All roads lead to thrombus formation : demystifying platelet signaling pathways

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ALL ROADS LEAD TO THROMBUS FORMATION

Demystifying platelet signaling pathways

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ALL ROADS LEAD TO THROMBUS FORMATION

Demystifying platelet signaling pathways

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ter verkrijging van de graad van doctor
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Nil volentibus arduum

Voor Willem

Contents

Chapter 1	General introduction	9
Chapter 2	Collagen-mimetic peptides mediate flow-dependent thrombus formation by high- or low-affinity binding of integrin $\alpha 2 \beta 1$ and glycoprotein VI	23
Chapter 3	Non-redundant roles of phosphoinositide 3-kinase isoforms α and β in glycoprotein VI-induced platelet signaling and thrombus formation	41
Chapter 4	PKC α regulates platelet granule secretion and thrombus formation in mice	65
Chapter 5	Functional divergence of platelet protein kinase C isoforms in thrombus formation on collagen	87
Chapter 6	Roles of platelet STIM1 and Orai1 in glycoprotein VI- and thrombin-dependent procoagulant activity and thrombus formation	109
Chapter 7	Regulation of integrin $\alpha \text{IIb} \beta 3$ closure in phosphatidylserine-exposing platelets	129
Chapter 8	General discussion	145
	Summary	161
	Samenvatting	165
	Dankwoord	171
	Curriculum Vitae	177
	Publications	181
	Addendum	185

Chapter 1

General introduction

Hemostasis assures the cessation of bleeding at sites of vessel injury, while thrombosis is the pathophysiological process of uncontrolled thrombus formation, potentially developing into vessel occlusion and subsequent heart attack or stroke. The cellular key role in these processes is carried out by platelets. These 1-3 μm discoid anuclear blood cell elements are shed from megakaryocytes in the bone marrow, and circulate in the human blood at concentrations of $150\text{--}400 \times 10^9/\text{L}$ during 7-10 days, after which they are cleared by the spleen, liver and bone marrow (1). This introductory chapter gives a general overview of key intracellular signaling processes and platelet activation in thrombus formation that are relevant for this thesis.

Platelet activation, aggregation and procoagulant activity in thrombus formation

Platelets are kept in a resting state because of their continuous encounter with vessel wall derived paracrine molecules like prostacyclin and nitric oxide. Upon vessel wall injury, exposed subendothelial extracellular matrix components, particularly collagen, together with von Willebrand factor (vWF), derived from endothelial cells and plasma (2), provide the main triggers for platelet adhesion and subsequent platelet aggregation and thrombus formation (Figure 1). Under arterial blood flow conditions, platelet adhesion and activation are mediated by the vWF receptor glycoprotein (GP)Ib-V-IX (3,4), and the collagen receptors, integrin $\alpha 2\beta 1$ and immunoglobulin-like receptor GPVI (5,6). In the context of this thesis, thrombus formation is defined as the overall result of platelet activation, aggregation, and procoagulant activity during whole blood flow over a thrombogenic surface *in vitro* or *in vivo* (Figure 1).

An essential step in platelet aggregation is activation of the integrin $\alpha \text{IIb}\beta 3$. In stimulated platelets, this abundantly expressed integrin changes in conformational state, in which its affinity increases for fibrinogen and vWF (7-10). By forming intercellular bridges, fibrinogen, and under high shear conditions also vWF, mediate platelet-platelet interactions. Interestingly, the activation of integrin $\alpha \text{IIb}\beta 3$ is a reversible process, and thus its continuous activation is required for stable thrombus formation (10).

Activated platelets release the contents of two types of secretory granules, namely dense granules (containing e.g. ADP, ATP, polyphosphates and Ca^{2+}) and alpha-granules (containing multiple proteins, among which membrane-bound P-selectin, vWF, fibrinogen and coagulation factor V). Secretion of the granule contents by collagen-adhered platelets is an important step in the recruitment of platelets from flowing blood. In addition, activated platelets produce thromboxane A_2 from arachidonic acid released from the membrane phospholipids by phospholipase A_2 . These platelet-derived secondary mediators bind to signaling receptors on nearby platelets, i.e. thromboxane A_2 to the TP receptor (11), ADP to the P_2Y_1 and P_2Y_{12} receptors (12), and ATP to the P_2X_1 receptor (13).

Figure 1. Schematic overview of platelet activation, aggregation and procoagulant activity in arterial thrombus formation. Collagen fibers exposed upon vascular damage form a thrombogenic surface for vWF binding, platelet adhesion and subsequent platelet integrin activation, secretion, aggregation, thrombin generation and fibrin clot formation. Initially, platelets transiently interact with collagen-bound vWF via GPIb-IX-V. They stably adhere and become activated via interaction with the collagen receptors, integrin $\alpha_2\beta_1$ and GPII. This activation triggers integrin $\alpha_{IIb}\beta_3$ to undergo a conformational transition into a high-affinity ligand binding state. The platelets aggregate via fibrinogen binding to the activated $\alpha_{IIb}\beta_3$, thereby forming a platelet thrombus. Concomitant release of autocrine mediators, like ADP and thromboxane A₂, results in the recruitment of additional platelets to the growing thrombus. A subpopulation of platelets, e.g. directly in contact with collagen, has a balloon-like shape and sustained high cytosolic Ca²⁺ levels. These platelets expose negatively charged PS at their outer membrane, and then become procoagulant. The PS serves as a catalytic surface for generation of activated factor Xa (FXa) and thrombin. The formed thrombin consolidates the thrombus, e.g. by conversion of fibrinogen into fibrin and by acting as a strong platelet agonist. Abbreviations: EC, endothelial cells; GPCR, G-protein-coupled receptor; Plt, platelet; SMC, smooth muscle cells.

Upon thrombus formation, a subpopulation of the platelets in the thrombus typically displays a high cytosolic Ca²⁺ concentration (14). These platelets are characterized by the expression of negatively charged phosphatidylserine (PS) at their outer surface. Earlier work has shown that segregation of platelet aggregatory and procoagulant microdomains in thrombus formation is regulated by transient activation of integrin $\alpha_{IIb}\beta_3$. Thrombin, generated by factors Xa and Va at the surface of procoagulant platelets, is a central connecting component between the activation of platelets and the coagulation system. Thrombin contributes to the coagulation process by activating other coagulation factors and by converting fibrinogen into fibrin, which consolidates the thrombus. In addition, thrombin acts as a potent platelet agonist by proteolytically activating the G-protein coupled, protease-activated receptors, PAR₁ and PAR₄ (15). Only very little is known yet on the causes, intracellular signaling processes and consequences of this heterogeneity in thrombus formation, although it is likely that thrombin can play an important role herein.

Collagen-based substrates for adhesive platelet receptors

The fibril-forming collagens type I and III are among the most potent platelet-activating components of the vascular sub-endothelium and atherosclerotic plaques (3,16). Platelet interaction to either collagen type involves GPVI and integrin $\alpha 2\beta 1$ receptors. While there is no doubt on the function of GPVI as the key platelet-activating collagen receptor, it is still controversial whether also integrin $\alpha 2\beta 1$ has a signaling role in collagen-induced platelet activation (17,18). GPVI binds to and is activated by (GPO)_n sequences within the triple-helical collagen chains (19,20). In contrast, integrin $\alpha 2\beta 1$ recognizes collagen motifs with a GXX'GER sequence, which are partly different for collagen type I (GFOGER, GMOGER, GLOGER and GASGER) and collagen type III (GMOGER, GLSGER and GAOGER) (21-23). Under static conditions, triple-helical collagen-like peptides containing such GXX'GER motifs indeed bind integrin $\alpha 2\beta 1$, albeit with different affinities (20,22). The variation of integrin-binding motifs has made it difficult to precisely dissect the function of integrin $\alpha 2\beta 1$ in platelet-collagen interactions. We reasoned that flow perfusion studies are needed, using the collagen-mimetic peptides with various integrin-binding motifs, to better understand the role of integrin $\alpha 2\beta 1$ in thrombus formation.

Platelet signaling through phosphoinositide 3-kinase isoforms

Human and mice platelets contain four different phosphoinositide 3-kinase (PI3K) isoforms, distinguished according to their catalytic subunit, e.g. p110 α , p110 β and p110 δ (class IA isoforms) and p110 γ (class IB isoform) (24). The class IA and IB isoforms use separate sets of regulatory subunits. For class IA, these are the subunits indicated as p85 α/β , p55 α/γ and p50 α , whereas for class IB the regulatory subunit is p101 γ (Figure 2A). Below, the isoforms will be named according to their catalytic subunit, i.e. PI3K α , β , γ and δ .

Platelet activation via GPVI depends on phosphorylation of the co-receptor, Fc receptor γ -chain (25), caused by the Src family kinases, Fyn and Lyn (26). Once phosphorylated, the Fc receptor γ -chain binds the tyrosine kinase Syk via its immunoreceptor tyrosine-based activation motif (ITAM) (27). This initiates a Syk-dependent signaling cascade, leading to formation of a signalosome around the transmembrane linker for activation of T cells (LAT), which serves as a docking site for other signaling proteins, including PI3K isoforms and phospholipase C $\gamma 2$ (28,29). This is briefly schematized in Figure 2B.

For GPVI-stimulated platelets, the general concept is that SH2 domain-containing PI3K regulatory subunits bind to the LAT signalosome, and recruit the corresponding catalytic subunits to the plasma membrane. This may lead to a positive feedback signal, since PI3K contributes to phospholipase C $\gamma 2$ activation downstream of GPVI activation (28). Other agonists that evoke recruitment of PI3K isoforms to the plasma membrane are ADP via the P2Y₁₂ receptor, and thrombin via the PAR_{1/4} receptors.

Figure 2. Domain structure and functions of phosphoinositide 3-kinases in platelet activation. A, Structure of class IA and IB PI3K isoforms, expressed in mouse and human platelets. Isoforms of both classes contain separate catalytic and regulatory subunits. In class IA isoforms, the inter-SH2 (Int-SH2) domains of the regulatory subunits *p85α/β*, *p55α/γ* or *p50α* interact with N-terminal domains of the catalytic subunits, *p110α*, *β*, or *δ*. Subsequent binding of the SH2 domains with the adaptor region (ADr) in the catalytic subunit is a prerequisite for PI3K activity. The class IB *p101* regulatory subunit contains neither Int-SH2 nor SH2 domains, and specifically interacts with the N-terminal of the *p110γ* catalytic subunit. B, Agonist-induced activation of unspecified PI3K isoforms can be triggered by: ADP-receptor P2Y₁₂ stimulation via the GTP-binding protein G_i; thrombin-receptor PAR_{1/4} stimulation via G_q and phospholipase Cβ (PLCβ); or by collagen-receptor GPVI stimulation via the LAT signalosome (involving the protein kinase Syk and Src-family kinases (SFK)) and PLCγ₂. The PI3K isoforms are considered to stimulate platelet secretion, integrin activation, and procoagulant activity. Which PI3K isoforms are involved in these key platelet responses and which isoform exactly becomes activated by which platelet agonist remains to be elucidated. Abbreviations: BH, Bcr homology domain; C₂, phospholipid binding domain; CATr, catalytic domain; PIK, helical phosphatidylinositol kinase homology domain; Pr, proline-rich domain; Ras-B, Ras-binding domain; SH2/3, Src homology domain 2/3.

