and plasma factors at the outer membrane. How the formation of this protein coat affects the binding, activation and activity of coagulation factors is unknown.

Aggregating and clot-retracting platelets

Aggregate-forming platelets in a thrombus are characterized by fibrinogen binding and the presence of active $\alpha_{IIb}\beta_3$ at their surfaces (determined with PAC-1 antibody for human platelets, and with JON/A for mouse platelets). As indicated above, this population of platelets differs from the PS-exposing platelets in that the latter do not show activated $\alpha_{IIb}\beta_3$ [53,55]. Interestingly, under coagulant conditions, the aggregated platelets with integrins in the active conformation play a predominant role in fibrin clot retraction. The latter process, which is dependent on platelet fibrin(ogen) binding and integrin outside-in signaling, translates the contractile forces generated in the platelet actin–myosin cytoskeleton to connecting fibrin fibers around platelets [65]. A diffuse fibrin network is thereby converted into a small and dense platelet–fibrin clot. Under flow conditions, this process is observed as contraction of platelet aggregates, and reflects the initial phases of coagulation (Video S1). It is seen as a mechanism for narrowing the gaps between platelets to allow contact-dependent signaling [66]. As the PS-exposing platelets are non-adhesive, have closed integrins, and have a disturbed actin cytoskeleton [56], it is quite unlikely that they will participate in clot retraction. This points to a major difference between the aggregating and PS-exposing platelet populations with respect to the regulation of coagulation processes. Whereas the former produce FXa and thrombin outside the platelet plug, the aggregated platelets consolidate the plug by clot retraction.

Apoptotic and necrotic platelets

In a pioneering paper, Schoenwaelder *et al*. [67] reported that the anti-tumor drugs ABT-737 and ABT-263 induce platelet PS exposure in response to relatively minor rises in cytosolic Ca^{2+}. These compounds inhibit the antiapoptotic proteins Bcl-2 and Bcl-x_L, and cause activation of the Bak–Bax pathway to start a mitochondrial-dependent route of apoptosis and subsequent PS exposure. The mechanism differs from that of agonist-induced PS exposure, as apoptotic PS exposure appears to rely on caspase activation. Other studies have indicated that the ABT compounds disrupt normal platelet activation responses, such as secretion, integrin activation, and platelet aggregation

[68]. Interestingly, the same or a similar apoptotic pathway is triggered in aging or stored platelets, which show a gradual decline in Bcl-x_L expression and also liberate the proapoptotic Bak/Bax proteins [69,70]. The aging process is slow, requiring a time span of several days to yield PS-positive platelets, as examined with the probes annexin A5 and lactadherin [71–73]. Although in vitro tests have shown that aging, apoptotic platelets have a coagulant potential and support thrombin generation, it is questionable whether these PS-exposing platelets play a role in coagulation under physiologic conditions, as they will be rapidly taken up by scavenging cells in the circulation.

Because of clear distinctions between apoptotic and agonist-stimulated PS-exposing platelets, e.g. with respect to caspase activation, it has been suggested that the latter are activated by a necrotic cell death pathway [74]. Given the common definition of necrosis as premature death of cells caused by external sources (e.g. hypoxia or injury), we do not prefer this terminology. Instead, we find the term 'procoagulant platelets' more appropriate, as it refers to their active participation in the support of thrombin and fibrin generation.

Platelet microparticles

Both procoagulant PS-exposing [75] and aging [76] platelets can release microparticles. As these microparticles, in part, expose PS, they have a thrombin-generating potential. This seems to be particularly relevant under pathophysiologic conditions, when elevated levels of platelet-derived microparticles have been measured [77]. Under normal conditions, their role may be restricted, because of rapid interaction with leukocytes and other scavenging cells [78].

Different platelet populations

This overview points to the presence of two types of activated platelet with fundamentally different roles in the coagulation process. On the one hand, there are PS-exposing and coated platelets (with partial overlap) that are characterized by a high activation state, binding of multiple coagulation factors, and the ability to stimulate thrombin generation. These platelets also actively form fibrin, with the coated platelets having a fibrin layer at their surface. On the other hand, present are aggregating platelets, which characteristically express activated integrins and have a role in fibrin clot retraction, which implies that they bind fibrin as well. Although apoptotic (aging) platelets also expose PS, it is unclear whether these have a physiologic role in coagulation. As reviewed elsewhere, the mechanism for this heterogeneity in platelet fate is still incompletely understood [53]. It seems that intrinsic platelet factors, such as platelet size and structure, protein composition, genetic factors, and platelet age, account for only part of the response heterogeneity. Platelet environmental factors, such as the local rheology, exposure to agonists, surrounding cells, and plasma, are probably at least as important. This is discussed further below.