Class IA and IB PI3K isoforms act by phosphorylating the 3-hydroxyl position of phosphoinositides, in particular phosphatidylinositol 4,5-bisphosphate, generating phosphatidylinositol 3,4,5-trisphosphate (30). Interestingly, they compete herein with phospholipase C isoforms, which catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol. With respect to platelets, there is limited evidence that individual class I PI3K isoforms have distinct cellular functions. For instance, P2Y₁₂ stimulation with ADP activates only the *p110β* (PI3Kβ) and *p110γ* (PI3Kγ) isoforms, which contribute to integrin αIIbβ₃ activation and platelet aggregation (31–34). It is still debated which of the PI3K isoforms are directly activated through the GPVI signaling path-

ways, and which isoforms are activated indirectly, e.g. following ADP/P2Y₁₂ receptor stimulation.

Also unclear are the downstream signaling events evoked by the various PI3K isoforms in platelets. A possible, though poorly explored, target of PI3K is the small GTPase Rap1b, which is considered to play a major role in α IIb β ₃ activation. Platelet agonists such as ADP and thrombin produce the active GTP-bound form of Rap1b, partly in a PI3K β -dependent manner (35,36). Whether and how Rap1b is activated via GPVI or P2Y₁₂, and which (other) PI3K isoforms are involved have remained vague. Given their central place in the platelet signaling network, PI3K isoforms likely play a role in granule secretion and procoagulant activity (Figure 2B). Again, precisely how is unclear. We addressed these issues by a combined pharmacological (PI3K isoforms specific inhibitors) and genetic approach. For the latter the only relevant mice available were deficient in p85 α , which is the main regulatory subunit of class IA isoforms, or in p110 γ , being the catalytic subunit of PI3K γ .

Platelet signaling through protein kinases C isoforms

The protein kinase C (PKC) family of serine-threonine kinases comprises multiple, abundantly expressed members. These PKC isoforms are grouped into three classes (37): conventional isoforms (α , β I, β II, γ), which are activated by Ca²⁺/diacylglycerol; novel isoforms (δ , ϵ , η , θ), activated by diacylglycerol alone; and atypical isoforms (ζ , ι/λ), which are diacylglycerol-independent (Figure 3A). All isoforms consist of one protein chain, containing both a catalytic and regulatory subunit. Human and mouse platelets highly express the conventional PKC isoforms α and β , and the novel isoforms δ and θ (38-42). Not much is known about atypical PKC isoforms in platelets.

Pharmacological studies have demonstrated an important positive role for PKC activity in key platelet responses, such as integrin α IIb β ₃ activation, secretion and aggregation (43-46). On the other hand, there is evidence for a negative regulatory role of PKC in other processes, e.g. lowering of the Ca²⁺ responses by promotion of Ca²⁺ extrusion (47,48), and suppression of platelet procoagulant activity. This raises the question whether perhaps the conventional and novel PKC isoforms may have distinct or even opposing roles in the general control of platelet activation (49). This is addressed in Figure 3B.

At the start of this thesis, only little was known about functions of individual PKC isoforms in platelets. Using biochemical approaches, PKC α had been identified as a stimulator of α -granule and dense-granule secretion (50) as well as platelet aggregation (51). With biochemical and pharmacological approaches, it was shown that PKC α interacts with two tyrosine kinases, Syk and Src, to regulate their activity (52). PKC β was reported to be a positive regulator of outside-in α IIb β ₃ signaling in platelets (53). In contrast, there was uncertainty about the functions of the novel PKC isoforms, PKC δ and PKC θ , because for both either positive or negative signaling functions have been reported (54-57).

Figure 3. Domain structure and functions of protein kinase C in platelet activation. A, Human and mouse platelets express PKC isoforms that are classified into three subgroups: the conventional (cPKC: PKC α,β,γ), novel (nPKC: PKC $\delta,\epsilon,\eta,\theta$), and atypical (aPKC: PKC $\zeta,\iota/\lambda$) PKC isoforms. All PKC isoforms contain a regulatory and catalytic region separated by a hinge sequence. Substrate and ATP-binding sites are located in the catalytic region near the C-terminal of the protein. The regulatory region differs among the three subgroups, and determines their response to agonists. cPKC isoforms have a diacylglycerol (DAG) and a Ca²⁺ binding site. nPKC isoforms lack the Ca²⁺ binding site, while the aPKC isoforms have a completely deviant regulatory region. B, In platelets stimulated with ADP, thrombin or collagen, unspecified cPKC or nPKC isoforms stimulate granule secretion and integrin $\alpha\text{IIb}\beta_3$ activation, whereas other(?) PKC isoforms suppress procoagulant activity by inhibiting PS exposure. See further, Figure 2.

Given the availability of mice deficient in PKC α , β , δ or θ , and the presence of several PKC inhibitors with isoform selectivity, we found it important to combine pharmacological and genetic approaches to study the individual contribution of these major PKC isoforms in platelet activation. For a complete insight, in addition to determining integrin activation, secretion and thrombus formation, also their role in Ca²⁺ signaling and procoagulant activity should be addressed.

Calcium flux-regulating proteins in platelet function

The majority of platelet agonists signal to elevation of cytosolic Ca²⁺ concentration (58,59), which is an indispensable event for secretion and integrin $\alpha\text{IIb}\beta_3$ activation, and, when this elevation is high and prolonged, for PS exposure and platelet procoagulant activity (Figure 4).

Increased cytosolic Ca²⁺ is one of the main triggers to provoke the active conformation of $\alpha\text{IIb}\beta_3$ in activated platelets. Currently, there are two models for the transition of this integrin from the resting to the high-affinity binding state, the latter of which is needed for

fibrinogen binding. One activation model involves unclasping of the α IIb and β 3 chains (10), the other model the tilting of β 3 (9) by intracellular binding of talin to the its transmembrane domain (8,60). Integrin α IIb β 3 receptors bound to fibrinogen molecules can form micro-clusters, allowing the formation of a cytosolic signaling complex that is capable to integrin outside-in signaling. In the signalosome, Src-family kinase members, including Src itself which is constitutively bound to the β 3 cytoplasmic tail, become activated to phosphorylate the protein kinase Syk, leading to phospholipase $Cy2$ activation. This effector enzyme causes reinforcement of the increase in Ca^{2+} , which may lead to PS exposure.

Strikingly, the process of PS exposure, which occurs at distinct sites in a thrombus, is accompanied by closure of activated α IIb β 3 integrins (Figure 4). How the latter process is regulated and whether Ca^{2+} is involved has remained unclear. An indirect indication for a role for Ca^{2+} in integrin closure is the finding that the Ca^{2+} -dependent thiol protease, calpain, cleaves the cytoplasmic tail of the β 3 chain, an event that is not compatible with the activated conformational state of α IIb β 3 (61). There may also be a role of mitochondrial membrane depolarization in Ca^{2+} -dependent integrin closure, since also PS exposure is suggested to be under mitochondrial control (62). Evidence in favour of this idea also comes from the observation that mouse platelets lacking cyclophilin D, which is a component of the mitochondrial permeability transition pore (mPTP), are deficient in PS exposure in response to receptor agonists (63,64). On the other hand, mouse platelets lacking other mitochondrial-associated proteins, i.e. the apoptosis-regulating factors Bax and Bad, have a normal rate of PS exposure, suggesting that the mitochondrial apoptosis pathway is regulated in a distinct way than agonist-induced PS exposure.

The platelet-activating conditions required for PS exposure and platelet procoagulant activity have extensively been investigated (65). Stimulation of the collagen receptor GPVI by itself results in a moderate procoagulant response (66,67), while the combined stimulation of collagen and thrombin receptors results in high PS exposure, likely because these agonists use different signaling pathways (58). In mouse and human platelets, the conventional route for cytosolic Ca^{2+} elevation is by activation of phospholipase $C\beta$ (evoked by G-protein coupled receptors for ADP and thrombin) and of phospholipase $Cy2$ (evoked by GPVI). Both isoforms of phospholipase C, as described above, catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate into diacylglycerol and IP_3 . The latter interacts with IP_3 receptor channels in the endoplasmic reticulum, which release Ca^{2+} from the intracellular stores. Mobilization of Ca^{2+} from these stores is accompanied by Ca^{2+} entry from the extracellular medium via a process, called store-operated Ca^{2+} entry (SOCE) (Figure 4). It has been recognized that SOCE is essential to reach sufficiently high Ca^{2+} levels for PS exposure (58,68,69). The entry of Ca^{2+} can also be facilitated by another process, called receptor-operated Ca^{2+} entry (ROCE) (70).

Figure 4. Central role of Ca^{2+} in platelet activation processes. Upon ADP, thrombin or collagen receptor activation, $\text{PLC}\beta$ and γ isoforms hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The interaction of IP_3 with its receptor (IP_3R) elicits Ca^{2+} mobilization from intracellular stores in the endoplasmic reticulum. Subsequently, the transmembrane Ca^{2+} -sensing protein STIM interacts with the plasma membrane Ca^{2+} channel, Orai_1 , to induce store-operated Ca^{2+} entry (SOCE), contributing to the Ca^{2+} rise. Mitochondria function as Ca^{2+} buffering systems, with the capacity for release, e.g. upon formation of the mitochondrial permeability transition pore (mPTP). Elevated Ca^{2+} stimulates integrin activation and secretion, while sustained high Ca^{2+} is required for PS exposure. The latter provides a procoagulant surface for activation of factor X (complex of factors VIIIa , IXa and X), and for activation of prothrombin (complex of factors II, Va and Xa). Procoagulant platelets have lost the capacity to bind fibrinogen through activated $\alpha\text{IIb}\beta_3$ integrins. How the integrins $\alpha\text{IIb}\beta_3$ are closed remains to be investigated. See further, Figure 2.

First studies to find the Ca^{2+} channel responsible for SOCE concentrated on members of the family of transient receptor potential channels (Trpc). In platelets, however, it soon appeared that Trpc6 acts as a diacylglycerol-activated non-SOCE channel (71). In work from other authors, the isoform Trpc1 was believed to be involved in SOCE (72), but this was negated later as described in this thesis. Major progress in this field was made with the discovery of the Orai class of plasma membrane Ca^{2+} channels. The isoform Orai_1 (also called CRACM_1) oligomerizes and opens following depletion of the Ca^{2+} stores, by interacting with the STIM_1 , which is a Ca^{2+} -sensing transmembrane protein located in the endoplasmic reticulum (73,74). Both Orai_1 and STIM_1 are expressed in human and mouse platelets, and both regulate platelet functions (Figure 4). Studies in other cell types suggest that the homologous protein STIM_2 may have a similar regulatory role in Ca^{2+} entry (75). The precise role of Ca^{2+} entry via Orai_1 and the STIM isoforms in Ca^{2+} -dependent thrombus formation and platelet PS exposure and procoagulant activity remains to be elucidated.

Outline of this thesis

This thesis aims to investigate different molecular signaling proteins and mechanisms, by which platelets contribute to thrombus formation and the hemostatic response. Particular attention is paid to the different signaling mechanisms involved in platelet aggregation and platelet procoagulant activity. The general introductory **chapter 1** gives a brief overview of relevant signaling pathways underlying both responses, with emphasis on the crucial roles of the platelet signaling proteins, PI3K and PKC, and the secondary mediator Ca^{2+} . Furthermore, this chapter addresses the property of collagen as an important platelet-activating component, exposed in the extracellular matrix. **Chapter 2** concentrates on the first stage of thrombus formation. Studied is how platelets at high (arterial) and low (venous) shear rates interact via their collagen receptors, GPVI and integrin $\alpha_2\beta_1$, using specifically designed triple-helical collagen-related peptides in the presence or absence of vWF. This work also assesses the suitability of collagen-related peptides as substitutes for native collagen surfaces to cause induction of thrombus formation under flow. The contribution of the various PI3K isoforms to collagen- and GPVI-induced thrombus formation is studied in **chapter 3**. Particular attention is paid to possible differences in functions between the class IA isoforms, PI3K α and β , and the class IB isoform, PI3K γ . It is also determined how PI3K isoforms contribute to activation of the small GTPase, Rap1b, and subsequent integrin $\alpha\text{IIb}\beta_3$ activation. The next two chapters focus on the PKC family of serine/threonine kinases with respect to platelet activation and thrombus formation. In **chapter 4**, a new knockout mouse is described to identify the function of the conventional isoform, PKC α , in platelet morphology, activation and thrombus formation. **Chapter 5** combines genetic and pharmaceutical approaches to compare the functions of the four major PKC isoforms in platelet responses and thrombus formation. Particular attention is paid to possible functional differences between the conventional, diacylglycerol-dependent PKC isoforms (PKC α , β) and the novel, diacylglycerol-independent PKC isoforms (PKC δ , θ). Given the key role of Ca^{2+} as a second messenger in platelet activation, **chapter 6** presents a study regarding the importance of Ca^{2+} mobilization and subsequent Ca^{2+} entry in thrombus formation. In particular, this chapter considers the contribution of STIM1, the key regulatory protein in SOCE, and, of Orai1, a well-recognized SOCE channel, to the thrombotic process under conditions where coagulation is either blocked or promoted. Whereas a moderate elevation in cytosolic Ca^{2+} promotes integrin $\alpha\text{IIb}\beta_3$ activation, a potent Ca^{2+} rise causes PS exposure along with integrin closure. The regulation of $\alpha\text{IIb}\beta_3$ closure in PS-exposing platelets is the central theme of **chapter 7**. The experiments concentrate on the role of the Ca^{2+} -dependent protease calpain and of relevant protein kinases. In addition, the role of mitochondria via mPTP formation is considered. The general discussion of **chapter 8** describes the most important findings of this thesis in relation to the current literature.

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Chapter 2

Collagen-mimetic peptides mediate flow-dependent thrombus formation by high- or low-affinity binding of integrin $\alpha 2\beta 1$ and glycoprotein VI

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Abstract

Collagen acts as a potent surface for platelet adhesion and thrombus formation under conditions of blood flow. Studies using collagen-derived triple-helical peptides have identified the GXX'GER motif as an adhesive ligand for platelet integrin $\alpha 2\beta 1$, and (GPO)_n as a binding sequence for the signaling collagen receptor, glycoprotein VI (GPVI). The potency was investigated of triple-helical peptides, consisting of GXX'GER sequences within (GPO)_n or (GPP)_n motifs, to support flow-dependent thrombus formation. At high shear rate, immobilised peptides containing both the high affinity $\alpha 2\beta 1$ -binding motif GFOGER and the (GPO)_n motif supported platelet aggregation and procoagulant activity, even in the absence of von Willebrand factor (vWF). With peptides containing only one of these motifs, co-immobilised vWF was needed for thrombus formation. The (GPO)_n but not the (GPP)_n sequence induced GPVI-dependent platelet aggregation and procoagulant activity. Peptides with intermediate affinity (GLSGER, GMOGER) or low-affinity (GASGER, GAOGER) $\alpha 2\beta 1$ -binding motifs formed procoagulant thrombi only if both (GPO)_n and vWF were present. At low shear rate, immobilised peptides with high- or low-affinity $\alpha 2\beta 1$ -binding motifs mediated formation of thrombi with procoagulant platelets only in combination with (GPO)_n. In conclusion, triple-helical peptides with specific receptor-binding motifs mimic the properties of native collagen I in thrombus formation by binding to platelet collagen receptors. At high shear rate, either GPIb or high-affinity (but not low-affinity) GXX'GER mediates GPVI-dependent formation of procoagulant thrombi. By extension, high-affinity binding for $\alpha 2\beta 1$ can control the overall platelet-adhesive activity of native collagens.

Introduction

Platelet interaction with subendothelial collagen is a key initial step in thrombus formation following vessel wall damage. At an intermediate or high shear rates, the von Willebrand factor (vWF) receptor, glycoprotein (GP) Ib-IX-V, tethers platelets to vWF that is bound to collagen fibres (1,2). Subsequent stable platelet adhesion and activation on collagen involves two collagen receptors, the immunoglobulin-like receptor GPVI and integrin $\alpha 2\beta 1$. We and others have shown that thrombus formation on collagen/vWF depends on concerted interplay between these receptors, where GPIb in synergy with $\alpha 2\beta 1$ or GPVI mediates adhesion, while $\alpha 2\beta 1$ reinforces signaling through GPVI to cause platelet activation (3-6). Release of the autocrine mediators, ADP and thromboxane A₂, plays a key supportive role in the recruitment and capture of passing platelets. Together with GPVI, these mediators induce affinity changes of integrins $\alpha IIb\beta 3$ and $\alpha 2\beta 1$, which are required for stable interactions of platelets with fibrinogen and collagen, respectively, and hence, control thrombus stability under flow conditions (7-9). In addition, increases in cytosolic Ca²⁺ caused by signaling via GPVI triggers the platelet procoagulant response, by stimulating surface exposure of phosphatidylserine (PS). This reaction strongly potentiates local formation of thrombin and establishes coagulation (5,10).

The precise role of integrin $\alpha 2\beta 1$ in collagen-induced thrombus formation however is still under debate. There is evidence from adhesion experiments that activated $\alpha 2\beta 1$ can stimulate platelets by triggering signaling events similar to those of GPVI (11). On the other

hand, it is published that binding to $\alpha 2\beta 1$ passively enforces platelet activation via GPVI, and perhaps GPIb (3,7,12). A complicating factor in establishing the precise function of $\alpha 2\beta 1$ in platelet-collagen interaction is the recent discovery that type I and III collagens contain various integrin-binding motifs with very different affinity for the resting and activated conformations of platelet $\alpha 2\beta 1$ (13). Thus, heterogeneity in adhesive sites may explain why unstimulated platelets can adhere to collagen in the apparent absence of activated $\alpha 2\beta 1$.

Identification of the minimal binding sequences for $\alpha 2\beta 1$ and GPVI within the collagen triple helix is a challenging task in hemostasis and thrombosis research. For more than a decade, it has been recognised that GPVI binds to and is activated by $(\text{GPO})_n$ sequences (where O is hydroxyproline) (14,15). Indeed, a cross-linked form of triple-helical $(\text{GPO})_n$, known as collagen-related peptide (CRP), is widely used as a specific GPVI-activating platelet agonist. Recent work has shown that two or more GPO triplets are required to promote platelet aggregation through GPVI (16). For integrin $\alpha 2\beta 1$, several recognition motifs have been identified within the platelet-activating collagens, types I, III and IV. These include GXX'GER motifs, of which GFOGER is found in collagens I and IV, while GMOGER, GLOGER and GASGER are present in the collagen type I $\alpha 1$ chain; and further, GMOGER, GLSGER and GAOGER are expressed in collagen type III (13,17,18). These GXX'GER motifs appear to interact with the metal-ion dependent adhesion site (MIDAS) of the integrin $\alpha 2$ chain (19). Earlier studies have shown that peptides containing GXX'GER motifs bind with different affinities to integrin $\alpha 2\beta 1$, and can be used to model collagen- $\alpha 2\beta 1$ interactions (13,16).

Here, it was investigated how triple-helical GXX'GER peptides with high, intermediate or low affinity to $\alpha 2\beta 1$ support flow-dependent platelet adhesion and thrombus formation. The various $\alpha 2\beta 1$ -binding GXX'GER sequences were synthesised within a $(\text{GPO})_n$ backbone to provide synergy with GPVI activation, or within a $(\text{GPP})_n$ backbone as a GPVI-independent control. These peptides were immobilised to glass in the presence or absence of vWF to establish potential synergism with platelet GPIb interactions under high shear.