Ligands and receptors mediating platelet PS exposure and fibrin formation

Most studies investigating the roles of platelets in coagulation have been carried out to determine the regulation of PS exposure and the ensuing thrombin generation. Less is known about the mechanisms controlling fibrin formation by platelets. An overview of the ligands and receptors involved in these two processes is given below.

VWF and GPIb–V–IX

A role of the GPIb–V–IX complex to platelet PS exposure, thrombin generation and subsequent fibrin formation has been reported by several groups, claiming that this relies stringently on the interaction of GPIb with VWF [34,79,80]. Particularly under coagulant conditions and with a low shear rate (implying the presence of thrombin), VWF binding to GPIb can lead to prolonged Ca^{2+} responses, PS exposure, assembly of coagulation factors, and formation of fibrin at the platelet surface [34]. The signaling mechanism downstream of GPIb may involve the actin cytoskeleton.

Initially, platelet procoagulant activity via GPIb was considered to rely on the presence of fibrin, interacting with GPIb via VWF [14]. However, studies with platelets from Bernard–Soulier patients pointed to a fibrin-independent signaling role of GPIb–V–IX to enhance thrombin-induced Ca^{2+} mobilization [81]. In mouse platelets, GPIbβ was found to potentiate the Ca^{2+} rises and PS exposure induced by either thrombin or collagen receptor agonists. This procoagulant effect was independent of the N-terminal part of GPIbα, as it was not affected by endopeptidase cleavage of this glycoprotein [82]. Interestingly, deficiency in GPV, which is also part of the GPIb–V–IX complex, increased rather than decreased the responses of mouse platelets to thrombin by a still undisclosed mechanism [83].

Thrombin and receptors

The thrombin receptors of human platelets, PAR1 and PAR4, belong to the class of G-protein-coupled receptors, and elevate cytosolic Ca^{2+} via Gq and phospholipase C (PLC)β stimulation [84]. The consequence is an oscillatory (spiking) Ca^{2+} signal, which, by itself, is insufficient to evoke PS exposure [85]. However, thrombin markedly enhances collagen-induced PS exposure, in which case binding to PAR1 appears to be the main activation mechanism [2,86], rather than binding of thrombin to GPIb [87]. Some authors, however, have reported a role for GPIb in PS exposure, e.g. in thrombin-stimulated gel-filtered platelets, where residual VWF may be present [79].

An explanation for the primary role of PAR1 in thrombin-mediated PS exposure comes from the recent observation that inhibition of protein kinase C (PKC) negatively regulates Ca^{2+} rises and PS exposure elicited by PAR1, but not by the other thrombin receptor, PAR4 [88]. However, PAR4 activation also has a known supporting role in potentiating Ca^{2+} rises and PS exposure [89]. In mouse platelets, where only PAR4 – and not

the other receptor, PAR3 – is cleaved by thrombin [90], thrombin-dependent PS exposure seems to rely on the presence of both isoforms.

The literature contains some evidence that the PAR isoforms on platelets can be cleaved by proteases other than thrombin. For example, PAR1 cleavage by ADAM-17, a related plasma protease [91], or by matrix metalloproteinase-1, has been reported [92]. At present, the physiologic relevance of these alternative mechanisms of thrombin receptor cleavage is unclear. The same holds for the reported cleavage of PAR4 by the fibrinolysis protease plasmin [93] or the neutrophil-derived cathepsin G [94].

Thromboxane and TP receptors

In spite of the prominent role of thromboxane A_2 in collagen-induced platelet aggregation, this autocrine agonist is only marginally effective in stimulating PS exposure. In fact, blockade of thromboxane production by aspirin affected PS exposure of collagen-adhered platelets only in combination with ADP receptor blockers [95]. Along the same line, aspirin treatment only slightly affected thrombin generation in coagulating platelet-rich plasma [96]. In agreement with these findings, stimulation of the platelet TP receptors is known to cause only limited Ca^{2+} mobilization.

ADP, fibrinogen, and receptors

The receptors for ADP (in particular $P2Y_{12}$) and fibrinogen ($\alpha_{IIb}\beta_3$) appear to have supporting and partially redundant roles in PS exposure and platelet-dependent thrombin generation. This was most clearly observed under coagulant conditions, e.g. in tissue factor-activated platelet-rich plasma. In this case, autocrine-produced ADP was found to prolong Ca^{2+} rises and to increase PS exposure via $P2Y_{12}$ mediated signaling towards phosphoinositide 3-kinase (PI3K) [97,98]. Blockade of $P2Y_{12}$ by intake of the prodrug clopidogrel significantly suppressed the procoagulant effect of platelets in coagulating plasma [99]. Similarly, blockade of this receptor by the active metabolite of prasugrel impaired ADP/collagen-induced Ca^{2+} rises, PS exposure and thrombin generation in whole blood [100,101].