Experimental procedures

Materials

Fibrillar type I collagen (Horm) from equine tendon was purchased from Nycomed (Munich, Germany). Bovine type III collagen was from Sigma (St. Louis, MO, USA); the preparation was dialyzed overnight against phosphate-buffered saline (PBS; in mmol/l: KCl 2.7, KH_2PO_4 1.5, NaCl 100, Na_2HPO_4 7.3, pH 8.0), and allowed to polymerize by a 5-min incubation at 35°C before use. D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (PPACK) and calcein were from Calbiochem (La Jolla, CA, USA). Human vWF isolated from plasma was from LFB (Les Ulis, France). Lyophilised vWF protein was used at a final concentration of 0.35 g L^{-1} in Hepes buffer, pH 7.45 (136 mmol L^{-1} NaCl, 10 mmol L^{-1} Hepes, 2.7 mmol L^{-1} KCl and 2 mmol L^{-1} MgCl_2 , 1 g L^{-1} bovine serum albumin, and 1 g L^{-1} glucose). Unfractionated and low-molecular weight heparin came from Sigma. The GPVI-specific single-chain variable fragment (scFv) antibody 10B12 was selected from the Cambridge Antibody Technology (MedImmune Cambridge) phage display libraries, as described (20). Fab fragments of the blocking monoclonal antibodies (mAb) 6B4 against GPIb α and 15D7 against integrin $\alpha 2\beta 1$

were generated as before (9,21). FITC-labelled annexin A₅ was from Nexins Research (Hoeven, the Netherlands); FITC-labelled monoclonal antibody against human P-selectin (α -CD62 mAb) from WAK Chemie (Steinbach, Germany). Polyclonal rabbit anti-human vWF antibody was from Dako (Glostrup, Denmark); FITC-labelled goat anti-rabbit IgG (GAR-FITC) from Southern Biotech (Birmingham, AL, USA). Sources of other chemicals were as described (5).

Peptide synthesis

Collagen-like peptides were synthesised as C-terminal amides on TentaGel R-RAM resin in a Applied Biosystems Pioneer automated synthesizer, using Fmoc [N-(9-fluorenyl) methoxycarbonyl] chemistry (22,23). Peptide melting temperature and peptide cross-linking of purified peptides were assessed as described (16). Cross-linked CRP, i.e. triple-helical (GPO)₁₀, was synthesised as before (22). The α 2 β 1-binding peptides had the general structure, H-GPC(GPO)₃-GXX'GER-(GPO)₃GPC-NH₂ or H-GPC(GPP)₅-GXX'GER-(GPP)₅GPC-NH₂, where amino acids X and X' may differ between peptides. Triple-helical conformation was maintained by the GPO (GPVI-binding) or GPP (non-GPVI-binding) triplets, introduced at each end of the sequence. In this paper, the triple-helical peptides are indicated as GXX'GER-(GPO)_n and GXX'GER-(GPP)_n. Table 1 gives the full amino acid composition.

Preparation of adhesive surfaces

Pre-cleaned glass coverslips were coated with one of the collagen-mimicking peptides. For static platelet adhesion, peptides were coated using a solution of 0.1–1000 μ g mL⁻¹. For flow studies, coating was mostly with 5 μ L of a 1 mg mL⁻¹ solution applied on a surface area of 15 mm². In case of (co-)coating with vWF, mixtures were prepared containing 0.5 mg mL⁻¹ of peptide and/or vWF, and 5 μ L of this mixture was applied to an area of 15 mm². Coating was during 1 h in a humid chamber, after which the coverslips were rinsed twice with saline to remove the unbound peptide (vWF). Blocking was with Hepes buffer pH 7.45 containing 1 g L⁻¹ bovine serum albumin. Markers at the bottom side of the coverslips indicated the site of coating. In order to quantify the immobilisation of peptides, coverslips were coated with multiple spots of one peptide (0.5 mg mL⁻¹), as described above. The adhered peptide was extracted from the glass by repetitive treatment with 6 mol L⁻¹ guanidine in 100 mmol L⁻¹ sodium acetate buffer. The collected material was reduced with 3% β -mercaptoethanol and applied to reversed-phase HPLC. Peptides were eluted in a linear gradient of water/acetonitrile with 0.1% trifluoroacetic acid, and peaks were analysed by electrospray ionization quadrupole mass spectrometry (ESI-MS). Calibration with purified reduced peptide on HPLC was used to determine the amount of immobilized peptide on coverslip.

Blood collection and flow experiments

Blood was taken from healthy volunteers and collected into 0.1 vol saline containing 40 μ mol L⁻¹ PPACK, and supplemented hourly with 20 μ mol L⁻¹ PPACK. Blood was used within 3.5 h (37°C), in which time interval platelet count and platelet adhesion tendency remained unaltered (5). No formation of fibrin (clot) was detected in the blood, and D-dimer levels

remained below the threshold value of 200 ng mL^{-1} . Furthermore, extra addition of low-molecular weight heparin (fragmin, 5 unit mL^{-1}) did not influence the experimental outcome. Donors had not taken aspirin or related drugs for at least two weeks, and gave full informed consent.

Whole-blood flow experiments were performed, as described in detail elsewhere (5,7). Briefly, blood was placed in a syringe and perfused over a peptide-coated coverslip through a transparent, $50 \text{ }\mu\text{m}$ deep and 3 mm wide parallel-plate flow chamber, using a pulse-free pump at a (wall) shear rate of 1000 s^{-1} for 4 min or 150 s^{-1} for 6 min . Corresponding blood flow rates were 4.5 mL h^{-1} or 0.675 mL h^{-1} . Where indicated, the blood was pre-incubated for 15 min with scFv mAb 10B12 (blocking GPVI) or with Fab fragments of 6B4 (blocking GPIb) or 15D7 (blocking $\alpha 2 \beta 1$) mAb. Coverslips were post-stained with FITC-annexin A5 ($0.5 \text{ }\mu\text{g mL}^{-1}$) or FITC- α -CD62 mAb ($1.25 \text{ }\mu\text{g mL}^{-1}$) by a 4-min perfusion at the same flow rate with Hepes buffer pH 7.45, supplemented with 1 unit mL^{-1} heparin and 2 mmol L^{-1} CaCl_2 . This staining did not change the size of platelet aggregates on the coverslip. A Visitech imaging system (Sunderland, UK), equipped with two intensified, charged-coupled device cameras, was used to collect phase-contrast and fluorescence images (24). Confocal fluorescence images were generated as described (10).

Image analysis

Size of recorded phase-contrast image was $120 \times 120 \text{ }\mu\text{m}$; size of fluorescence images was $150 \times 150 \text{ }\mu\text{m}$. Surface area coverage was analysed with Image-Pro software (Media Cybernetics) and Quanticell software (Visitech), respectively.

Static platelet adhesion

Adhesion of washed platelets ($1.25 \times 10^8 \text{ mL}^{-1}$) to coated surfaces was determined colorimetrically (absorbance 405 nm), as described (25).

Statistical analysis

Differences were tested on significance with the Mann-Whitney U test using the statistical package for social sciences (SPSS 11.0, Chicago, IL, USA). Results are given as mean values $\pm \text{SEM}$, unless otherwise indicated.

Results

Efficacy of coating with triple-helical GXX'GER-(GPO)_n and GXX'GER-(GPP)_n peptides

Triple-helical collagen-mimetic peptides were synthesised with high-affinity (GFOGER, GLOGER), intermediate affinity (GLSGER, GMOGER) or low affinity (GASGER, GAOGER) binding to $\alpha 2 \beta 1$; these motifs were either within a GPVI-binding (GPO)_n backbone or a non-GPVI-binding (GPP)_n backbone (Table 1). These peptides were coated at various concentrations onto a glass surface in order to optimize the coating method. After wash of non-bound material, washed human platelets were added to determine the ability of the immobilised peptides to support static platelet adhesion (Fig. 1). In general, the adhesion was higher to (GPO)_n than to (GPP)_n peptides, and it decreased in the order of GFOGER > GLOGER >

GLSGER > GAOGER, such in agreement with the lower binding affinity for $\alpha 2\beta 1$. For all peptides, a coating concentration of $500 \mu\text{g mL}^{-1}$ was sufficient for optimal platelet adhesion.

Table 1. Properties of the synthesised triple-helical, collagen-mimetic GXX'GER peptides. Shown are the abbreviated hexapeptide sequences, incorporated into GPO or GPP triple helices; further the number of loci of these sequences in collagen types I, III and IV. IC₅₀ values are given of replacement of platelet binding to monomeric collagen (13).

Abbreviation	Full sequence	Collagen loci	IC ₅₀ (μM)
GFOGER-(GPO) _n	H-GPC(GPO) ₃ GFOGER(GPO) ₃ GPC-NH ₂	5	1.3
GFOGER-(GPP) _n	H-GPC(GPP) ₃ GFOGER(GPP) ₃ GPC-NH ₂		0.2
GLOGER-(GPO) _n	H-GPC(GPO) ₃ GLOGER(GPO) ₃ GPC-NH ₂	3	3.9
GLOGER-(GPP) _n	H-GPC(GPP) ₃ GLOGER(GPP) ₃ GPC-NH ₂		6.3
GLSGER-(GPO) _n	H-GPC(GPO) ₃ GLSGER(GPO) ₃ GPC-NH ₂	1	4.8
GLSGER-(GPP) _n	H-GPC(GPP) ₃ GLSGER(GPP) ₃ GPC-NH ₂		34.6
GMOGER-(GPO) _n	H-GPC(GPO) ₃ GMOGER(GPO) ₃ GPC-NH ₂	2	16.5
GMOGER-(GPP) _n	H-GPC(GPP) ₃ GMOGER(GPP) ₃ GPC-NH ₂		95.8
GASGER-(GPO) _n	H-GPC(GPO) ₃ GASGER(GPO) ₃ GPC-NH ₂	1	109
GASGER-(GPP) _n	H-GPC(GPP) ₃ GASGER(GPP) ₃ GPC-NH ₂		>200
GAOGER-(GPO) _n	H-GPC(GPO) ₃ GAOGER(GPO) ₃ GPC-NH ₂	1	153
GAOGER-(GPP) _n	H-GPC(GPP) ₃ GAOGER(GPP) ₃ GPC-NH ₂		>200

To determine the coating efficiency, glass coverslips containing multiple spots of two representative peptides, i.e. GLSGER-(GPO)_n and GAOGER-(GPP)_n (coating concentration $500 \mu\text{g mL}^{-1}$), were analysed for actually bound material. Extraction of the immobilized peptides required a treatment with chaotropic agent, 6 mol L^{-1} guanidine in acetate buffer. The extracted material was reduced with β -mercaptoethanol, and applied to reversed-phase HPLC. As shown in Fig. 2A, the eluted GLSGER-(GPO)_n appeared as a double peak at 25-26% acetonitrile, representing its reduced form (peak 1) and a β -mercaptoethanol adduct (peak 2). ESI-MS showed that the eluted peptide had a molecular mass of 2718.6 Da (Fig. 2A), which approximated the theoretical mono-isotopic mass (2717.2 Da) and the average mass (2719.0 Da). Using the same procedure, the recovered GAOGER-(GPP)_n peptide eluted in HPLC as one major peak at 34% acetonitrile, which was identified as the reduced peptide and β -mercaptoethanol adduct (Fig. 2B, peak 3). The observed molecular mass was 3643.8 Da, which fitted well between the theoretical mono-isotopic mass (3641.7 Da) and the average mass (3644.2 Da). The mass spectrum showed an additional m/z value

of 1240.8, representing the triple protonated state of the β -mercaptoethanol adduct of GAOGER-(GPP)_n.