Recent work has indicated that $\alpha_{IIb}\beta_3$ outside-in signaling via PLC and the tyrosine kinase Syk also support thrombin-induced Ca^{2+} rises and PS exposure, along with platelet-dependent thrombin generation [102]. The contributory role of $\alpha_{IIb}\beta_3$ partly overlaps with that of autocrine mechanisms via $P2Y_{12}$, which not surprising, given the prominent role of $P2Y_{12}$ in integrin activation. These findings explain why integrin antagonists cause marked suppression of tissue factor-induced thrombin generation, and prolong the clotting times in platelet-rich plasma [103,104].

Immune receptor FcγRIIA

There is limited evidence that stimulation of the immune receptor FcγRIIA on human platelets induces PS exposure and microparticle formation, e.g. via antibodies against the heparin–platelet factor 4 complex [105]. Platelet activation via this receptor, which probably signals via the Syk–PLCγ2 pathway, is considered to contribute to the pathology of heparin-induced thrombocytopenia.

Collagen and GPVI

Circulating platelets will encounter vascular collagen only after endothelial damage and luminal exposure of the extracellular matrix. Under in vitro conditions, stable adhesion of platelets to immobilized fibers of collagen type I is an effective trigger of PS exposure via the immunoglobulin-type collagen receptor GPVI [106]. Studies of platelet interaction with collagen fibers under flow have indicated that all key constituents of the GPVI signalosome contribute to GPVI-induced PS exposure. In line with this, platelets from mice lacking the FcR γ-chain, LAT, Syk or PLCγ2, or with blocked GPVI, showed a marked reduction in collagen-dependent PS exposure and thrombin generation [107,108]. On the other hand, mouse platelets with a gain-of-function mutation in PLCγ2 were more active in collagen-dependent PS exposure and thrombus formation [109]. The adhesive collagen receptor $\alpha_2\beta_1$ was found to support procoagulant activity in an indirect way, by enforcing collagen–platelet interactions via GPVI [110].

In line with this, inhibition of the protein tyrosine kinase Syk, which, together with Src family kinases, controls PLCγ2 activity, has been found to suppress GPVI-induced PS exposure, whereas inhibition of protein phosphatases increases this response [16,111]. The responsible phosphatase has been unknown for a long time, but recent data point to involvement of the phosphatase TULA2, which associates with Syk and can negatively regulate GPVI-induced signaling events [112]. This phosphatase might also control PS exposure.

Platelet adhesion to immobilized collagen causes appreciable PS exposure. However, costimulation with collagen and other agonists such as thrombin is needed to obtain substantial fractions of PS-exposing platelets [2,113]. This costimulation probably has a dual effect: it potentiates the Ca^{2+} rises in platelets, and also activates the integrins ($\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$) needed for stable platelet adhesion to collagen fibers and for signaling via the low-affinity GPVI receptors. This is in contrast to the process of collagen-induced platelet aggregation, which is less dependent on high Ca^{2+} rises, and only requires $\alpha_{IIb}\beta_3$ activation via autocrine agonists such as ADP and thromboxane. Other ligands of GPVI, such as cross-linked collagen-related peptide (low affinity) and, particularly, the snake venom convulxin (high affinity) also provoke PS exposure, but again this response is markedly enhanced by costimulation with thrombin [106].

Other ligands and receptors

A newly described platelet receptor acting via a similar activation pathway of LAT–Syk–PLCγ2 is the protein CLEC2, which can be activated by the snake venom rhodocytin and the

lymphatic protein podoplanin [114]. There are no reports yet on platelet procoagulant activity induced by CLEC2 activation.

In addition to collagen, a number of other adhesive proteins have been found to promote PS exposure, especially after immobilization at a surface. For instance, immobilized thrombospondin-1 and oxidized LDL can stimulate PS exposure of adhered platelets to a limited extent via the receptor CD36 [115]. Furthermore, under coagulant conditions with thrombin present, surface-immobilized fibrinogen [17] and laminin [116] can stimulate PS exposure of adhered platelets via $\alpha_{IIb}\beta_3$ and FXII, respectively. The findings that platelet adhesion favors procoagulant activity are clearly relevant, not only because stable adhesion permits more continued signaling, but also because this provides a way to locally confine the coagulation process to sites of platelet adhesion and activation.

In summary, only a few physiologic agonists, particularly those stimulating GPVI, are capable of inducing PS exposure by themselves. On the other hand, a larger number of agonists, including VWF, fibrinogen, thrombin, ADP, antibodies, and thrombospondin, appear to enhance the process of (GPVI-induced) PS exposure by stimulating their respective receptors. In most cases, the co-agonists act by increasing the Ca^{2+} signal, leading to a prolonged high Ca^{2+} rise (Fig. 1). Apparently, to generate a sufficiently high Ca^{2+} signal for PS exposure, costimulation is needed of both receptors linked to PLCγ isoforms ($\alpha_{IIb}\beta_3$, FcγRIIA, and GPVI) and receptors linked to PLCβ isoforms (GPIb and PAR1/4). The requirement for 'full' PLC stimulation is supported by the observation that PI3K isoforms support PLC-dependent Ca^{2+} mobilization by producing phosphatidylinositol trisphosphate, which acts as membrane-binding site and enhancer of PLCγ isoforms [117]. Regarding the control of fibrin formation, a contribution of the VWF–GPIb axis has been clarified so far. Given the high-activation conditions required for PS exposure, it is reasonable to assume that not all platelets in a thrombus encounter

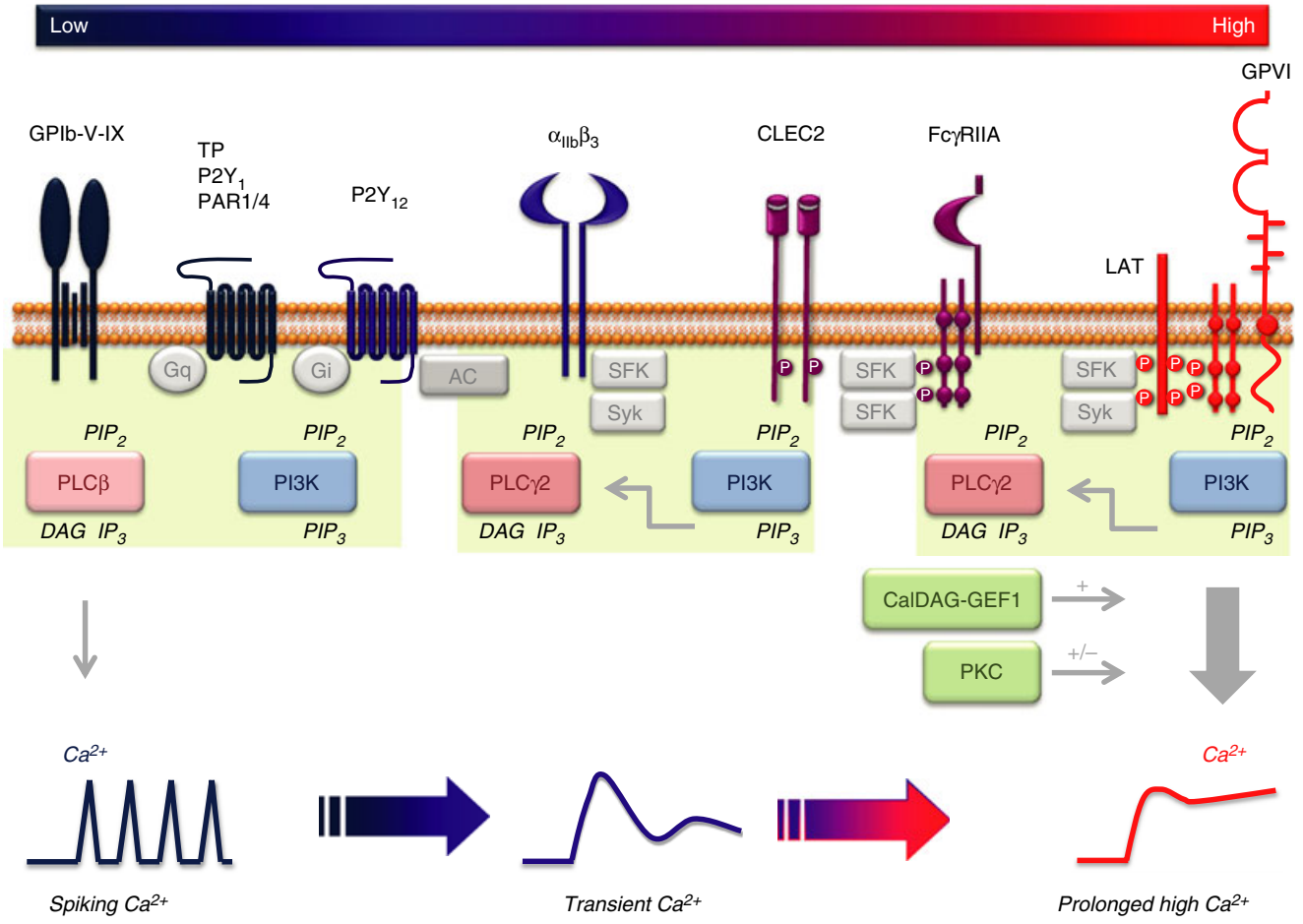

Fig. 1. Platelet-based coagulation: signaling for prolonged high Ca^{2+} rises, which are required for phosphatidylserine (PS) exposure. The gradient color bar indicates the range of receptors with low Ca^{2+}-mobilizing potency, owing to phospholipase C (PLC)β activation, causing spiking Ca^{2+} signals (blue), to receptors with high Ca^{2+}-mobilizing potency, owing to PLCγ2 activation, causing prolonged Ca^{2+} signals (red). Isoforms of phosphoinositide 3-kinase (PI3K) produce the phosphoinositide phosphatidylinositol trisphosphate (PIP_3), which can stimulate PLCγ2 activity. Co-signaling via multiple receptors will result in accumulated Ca^{2+} signals, reaching the threshold levels of prolonged high Ca^{2+} required for PS exposure. The diacylglycerol (DAG) and Ca^{2+}-dependent effector proteins protein kinase C (PKC) and CALDAG-GEFI act as modulators of the Ca^{2+} signal. See also text. AC, adenylyl cyclase; GP, glycoprotein; IP_3, inositol 1,4,5-trisphosphate; PAR, protease-activated receptor; PIP_2, phosphatidylinositol bisphosphate.