Figure 1. Effect of coating density of collagen-mimetic peptides on platelet adhesion. Glass coverslips were coated with solutions of 0.1–1000 $\mu\text{g L}^{-1}$ of GXX'GER-(GPO)_n or GXX'GER-(GPP)_n peptide, as indicated. After wash of all unbound peptide, static adhesion of washed platelets (1 h) was determined colorimetrically (absorbance 405 nm). Data are means $\pm$ SEM (n=3).

By calibrating with pure reduced peptides, peak areas from the HPLC profiles allowed semi-quantitative determination of the recovery of the peptides from glass coverslips. For GLSGER-(GPO)_n and GAOGER-(GPP)_n the mean recovery was about 20% (n=7–10 spots), corresponding to $\sim 1.1 \mu\text{g/spot}$ or a density of $\sim 70 \text{ ng/mm}^2$. Since the peptides could only be eluted from coverslips by guanidine and not by acetate buffer alone, this pointed to a firm and high-density coating to the glass surface.

Coated triple-helical GFOGER-(GPO)_n peptide with vWF mediates thrombus formation at high shear rate

The coated triple-helical GXX'GER peptides were used to study platelet adhesion and thrombus formation under high-shear flow conditions, using a validated experimental setup (5,7,10). Human blood was anticoagulated with PPACK, to completely prevent fibrin formation, and was perfused over the various surfaces at a moderately high shear rate of 1000 s^{-1} . Coverslips coated with native collagen types I and III were used as reference surfaces. Flow over collagen type I and III resulted in rapid platelet adhesion and aggregate formation (Fig. 3A a, b). Staining for PS-exposing platelets (FITC-annexin A5) or for platelets expressing P-selectin (FITC anti-CD62 mAb) gave many single platelets which exposed PS and aggregates of P-selectin positive platelets. This heterogeneity in platelet activation in flow-dependent thrombus formation has been described elsewhere (26). In the absence of coating, no platelets adhered, as reported before (5).

Figure 2. Recovery and analysis of coated GLSGER-(GPO)_n and GAOGER-(GPP)_n peptides by HPLC and ESI-MS. Coated GLSGER-(GPO)_n (A) and GAOGER-(GPP)_n (B) peptides were extracted from glass coverslips, and analysed using HPLC (left panels) and ESI-MS (right panels). Coating was at 500 µg mL⁻¹, followed by wash. ESI-MS showed a double protonated state of reduced GLSGER-(GPO)_n (1) and a triple protonated state of reduced GAOGER-(GPP)_n (3), correlating well with the theoretical peptide masses of 2718.2 and 3641.7 Da, respectively. When calibrated against pure reduced peptides by HPLC, coating efficiencies on coverslips were determined at about 20% for GLSGER-(GPO)_n and GAOGER-(GPP)_n.

As vWF is required for collagen-dependent platelet adhesion under shear (27), coverslips were also coated with vWF, alone or in combination with triple-helical peptides. Staining of the coated coverslips with anti-vWF mAb showed 5-fold (co-coating with peptide and vWF) or 10-fold (coating with vWF alone) increased signal intensities in comparison to uncoated coverslips. This points to adequate immobilisation of vWF on the glass surface. Perfusion of blood (1000 s⁻¹) over coverslips coated with only vWF resulted in adhesion of mostly single platelets with limited spreading (Fig. 3A c), such in agreement with published data (27,28). In contrast, using GFOGER-(GPO)_n as coating, platelets stably adhered and formed compact aggregates (Fig. 3A d). Co-coating of GFOGER-(GPO)_n and vWF resulted in increased adhesion of platelets, which again formed aggregates (Fig. 3A e). Staining of activated platelets on coverslips with GFOGER-(GPO)_n ± vWF showed clusters of single PS-exposing platelets and aggregates of P-selectin positive platelets (Fig. 3A c-e).

The adhesion of platelets on GFOGER-(GPO)_n was linear in time, up to a period of 4 min, which is consistent with previous observations for collagen type I surface (7). At this time point, confocal image stacks showed that thrombi of calcein-labelled platelets had an average volume of 140 and 110 µm³ per 1000 µm³ on coated GFOGER-(GPO)_n and collagen I, respectively.

Quantitative analysis of platelet deposition was performed at the 4-min end point, by recording phase-contrast and fluorescence images from the coated surfaces. Single platelets on collagen, but not on vWF alone, did not bind FITC-annexin A5 (Fig. 3B). The platelet aggregates formed on immobilised GFOGER-(GPO)_n with(out) vWF occupied a surface area of 23–27% and were somewhat reduced in PS exposure in comparison to platelets on collagen. The activated state of platelets on coverslips coated with GFOGER-(GPO)_n/vWF was confirmed by measuring single-cell Ca^{2+} fluxes, which showed strong and prolonged increases in cytosolic Ca^{2+} , similar to those in platelets on type I collagen (I. Munnix, unpublished data, 2008).

Figure 3. Flow-induced thrombus formation on collagen or coated GFOGER-(GPO)_n in the presence or absence of vWF. Coverslips were coated with collagen type I (a), type III (b), vWF (c), GFOGER-(GPO)_n alone (d) or with vWF (e). Blood was flowed over these coatings for 4 min at 1000 s⁻¹, followed by stain with FITC-annexin A5 or FITC-α-CD62 mAb. (A) Representative phase-contrast and fluorescence images after perfusion. (B) Platelet deposition and PS exposure. (C) Effects of inhibiting antibodies: scFv 10B12 to block GPVI (α-GPVI, 100 μg mL⁻¹), 15D7 mAb to block α2β1 (α-α2β1, 20 μg mL⁻¹) or 6B4 to block GPIb (α-GPIb, 20 μg mL⁻¹). Bars indicate coverage of all platelets (white) and annexin A5-binding platelets (black). Mean ± SEM (n=6–8), * p<0.05 compared to vehicle control.

To dissect specific involvement of the collagen and vWF receptors in platelet adhesion to the coated peptide surfaces, blood was preincubated with maximally effective concentrations of blocking mAbs against GPVI, $\alpha_2\beta_1$ or GPIb. Inhibition of GPVI with scFv 10B12 (5) permitted stable adherence of mostly single platelets to coated GFOGER-(GPO)_n ± vWF, but large platelet aggregates and PS-exposing platelets were no longer present (Fig. 3C). Blocking of $\alpha_2\beta_1$ integrin by 15D7 mAb (9) markedly reduced the area coverage and PS exposure of platelets on GFOGER-(GPO)_n ± vWF. When GPIb α was inhibited by 6B4 mAb (21), area coverage of platelets again markedly decreased on GFOGER-(GPO)_n/vWF, and to a lesser degree on GFOGER-(GPO)_n alone. Together, these results indicate that immobilised triple-helical GFOGER-(GPO)_n peptide in combination with vWF effectively mimics native type I collagen in mediating $\alpha_2\beta_1$ -, GPVI- and GPIb-dependent platelet adhesion and aggregation under flow.

Coated triple-helical peptides with either GFOGER or (GPO)_n sequences require vWF for full thrombus formation at high shear rate

To distinguish between the contribution of the $\alpha_2\beta_1$ - and GPVI-binding motifs of GFOGER-(GPO)_n, homologous peptides were used that contained only one of these motifs. Coverslips were coated with CRP, i.e. the classical triple-helical (GPO)_n peptide which activates platelets exclusively via GPVI. At 1000 s⁻¹ platelets did not adhere to coated CRP, similarly as to vWF (Fig. 4A a-b). In contrast, on coverslips coated with CRP plus vWF, platelets adhered well and formed aggregates, although these were smaller in size than those on GFOGER-(GPO)_n/vWF surface (Fig. 4A c). Staining with FITC-annexin A5 showed many PS-exposing platelets on CRP/vWF, while again platelet aggregates were P-selectin positive (Fig. 4A, B).

Flow experiments performed at a higher shear rate of 1700 s⁻¹, representative for that in middle-sized arteries, indicated formation of single platelets and micro-aggregates on coated vWF alone (area coverage <2%). Further, increase in shear rate from 1000 to 1700 s⁻¹ increased platelet deposition and aggregation on coated CRP/vWF (area coverage from 19.2±3.9 to 47.6±1.1%) and on coated GFOGER-(GPO)_n/vWF (area coverage from 26.5±3.4 to 47.3±1.2%), thus underlining the cooperative activity of poly-GPO peptides with vWF.

Figure 4. Flow-induced thrombus formation on coated CRP in the presence or absence of vWF. Blood was flowed for 4-min at 1000 s^{-1} using coverslips coated with vWF (a), CRP (b) or combination of both (c). Staining was with FITC-annexin A5 or FITC- α -CD62 mAb. (A) Representative phase-contrast and fluorescence microscopic images after perfusion. (B) Surface area coverage of all platelets (white bars) and annexin A5-binding platelets (black bars) for each surface type (n=6-8).

Complementary experiments were performed using coated GFOGER-(GPP)_n peptide in which the GPVI-activating GPO stretches were replaced by inactive GPP triplets. At high shear, platelets did adhere to GFOGER-(GPP)_n but formed only small aggregates (Fig. 5A a). However, large aggregates were formed on coverslips co-coated with GFOGER-(GPP)_n/vWF (Fig. 5A b). These aggregates were typically loose in structure and disintegrated upon prolonged rinse with Hepes buffer. Platelets which adhered to GFOGER-(GPP)_n ± vWF showed little, but still detectable staining with FITC-annexin A5 (Fig. 5B).

On GFOGER-(GPP)_n/vWF surface, blocking of GPVI with scFv 10B12 substantially reduced platelet deposition, aggregate formation and, interestingly, PS exposure (Fig. 5A c). Blocking of $\alpha 2\beta 1$ (15D7 mAb) had similar effects (Fig. 5A, d), but blocking of GPIb (6B4 mAb) did not affect PS exposure (Fig. 5A, B). Furthermore, GPVI blocking reduced numbers of annexin A5-binding platelets on GFOGER-(GPP)_n surface. Together, this suggests residual involvement of GPVI next to $\alpha 2\beta 1$ in platelet aggregation and PS exposure on coated GFOGER-(GPP)_n.