sufficient levels of agonists, thus explaining part of the response heterogeneity. On the other hand, intrinsic platelet factors may contribute as well, e.g. different numbers of receptors or signaling molecules [53].

Platelet signaling to PS exposure and fibrin formation: central role of Ca^{2+}

Since the early finding that Ca^{2+} ionophores (A23187 or ionomycin) cause rapid and full PS exposure in essentially all platelets, owing to phospholipid membrane scrambling, it has become clear that a prolonged high Ca^{2+} signal is a sufficient trigger for this platelet response [2,58]. This is confirmed by the observation that most physiologic agonists or combinations provoking PS exposure also induce sustained Ca^{2+} rises. The recent literature provides novel insights into the signaling pathways involved (Fig. 2).

Store-operated Ca^{2+} entry (SOCE)

An initial finding was that Ca^{2+} entry via SOCE not only prolongs the platelet Ca^{2+} signal, but also promotes PS exposure [118]. In the last few years, by the use of murine knockout and pharmacologic approaches, a central role of two membrane proteins in SOCE has been demonstrated [9]. Stromal interaction molecule (STIM)1 was identified as a central Ca^{2+} sensor in the platelet reticular membrane, monitoring Ca^{2+} store depletion, and Orai1 was identified as a Ca^{2+} entry channel that is activated through coupling to STIM1 [119,120]. The chaperone protein cyclophilin A is proposed to act as a positive Ca^{2+} modulator of STIM1 [121]. With mouse platelets lacking STIM1 or Orai1, collagen-dependent thrombus formation was greatly impaired, along with GPVI-induced PS exposure [119,120,122,123]. In contrast, no role was found for the paralogs STIM2 and Orai3, which are both present in platelets. Interestingly, Orai1 makes only a small contribution to Ca^{2+} entry in platelets activated by thrombin and PLCβ. This may explain why, in the presence of high thrombin concentrations, PS exposure is only marginally dependent on STIM1 and Orai1 [122]. Observations such as these have raised the question of whether another Ca^{2+} entry mechanism (receptor-operated Ca^{2+} entry) could operate downstream of thrombin receptors to facilitate PS exposure [124]. Recent findings with human and mouse platelets suggest that transient receptor potential cation channel (TRPC)6 could play such a role [125,126]. Earlier reports on a role of the