Figure 5. Flow-induced thrombus formation on GFOGER-(GPP)_n surfaces in the presence or absence of vWF. Blood was flowed for 4-min at 1000 s⁻¹ over coverslips coated with GFOGER-(GPP)_n alone or in combination with vWF. (A) Representative phase-contrast and fluorescence images after flow in the presence of (a-b) vehicle, (c) 100 μg mL⁻¹ scFv 10B12 to block GPVI (α-GPVI), or (d) 20 μg mL⁻¹ 15D7 mAb to block α2β1 (α-α2β1). (B) Effect of the blocking antibodies including 6B4 (α-GPIIb) (20 μg mL⁻¹) on platelet deposition. Bars show percentages of surface area coverage of all platelets (white) and annexin A5-binding platelets (black) (n=3-5, * p<0.05 compared to vehicle control).

Coated lower-α2β1 affinity GXX'GER-(GPO)_n and GXX'GER-(GPP)_n peptides differ in potency to induce thrombus formation at high shear rate

To determine how modulation of integrin α2β1 binding affects the adhesive properties of the triple-helical peptides, lower affinity homologues of GFOGER-(GPO)_n and GFOGER-(GPP)_n were used. As indicated in Table 1, triple-helical peptides containing a GLOGER, GLSGER or GMOGER sequence, have a reduced, moderate affinity for α2β1. Furthermore, peptides with a GASGER or GAOGER sequence are low in affinity for the integrin (IC₅₀ > 100 μM) (13).

Unlike GFOGER-(GPO)_n and GFOGER-(GPP)_n, coated peptides with low-affinity GASGER or GAOGER motifs, in either a (GPO)_n or a (GPP)_n backbone, were unable to support platelet adhesion, aggregation or PS exposure under shear conditions (Fig. 6A, B). However, when coated together with vWF, the GASGER and GAOGER peptides supported

platelet adhesion particularly when incorporated into (GPO)_n sequences (Fig. 6C). Staining with FITC-annexin A₅ indicated significant PS exposure when both vWF and (GPO)_n were present. Together, these results suggest that peptides with low affinity $\alpha 2\beta 1$ -binding motifs (GASGER and GAOGER) are highly platelet adhesive only in combination with binding sites for GPVI (poly-GPO) and GPIb (vWF).

Figure 6. Flow-induced thrombus formation on surfaces of various $\alpha 2\beta 1$ -binding triple helical peptides. Blood was flowed over coverslips with coated GXX'GER-(GPO)_n or GXX'GER-(GPP)_n and vWF, as indicated. (A) Representative phase-contrast and fluorescence images are given after perfusion over indicated coatings for 4 min at 1000 s⁻¹. (B) Surface area coverage of all platelets (white bars) and PS-exposing platelets (black bars) with each type of surface. (C) Effect of co-coated vWF on surface area coverage of all platelets in the absence (white bars) or presence (crossed bars) of vWF (n=3-7).

Coated GXX'GER-(GPO)_n and GXX'GER-(GPP)_n peptides differ in platelet adhesion and aggregation at low shear rate

The platelet-adhesive potency of the triple-helical peptides was also studied under low shear conditions, where vWF and GPIb do not play a role. On coverslips with GFOGER-(GPO)_n few large aggregates with annexin A₅-binding platelets were formed (Fig. 7A). In contrast, on GFOGER-(GPP)_n only single platelets and small aggregates were formed, although some platelets stained with annexin A₅. Coated CRP was moderately effective. For the other (GPO)_n peptides, the potency of platelet adhesion decreased in the order of GFOGER, GLOGER > GMOGER, GAOGER, GLSGER > GASGER (Fig. 7B). Annexin A₅ staining also followed this order. Interestingly, the low-affinity $\alpha 2\beta 1$ binding peptide GAOGER-(GPO)_n supported platelet adhesion/aggregation and PS exposure better than its homologue GASGER-(GPO)_n. For the (GPP)_n containing peptides, adhesion was in the order of predicted $\alpha 2\beta 1$ affinities: GFOGER > GLOGER > GMOGER, GLSGER, GASGER, GAOGER

(Fig. 7B). Annexin A5 staining was negligible. Together, the data show that, at low shear rate, only the combination of GFOGER and (GPO)_n –but not (GPP)_n– motifs leads to formation of aggregates with procoagulant platelets.

Figure 7. Flow-induced thrombus formation on surfaces of various $\alpha_2\beta_1$ -binding triple helical peptides at low shear rate. Blood was flowed for 6 min at 150 s^{-1} over indicated coatings. (A) Representative phase-contrast and fluorescence images of coverslips coated with (a) GFOGER-(GPO)_n, (b) GFOGER-(GPP)_n, or (c) CRP. (B) Representative phase-contrast and fluorescence images of coverslips coated with GXX'GER-(GPO)_n or GXX'GER-(GPP)_n peptides. Bars give surface area coverage of all platelets (white) and annexin A5 binding platelets (black) per type of surface ($n=3-5$).

Discussion

The presented findings indicate that collagen-mimetic, triple-helical peptides, immobilised to glass, effectively and efficiently can take over the functions of native collagens in mediating platelet adhesion and activation via the receptors GPVI and $\alpha_2\beta_1$. At high shear rate and with vWF present, peptides with the GPVI-binding (GPO)_n sequence or the high-affinity $\alpha_2\beta_1$ -binding GFOGER motif caused stable adhesion, activation and aggregation of platelets. Adhesion and aggregation were sensitive to blockade of the vWF receptor GPIb. In the absence of vWF, the combination of GFOGER and (GPO)_n sequences was required for platelet adhesion and activation. In general, on (GPO)_n containing surfaces, including CRP, platelet activation was detected as P-selectin expression and exposure of PS, measured by binding of FITC-annexin A5. The GFOGER sequence has been identified as the major high-affinity $\alpha_2\beta_1$ -binding stretch in type I collagen (13) but not type III collagen (29), which given the present results implicates that it is readily exposed for platelet adhesion in native type I collagen fibers.

The integrin-binding peptides used in this study consisted of one copy of high-affinity (GF/LOGGER), intermediate affinity (GLO/SGER) or low-affinity (GAO/SGER) $\alpha 2\beta 1$ recognition site within the backbone of repeated GPP or GPO sequences to provide triple-helical conformation (13,30). The GER part functions to recruit platelets via $\alpha 2\beta 1$, and the GPO part acts to activate GPVI. The peptides were synthesised based on the assumption that the integrin-binding motif spans a 6 amino acid sequence, as demonstrated in the co-crystal model of $\alpha 2$ I-domain and GFOGER (19). Surprisingly, when immobilised on a surface, especially the high-affinity GFOGER peptides are active in trapping platelets and forming aggregates under flow. At high-shear conditions they synergise with GPIb-vWF interactions. In addition, combination of the $\alpha 2\beta 1$ -binding domain and (GPO)_n sequences markedly increases platelet adhesion and activation via GPVI under flow.

When comparing the various triple-helical peptides, the most potent surface is provided by GFOGER-(GPO)_n, which mediates platelet adhesion and aggregate formation via $\alpha 2\beta 1$ as well as GPVI. Platelet adhesion to this peptide leads to full GPVI-induced platelet responses, including secretion (P-selectin exposure) and procoagulant activity (PS exposure), both of which rely on stimulation of the LAT/phospholipase C $\gamma 2$ activation pathway (7,26).

Co-coating with vWF further stimulated adhesion and increased platelet accumulation into aggregates. Experiments with blocking antibodies confirmed the involvement of $\alpha 2\beta 1$, GPVI and GPIb in platelet adhesion to the peptide surfaces, but also supported the concept that interplay between these adhesive receptors is required for stable platelet adhesion and activation under high shear conditions (3-6). An interesting observation is that the previously observed heterogeneity of platelet aggregates, containing discrete populations of aggregated (P-selectin positive) and PS-exposing platelets (26) is not restricted to thrombus formation on native collagen fibres, but also extends to immobilised GFOGER-(GPO)_n as, in aggregates, only platelets which were in direct contact with the coated peptide, bound annexin A5.

At high shear rate, CRP, which contains just (GPO)_n sequences, mediated platelet adhesion and thrombus formation only when co-immobilised with vWF. The same was true for (GPO)_n-containing peptides with low-affinity $\alpha 2\beta 1$ binding motifs, GAOGER and GASGER. These results confirm and extend earlier evidence that combined adhesion via GPIb and GPVI is sufficient to induce thrombus formation, not only on native collagen (5), but also on synthetic peptides (this chapter).

An unexpected finding was that the $\alpha 2\beta 1$ -binding GFOGER-(GPP)_n peptide, lacking GPVI-activating (GPO)_n stretches, caused low, though readily-detectable platelet aggregation and PS exposure. This was clear in both high- and low-shear experiments. This is in good agreement with the earlier determined high binding affinity to $\alpha 2\beta 1$ on platelets (Table 1). Since these platelet responses are suppressed by blocking GPVI with scFv 10B12, it was concluded that the GFOGER-(GPP)_n surface has limited GPVI-dependent activity. This is not without precedence, as others have shown that static adhesion of platelets to the GFOGER peptide results in tyrosine phosphorylation of proteins of the GPVI-FcR γ -chain cascade, including Src, Syk, SLP76 and phospholipase C $\gamma 2$ (11). The authors suggested that

$\alpha 2\beta 1$ engagement via outside-in signaling leads to a similar activation pathway as GPVI ligation. Effects of GPVI-blocking agents were not determined in this study, but it was demonstrated that platelets deficient in GPVI/FcR γ spread normally on GFOGER-(GPP) $_n$, while phospholipase C $\gamma 2$ deficient platelets showed impaired spreading. It is possible that the GFOGER peptide signals differently under static and flow conditions. However, one possibility is that $\alpha 2\beta 1$ engagement with GFOGER does lead to activation (clustering?) of GPVI, as these receptors have been shown to interact during collagen activation of platelets (3). This needs further investigation.

The results of low-shear experiments further point to marked synergism between GPVI (GPO) and high-affinity $\alpha 2\beta 1$ binding (GFOGER) in adhesion and aggregation, but not between GPVI and low-affinity $\alpha 2\beta 1$ binding. In other words, the low-affinity $\alpha 2\beta 1$ GAO/SGER peptides were poorly adhesive within a (GPO) $_n$ backbone, and not at all active if presented within a (GPP) $_n$ backbone unless vWF was also present. It is known that type I and III collagens contain more low-affinity than high-affinity $\alpha 2\beta 1$ binding sites (13,29). Hence, the current study with model peptides would predict that, in platelet interaction with native collagens, especially the high-affinity integrin binding sites contribute to shear-dependent thrombus formation.