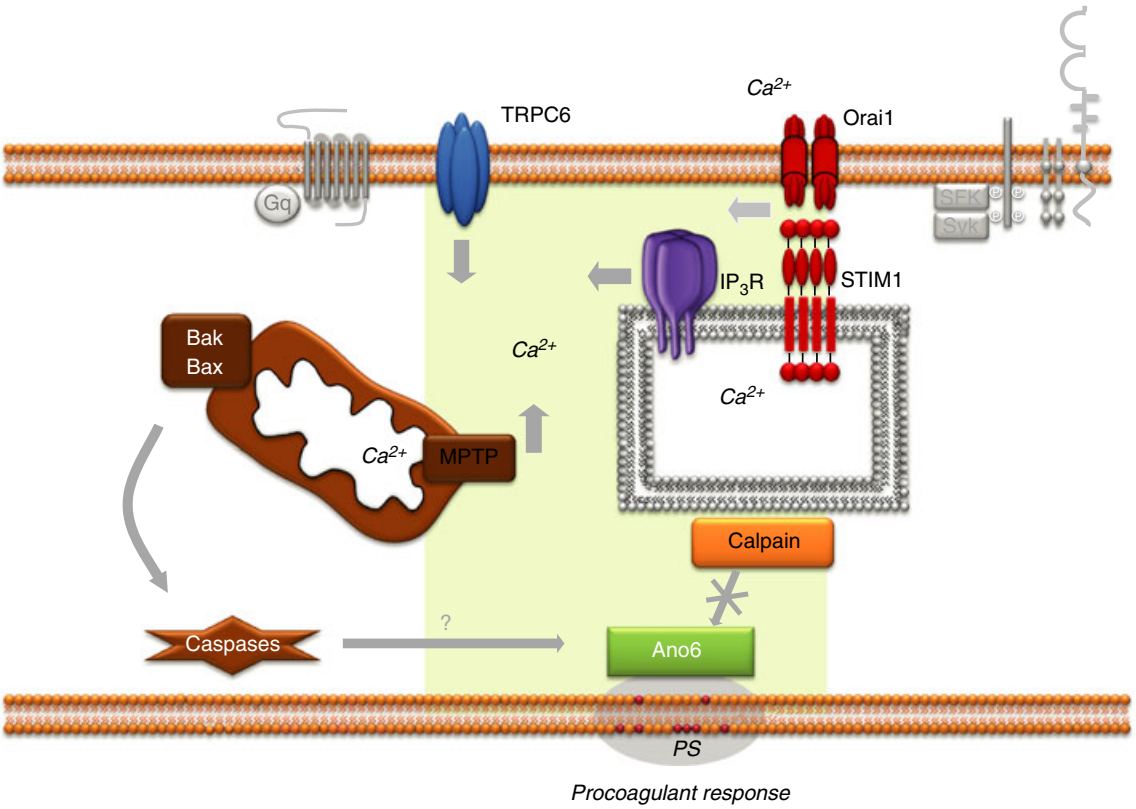

Fig. 2. Platelet-based coagulation: central role of elevated Ca^{2+} in phosphatidylserine (PS) exposure. The mechanisms for Ca^{2+} elevation contributing to PS exposure include: Ca^{2+} mobilization from the endoplasmic reticulum via IP_3 receptors (IP_3Rs); Ca^{2+} entry by store-operated Ca^{2+} entry via stromal interaction molecule 1 (STIM1) and Orai1 channels; and release of mitochondrial Ca^{2+} via mitochondrial permeability transition pore (MPTP) formation. Elevated Ca^{2+} activates the protease calpain, and triggers phospholipid scrambling via anoctamin 6 (Ano6), thereby resulting in PS exposure. The apoptotic pathway of PS exposure via Bak/Bak-induced caspase activation is relatively independent of Ca^{2+}. TRPL6, transient receptor potential cation channel 6.

isoform TRPC1 in Ca^{2+} entry [127] were ruled out because of the complete lack of a phenotype in mouse *Trpc1*$^{-/-}$ platelets [126,128].

Transmembrane fluxes of ions other than Ca^{2+} may contribute to the procoagulant activity. Prolonged platelet treatment with ouabain, which inhibits the Na^+/K^+-ATPase, was found to provoke limited PS exposure and thrombin generation via a small but persistent Ca^{2+} rise that was dependent on Na^+ entry [129]. In addition, Na^+ loading via the Na^+/H^+ exchanger [130], or Ca^{2+}-activated K^+ currents (Gardos channels) [131] may increase platelet PS exposure and prothrombinase activity. The precise mechanisms are not understood.