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Chapter 3

Non-redundant roles of phosphoinositide 3-kinase
isoforms α and β in glycoprotein VI-induced platelet
signaling and thrombus formation

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Abstract

Platelets are activated by adhesion to vascular collagen via the immunoglobulin receptor, glycoprotein VI (GPVI). This causes potent signaling towards activation of phospholipase Cy_2 , which bears similarity to the signaling pathway evoked by T- and B-cell receptors. Phosphoinositide 3-kinase (PI3K) plays an important role in collagen-induced platelet activation, as this activity modulates the autocrine effects of secreted ADP. Here, we identified the PI3K isoforms directly downstream of GPVI in human and mouse platelets, and determined their role in GPVI-dependent thrombus formation. The targeting of platelet PI3K α or β strongly and selectively suppressed GPVI-induced Ca^{2+} mobilization and inositol 1,4,5-triphosphate production, thus demonstrating enhancement of phospholipase Cy_2 by PI3K α/β . That PI3K α and β have a non-redundant function in GPVI-induced platelet activation and thrombus formation was concluded from measurements of: (i) serine phosphorylation of Akt, (ii) dense granule secretion, (iii) intracellular Ca^{2+} rises and surface expression of phosphatidylserine under flow and, (iv) thrombus formation, under conditions where PI3K α/β was blocked or p85 α was deficient. In contrast, GPVI-induced platelet activation was insensitive to inhibition or deficiency of PI3K δ or γ . Furthermore, PI3K α/β but not PI3K γ , contributed to GPVI-induced Rap1b activation and, surprisingly, also to Rap1b-independent platelet activation via GPVI. Together, these findings demonstrate that both PI3K α and β isoforms are required for full GPVI-dependent platelet Ca^{2+} signaling and thrombus formation, partly independently of Rap1b. This provides a new mechanistic explanation for the anti-thrombotic effect of PI3K inhibition, and makes PI3K α an interesting new target for antiplatelet therapy.

Introduction

Exposed collagen in a damaged vessel wall activates platelets via their immunoglobulin family receptor, glycoprotein VI (GPVI), by using a complex signal transduction pathway, which is reminiscent to the pathway employed by immune receptors in T- and B-cells (1,2). In platelets, tyrosine phosphorylation of the Fc receptor γ -chain, linked to GPVI via Src family kinases, leads to a cascade of protein phosphorylation events, cumulating in the activation of phospholipase Cy_2 (PLC γ_2). This key effector enzyme triggers many downstream events including: production of inositol 1,4,5-trisphosphate (InsP $_3$); mobilization of cytosolic Ca^{2+} ; activation of integrin $\alpha IIb\beta_3$; secretion of platelet granules loaded with autocrine stimulating agents (ADP, ATP); and exposure of negatively charged phosphatidylserine (PS) at the platelet surface to ensure coagulation (1,3,4). All these responses are potently triggered by GPVI ligands which, besides collagen, include collagen-related peptides and the snake venom convulxin (5-7).

One of the GPVI-induced signaling events contributing to PLC γ_2 activation is activation of the protein/lipid kinase, phosphoinositide 3-kinase (PI3K) in both human and mouse platelets (8-11). Evidence for this role came from the finding that in platelets stimulated with GPVI agonists, the p85 α regulatory subunit of PI3K co-precipitates with the Fc receptor γ -chain and the LAT adaptor protein (8). The p85 α subunit pulls p110 catalytic subunits to the membrane, where they catalyze the formation of 3-phosphorylated inositol phospholipids, primarily the phosphoinositide 3,4,5-trisphosphate [PI(3,4,5)P $_3$] (10).

Currently, there is evidence that individual class I PI3K isoforms, which are distinguished according to their catalytic subunits, have specific cellular and physiological functions. For in-

stance, the p110 α isoform (PI3K α) has been implicated in oncogenesis, and isoform-selective PI3K α inhibitors can reduce tumor formation (12). The p110 γ isoform (PI3K γ) is involved in innate immunity and various inflammatory diseases (13), while p110 δ has a more important role in adaptive immunity, e.g. in T- and B-cells (14). Human and mouse platelets contain four different PI3K isoforms, among which the class IA catalytic subunits, p110 α , β and δ (PI3K α , β and δ) and the class IB catalytic subunit, p110 γ (PI3K γ) (15-17). For class IA, the corresponding regulatory subunits are p85 α / β p55 α / γ and p50 α , while for class IB the regulatory subunit is p101 γ . Structural studies in other cells have indicated that the regulatory class IA subunits, particularly p85 α , can interact with tyrosine kinase-linked receptors via the SH2 domains (18). In contrast, class IB isoforms may rather interact with G-protein coupled receptors (16). This concept was recently challenged by the observation that, in platelets, both PI3K β and γ are activated via the P2Y₁₂ receptor for ADP, which is coupled to G_i, and that both isoforms contribute to integrin α IIb β ₃ activation and platelet aggregation (17,19-21). Hence, it is clear that PI3K isoforms can be activated by other platelet receptors than only GPVI.

To date, it is debated which of the PI3K isoforms become directly activated by GPVI signaling, and which are activated indirectly, e.g. following ADP receptor stimulation. Also unclear is which are the downstream events mediated by the various isoforms. Reasons for this lack of clarity include: (i) the large contribution to GPVI-induced responses of secondary, autocrine stimulators particularly ADP and thromboxane (1,17,22); (ii) the proposed stimulation by PI3K and its product PIP₃ to Ca²⁺ entry rather than to PLC activity (23,24); (iii) the limited knowledge on the effector targets of PI3K and PIP₃ in platelets, of which only PDK and Akt are well studied (21,25); (iv) the observation that p110 δ is not a major isoform implicated in GPVI-induced platelet activation (26); (v) the limited availability of mice deficient in PI3K subunits.

One possible, poorly explored target of PI3K in platelets is the small GTPase Rap1b, which is highly expressed in these cells, and is considered to play a key role in the activation process particularly towards α IIb β ₃ activation. Platelet agonists such as ADP and thrombin produce the active GTP-bound form of Rap1b, partly in a PI3K-dependent manner (27,28). Recent findings suggest that PI3K β is the main isoform responsible for the ADP-induced activation of Rap1b and Akt, while PI3K γ contributes to α IIb β ₃ activation by a separate pathway (21). However, whether and how Rap1b is activated by GPVI is unresolved, as is the role of different PI3K isoforms herein.

In the present paper, we studied the identity of the PI3K isoforms downstream of GPVI, and determined their role in platelet activation and thrombus formation. To discriminate between direct and indirect GPVI-induced effects, the cells were stimulated in the presence of autocrine stimulation inhibitors, blocking the signaling contributions of both ADP and thromboxane. For the studies, we combined a pharmacological approach using a panel of isoform-specific PI3K inhibitors with experiments using mice deficient in distinct PI3K catalytic or regulatory subunits. We find that PI3K α and β as key mediators of GPVI-induced thrombus formation, and show that these isoforms affect the activity of both PLC γ ₂ and Rap1b.

Experimental procedures

Mouse strains

Mice deficient in the p85 α regulatory PI3K subunit had a C57BL/6 genetic background (11,29). Mice deficient in p110 γ or p110 δ (17) or deficient in Rap1b (30) were from sources previously described. All knock-out mice had normal platelet counts. Wildtype mice were used of the same background and same breeding program. Animal experiments were approved by the local animal experimental committees.

Materials

Fura-2 and Fluo-3 acetoxymethyl esters, Pluronic F-127 and Oregon green 488-labeled fibrinogen (OG-fibrinogen) were from Molecular Probes; OG-annexin A5 was from Nexins Research; FITC-labeled anti-human CD62 monoclonal antibody (mAb) was from Sanquin. Antibodies against Akt were from Cell Signaling Technology; anti-Rap1 antibody was from BD Biosciences. LY294002 and H-Phe-Pro-Arg chloromethyl ketone (PPACK) were from Calbiochem. Wortmannin, apyrase (grade V), α -thrombin and MRS-2179, a P2Y1 antagonist were from Sigma. Inhibitors of PI3K isoforms (Suppl. Table 1) and control substance PIK-112 were kind gifts from the Baker Heart Research Institute, and synthesized as described (17,31). Cangrelor (AR-C69931MX), a P2Y12 antagonist, was kindly provided by The Medicines Company. Other reagents were from sources indicated before (32,33).

Blood collection and platelet isolation

For platelet isolation, blood was collected into one-sixth volume of acid-citrate-dextrose anti-coagulant (85 mM sodium citrate, 78 mM citric acid, 11 mM D-glucose). Donors gave full informed consent according to the Helsinki declaration, and had not taken medication for 2 weeks. Platelet-rich plasma (PRP) and washed platelets were obtained by centrifugation, as described for human (7) and mouse (32,33) platelets. Washed human platelets were resuspended in Hepes buffer pH 7.45 (137 mM NaCl, 10 mM Hepes, 2.7 mM KCl, 2 mM MgSO₄, 0.42 mM D-glucose, 0.2 U/ml apyrase and 0.1% bovine serum albumin, pH 7.45). Mouse platelets were resuspended in a modified Hepes buffer (33). Platelets were counted with a Coulter counter. For whole-blood flow experiments, human blood was collected into 40 μ M PPACK (34), while mouse blood was collected into 40 μ M PPACK plus 5 U/ml heparin (32).

Intracellular Ca²⁺ measurement in platelet suspensions

Human PRP (2×10^8 platelets/ml) was loaded with 2.5 μ M Fura-2 acetoxymethyl ester in the presence of aspirin (100 μ M) and apyrase (0.2 U ADPase/ml) at 37°C for 45 min (35). The loaded platelets were resuspended in Hepes buffer pH 7.45 (1×10^8 /ml), and were used within 90 min. Before addition of agonist, the cells were preincubated with apyrase (0.1 U/ml) and ADP receptor blockers (40 μ M MRS-2179 for P2Y1 and 10 μ M ARC-69931MX for P2Y12) to inhibit auto-crine stimulation. In some experiments, apyrase was added at a high concentration (0.6 U/ml). Platelet preincubation with indicated PI3K inhibitors or Me2SO vehicle was for 10 min (37°C). Calcium responses were recorded under stirring with an SLM-Aminco or a Cairn Research spectrofluorometer, at alternate excitation wavelengths of 340 and 380 nm (37°C). The 340/380-nm ratio values were converted into nanomolar concentrations of [Ca²⁺]_i, as described (35). Sepa-

rate calibrations were done for incubations containing colored PI3K inhibitors. Rises in $[Ca^{2+}]_i$ were expressed as 5 min-time integrals, to quantify prolonged Ca^{2+} signaling effects (36).

Platelet aggregation and flow cytometry

Human PRP was pretreated with aspirin, and platelets were washed. The washed platelets were preincubated for 10 min with autocrine stimulation inhibitors (see above) and PI3K blockers or Me2SO vehicle. After 10 min of activation, samples were analyzed by flow cytometry for P-selectin expression using FITC-anti-CD62 mAb (1:100), or for $\alpha IIb\beta_3$ activation using FITC-labeled PAC1 mAb (37).