Mitochondrial collapse

Mitochondrial collapse caused by MPTP formation can be induced by various forms of cellular stress, e.g. free oxygen radicals. Several groups have examined a role for this mitochondrial process in platelet procoagulant activity. In Ca^{2+} ionophore-stimulated platelets, inhibition of MPTP formation with cyclosporin A was found to prevent depolarization of the mitochondrial inner membrane potential, as well as microparticle formation. However, PS exposure was only moderately inhibited, indicating that mitochondrial collapse as such is not a strict requirement for PS exposure [132]. In agreement with this, Ca^{2+} ionophore-induced PS exposure was unchanged in platelets from mice deficient in cyclophilin D, which is a component of the MPTP [62]. However, in the cyclophilin D-deficient platelets, PS exposure and thrombin generation induced by the receptor agonists convulxin and thrombin were strongly diminished. Along the same line, cyclosporin A suppresses the formation of coated platelets in response to convulxin/thrombin [63]. This implies a role of mitochondrial collapse in fibrin (coat) formation. In other cells, MPTP formation abolishes the sequestration of Ca^{2+} in mitochondria [133]. Hence, also in platelets, mitochondrial collapse may lead to a loss of Ca^{2+} flux control, thus provoking agonist (convulxin/thrombin)-induced PS exposure. In contrast, the apoptosis-induced PS exposure, while also involving mitochondria, is essentially Ca^{2+}-independent, and specifically relies on Bak/Bax and caspase activation [74].

Signaling downstream of Ca^{2+}

The majority of platelet agonists signal via PLCβ or PLCγ isoforms, which produce diacylglycerol (DAG) and the Ca^{2+}-mobilizing second messenger inositol 1,4,5-trisphosphate, resulting in activation of PKC and CalDAG-GEFI (Fig. 1). Detailed studies using knockout mice and specific inhibitors point to a two-sided role for PKC isoforms in platelet activation and PS exposure [134]. The conventional isoforms PKCα and PKCβ, which are activated by DAG and Ca^{2+}, positively contribute to platelet secretion, Ca^{2+} signaling, and PS exposure [135–137]. The novel isoform PKCθ, being activated by DAG alone, acts differently, in that it suppresses GPVI-induced Ca^{2+} mobilization and PS exposure [136]. This negative role of PKCθ could explain why general PKC stimulation with phorbol ester downregulates these platelet responses [134]. However, some authors have reported slight stimulation of platelet function by PKCθ [138]. In summary, the role of PKC isoforms in platelet PS exposure (procoagulant response) can be understood in terms of PKC-dependent fine-tuning of the Ca^{2+} signal.

Mouse experiments also point to a separate role in procoagulant activity of the regulatory protein CalDAG-GEFI [139]. This activates the small GTPase Rap1b, thereby supporting integrin activation and other platelet functions. Platelets from CalDAG-GEFI-deficient mice were found to be substantially impaired in PS exposure during thrombus formation on collagen, by a mechanism that is not yet understood [140]. It was concluded that CalDAG-GEFI, like STIM1, contributes to the first wave of thrombin generation catalyzed by PS-exposing procoagulant platelets. Furthermore, platelet CalDAG-GEFI appeared to regulate fibrin clot formation.

With the recent discovery of the platelet protein anoctamin 6 (gene *TMEM16F*) as a key regulator of Ca^{2+}-dependent phospholipid scrambling and hence PS exposure [141], other speculations on protein PS translocators have been ruled out [77]. In platelets from patients with Scott syndrome, a moderate bleeding disorder, anoctamin 6 is lacking, and ionomycin-induced and agonist-induced PS exposure are greatly impaired [141,142], in spite of normal Ca^{2+} rises [143]. Anoctamin 6 is a multiple membrane-spanning protein with a supposed Ca^{2+}-binding site, but whether it operates alone is unclear. Recent data obtained with mice containing inactivated anoctamin 6 support a role of this protein in platelet PS exposure and arterial thrombus formation [144]. Another Ca^{2+}-dependent protein contributing to microparticle formation is the protease calpain, but inhibitor studies have indicated that its role in PS exposure is moderate at best [145].

Taken together, the available evidence indicates that PS exposure in response to agonist stimulation is accomplished by multiple pathways acting synergistically to cause a rise in Ca^{2+} (Fig. 2). In other words, platelet stimulation with multiple agonists that cause massive Ca^{2+} store depletion and SOCE, owing to the STIM1–Orai1 interaction, may result in a loss of the normal control of Ca^{2+} handling by Ca^{2+} pumps, PKCθ, and MPTP formation, so that cytosolic Ca^{2+} remains high, and Ca^{2+}-dependent phospholipid scrambling via anoctamin 6 becomes activated. How this facilitates extracellular fibrin formation is unclear.

Platelet signaling for clot retraction: central role of integrins

The regulation of clot retraction by platelets has been less extensively studied than that of PS exposure. Despite the only sparse mechanistic studies, clot retraction is generally considered to be an $\alpha_{IIb}\beta_3$-dependent event and a marker of integrin

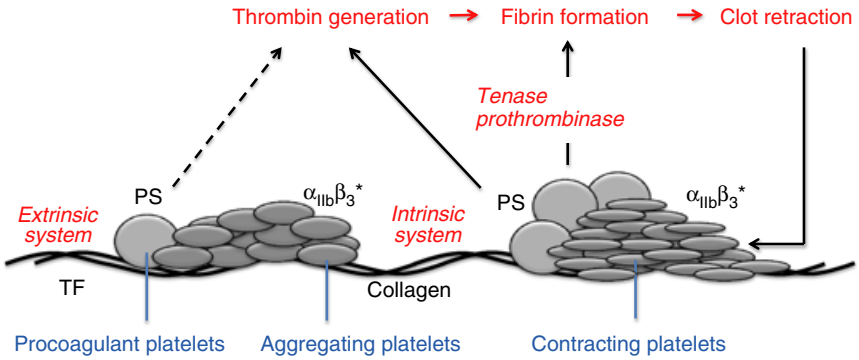

Fig. 3. Model of platelet-based coagulation. Vascular damage initiates the extrinsic coagulation pathway via tissue factor (TF) and the intrinsic pathway via collagen. Collagen-adhered platelets and, in a later stage, patches of platelets in a thrombus expose phosphatidylserine (PS), and thereby serve as a membrane substrate for the tenase and prothrombinase complexes, resulting in massive thrombin generation. These platelets can also support fibrin formation and form a fibrin coat. Populations of PS-exposing platelets are characteristically high in cytosolic Ca^{2+}, whereas the remaining aggregated platelets in a thrombus display activated $\alpha_{IIb}\beta_3$.* By interacting with fibrin in the presence of thrombin, the latter platelets contract and cause clot retraction.

outside-in signaling. While we do not aim to repeat other reviews on platelet integrin signaling [13], here we summarize what is known of the signaling processes implicated in platelet-dependent clot retraction.

Signaling downstream of $\alpha_{IIb}\beta_3$

Several investigations, mostly using mouse blood, have indicated that clot retraction is mediated by signaling proteins that also contribute to $\alpha_{IIb}\beta_3$ outside-in signaling. Specific platelet proteins implicated in clot retraction include the protein tyrosine kinases Src and Fyn, the adapter protein Lnk, AMP-activated protein kinase, and the effector proteins PLCγ2 and PI3Kβ [146–148]. Recent studies point to two phases in platelet-dependent clot retraction upon stimulation with thrombin: the initial formation of high-adhesion $\alpha_{IIb}\beta_3$ contacts with fibrin, and a subsequent phase in which the platelet contractile forces are transmitted to the fibrin clot [65,149]. The force generation occurs via the actin cytoskeleton, and appears to depend on activation of myosin IIA by Rho kinase and myosin light-chain kinase [150]. An extracellular process required for clot retraction is the cross-linking of platelet-associated fibrin fibers by the transglutaminase FXIIIa [151]. The Ca^{2+}-dependent protease calpain suppresses clot retraction, probably by cleaving cytoskeletal proteins [152]. In this context, it is relevant to note that calpain causes considerable degradation of cytoskeletal proteins in PS-exposing platelets [153], thus providing another explanation for why the PS-exposing platelet population is not involved in clot retraction. Next to the already established role of thrombin in platelet PS exposure [154], these data also point to a physiologic role of thrombin in integrin-dependent clot retraction.

Hence, the signaling processes contributing to platelet-regulated clot retraction, as far as they have been unraveled, rely on $\alpha_{IIb}\beta_3$-dependent signaling processes controlling actin–myosin interactions, which are strikingly different from those leading to PS exposure.

Conclusion

Taken together, all of these findings lead to an adapted model of platelet-based coagulation, as schematized in Fig. 3. Collagen-adhered platelets and, in a later stage, patches of platelets in a thrombus, activated by thrombin and other agonists, display with prolonged high Ca^{2+}, and expose PS. PS-exposing platelets serve as membrane substrate for multiple coagulation factors, with massive propagation of thrombin generation as a result. These platelets can also support fibrin formation and form a fibrin coat. The remaining aggregated platelets with active $\alpha_{IIb}\beta_3$ contract and cause clot retraction by interacting with fibrin in the presence of thrombin. This scheme may help to resolve many unanswered questions regarding platelet and coagulation activation in arterial and venous thrombosis.

Disclosure of conflict of interests

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Video S1. Whole-blood thrombus formation on collagen under coagulating conditions.

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