Washed mouse platelets were prepared from PRP (21), and preincubated with a cocktail of autocrine stimulation inhibitors (100 μ M MRS-2179, 10 μ M AR-C69931MX and 10 μ M indomethacin). Platelet aggregation was measured, as described (21). OG488-fibrinogen binding to the platelets was assessed by flow cytometry (33).

InsP measurement

Accumulation of InsP1 due to InsP3 production was measured with a IP-One Elisa kit from Cis-Bio, determining InsP1 levels. Aspirin-treated platelets (1×10^8 /ml) were preincubated with ADP receptor blockers, LiCl (1 mM), and Me2SO vehicle or PI3K blocker. After stimulation for 5-10 min with convulxin or thrombin, the platelets were lysed. Cell lysates were incubated with InsP1-HRP conjugate and anti-InsP1 mAb, according to the manufacturer's instructions.

Activation of Akt and Rap1 by western blotting

Akt activation was measured by Western blot analysis of platelet lysates. A polyclonal anti-phosphoserine-473 Akt Ab was used to detect active Akt, and a polyclonal anti-Akt Ab to determine total Akt (19). Activation of Rap1 was measured in platelet lysates by selective precipitation of GTP-bound Rap1 using GST-RalGDS bound to glutathione-Sepharose (27). Western blotting was performed with anti-Rap1 Ab (250 ng/ml) and a sheep anti-mouse HRP-coupled secondary Ab (1:5000).

Thrombus formation and procoagulant activity under flow

Perfusion of PPACK-anticoagulated human blood (34) and of PPACK/heparin-anticoagulated mouse blood (32) was done in the absence of coagulation as described. Blood samples were preincubated with PI3K inhibitor or Me2SO vehicle for 10 min. Blood was flowed through a transparent, parallel-plate flow chamber, containing a collagen-coated coverslip, at defined shear rate for 4 min. The thrombi in flow chambers were stained by rinse with Hepes buffer pH 7.45, supplemented with 1 U/ml heparin, 2 mM $CaCl_2$ and OG488-annexin A5 (1 μ g/ml). Microscopic images were recorded in real-time using a Visitech imaging system, equipped with two intensified CCD cameras (34). Bright-field contrast and fluorescence digital images were taken from >10 randomly chosen fields. ImagePro software (Media Cybernetics) was used to quantify platelet deposition (38).

Intracellular Ca^{2+} measurement under flow

Human PRP ($2 \times 10^8/\text{ml}$) was incubated with $7 \mu\text{M}$ Fluo-3 acetoxymethyl ester for 45 min at 20°C under gentle rotation. Washed mouse platelets ($2 \times 10^8/\text{ml}$) were incubated with $5 \mu\text{M}$ Fluo-3 acetoxymethyl ester plus 0.2 mg/ml Pluronic F-127. The dye-loaded cells were added to autologous blood (10% labeled platelets), and used for flow experiments within 1 h. Fluorescence images were recorded during high-speed (5 Hz) perfusion of blood over a collagen surface (39). Off-line, regions-of-interest representing 1 adhered cell were analyzed for fluorescence changes. Raw fluorescence data were converted into F/Fo values by pseudo-ratio analysis, and then into nanomolar concentrations of $[\text{Ca}^{2+}]_i$ (40). For quantification, traces from individual cells were superimposed, so that frame numbers of initial $[\text{Ca}^{2+}]_i$ rises coincided.

Statistics

Differences between groups were tested with a Mann-Whitney U test or ANOVA. Effects of inhibitors were tested with a Student's paired t test. The statistical package for social sciences was used (SPSS 15.0).

Results

Prominent roles of $\text{PI3K}\alpha$ and β isoforms in GPVI-induced Ca^{2+} responses

Although PI3K activity has been implicated in GPVI-mediated platelet activation, it is unclear whether its role is direct or indirect, e.g. via autocrine ADP release and P2Y_{12} signaling. Another proposal is that PI3K may specifically stimulate GPVI-induced Ca^{2+} entry rather than mobilization of Ca^{2+} from internal stores (23). To investigate this further, human Fura-2-loaded platelets were activated with GPVI agonist under conditions where indirect effects of autocrine stimulators (thromboxane A_2 and ADP) were prevented with aspirin and a high concentration of ADP-degrading apyrase (0.6 U/ml). When platelets were stimulated with CRP in the presence of extracellular EGTA (where Ca^{2+} is only mobilized from internal stores), the general PI3K inhibitor wortmannin ($0.1 \mu\text{M}$) caused ~60% suppression of the Ca^{2+} signal (Fig. 1A). In the presence of extracellular CaCl_2 (where Ca^{2+} entry takes place in addition), wortmannin had a similar inhibitory effect on the Ca^{2+} signal. The treatment of platelets with another general PI3K inhibitor, LY-294002 ($25 \mu\text{M}$), was as effective as wortmannin. Furthermore, similar effects were obtained with both inhibitors, when platelets were stimulated with the GPVI ligand, convulxin (Fig. 1B). To directly determine the inhibitor effect on Ca^{2+} entry, platelets were first stimulated with GPVI ligand causing Ca^{2+} mobilization, after which CaCl_2 was added and Ca^{2+} entry was allowed. Strikingly, wortmannin suppressed both parts of the Ca^{2+} signal at similar extent (Fig. 1C). A control experiment indicated that blocking anti-GPIb antibodies did not affect the convulxin-induced Ca^{2+} response (not shown), thus excluding involvement of GPIb-induced Ca^{2+} signal generation with this agonist.

Interestingly, treatment of the platelets with wortmannin or LY-294002 caused only small inhibition of the thrombin-evoked Ca^{2+} responses in both EGTA and CaCl_2 medium (Fig. 1B). Together, these data suggest that PI3K activity primarily enhances $\text{PLC}\gamma_2$ -evoked Ca^{2+} mobilization from stores, whereas it only secondarily affects Ca^{2+} entry.

Supplementary table 1. Inhibition of PI3K lipid kinase activity for purified enzymes in vitro. Listed are IC_{50} values for the indicated compounds, regarding inhibition of the catalytic activity of recombinant class I PI3K isoforms in cell-free system. Data were drawn from appropriate patent and publication information, see Refs. (31,41).

Compound	Relative specificity	IC_{50} (μ M)			
		α	β	γ	δ
PIK-112	none	>100	>100	>100	>100
PIK-75	α	0.008	0.34	0.9	>20
PI-103	α/β	0.004	0.018	>500	>20
YM-024	α/δ	0.30	2.65	9.1	0.33
TGX-221	β	7.0	0.008	3.5	0.2
AS-252424	γ	1.1	>20	0.035	>20
IC-87114	δ	>100	75	29	0.1
LY-294002	$\alpha-\delta$	0.50	1.0	n.d.	0.57
Wortmannin	$\alpha-\delta$	0.001	0.003	n.d.	0.001

Various new isoform-specific catalytic site inhibitors of class I PI3Ks have been developed and used for functional studies in other cell types (31,41). The selectivity of these inhibitors for PI3K α - δ isoforms is mostly determined in cell-free in vitro studies using isolated enzymes (IC_{50} values are given in Supplementary Table I). The compound PIK-75 has a far higher affinity for PI3K α (IC_{50} 8 nM) than for other isoforms. At nanomolar concentrations, the compound PI-103 inhibits both PI3K α and β (PIK-112 is an inactive analog). YM-024 has a relatively high affinity for PI3K α and δ whilst TGX-221 is most active against PI3K β (IC_{50} 7 nM). The compounds AS-252424 and IC-87114 show highest activity towards PI3K γ and PI3K δ isoforms, respectively. It should be remarked that effective doses of these compounds for complete inhibition of the kinase activity in adipocytes, hepatoma cell lines and platelets are reported to be in the low micromolar range (17,21,41). Hence, for adequate inhibition of PI3K activities in intact platelets, higher doses are needed than apparent from the IC_{50} values. Reasons for this are the high platelet counts in experiments and the high ATP concentration in cells (at least 10-fold higher than of in vitro kinase assays).

For the above-mentioned inhibitors, dose-effect relations were determined for convulxin-induced Ca^{2+} responses in $CaCl_2$ medium. In order to prevent autocrine stimulatory effects of thromboxane and ADP, all experiments were carried out with aspirin-treated platelets and blockers of both ADP receptors were added. Time integrals of increases in $[Ca^{2+}]_i$ served as a read-out of the extent of GPVI-induced PLC activation (36). Strikingly, those inhibitors with a high-affinity towards PI3K α (PIK-75, PI-103 and YM-024, 0.5-1 μ M) reduced the Ca^{2+} signal with about 50%, while the control substance PIK-112 was completely inactive (Fig. 2A,B). In addition, the PI3K β selective inhibitor TGX-221 (0.1-2.5 μ M) caused a similar reduction in Ca^{2+} signal. Combined application of PIK-75/TGX-221 or PI-103/TGX-221 did not further suppress the response. In general, the isoform specific PI3K α or β inhibitors were similarly effective as the general PI3K inhibitors, wortmannin and LY-294002. On the other hand, AS-252424 (0.1-1 μ M), inhibiting PI3K γ , was without any effect, while the PI3K δ inhibitor IC-87114 only slightly reduced the Ca^{2+} response.

Figure 1. Major contribution of PI3K to GPVI-induced versus thrombin receptor-induced Ca^{2+} responses in absence of autocrine mediators. Human platelets, treated with aspirin and loaded with Fura-2, were preincubated for 10 min with Me2SO vehicle (control), wortmannin (0.1 μM) or LY-294002 (10 μM) in the presence of apyrase (0.6 U/ml). The platelets were stimulated with GPVI ligand, CRP (5 $\mu\text{g}/\text{ml}$) or convulxin (70 ng/ml), or with thrombin (10 nM), in the presence of 1 mM EGTA or 1 mM CaCl_2 . A. Effect of wortmannin (WT) on CRP-induced $[\text{Ca}^{2+}]_i$ rises. B. Quantitative effect of wortmannin and LY-294002 on integrated Ca^{2+} responses in the presence of EGTA or CaCl_2 . Per agonist, time- $[\text{Ca}^{2+}]_i$ integrals of the relevant control condition were set at 100%. C. Effect of wortmannin on convulxin-induced Ca^{2+} mobilization and secondary Ca^{2+} entry (1 mM CaCl_2). Representative traces are shown. Means $\pm$ S.E. ($n=4-5$); * $p<0.05$ compared to control.

For comparison, the same panel of inhibitors was tested in measurements of thrombin-induced Ca^{2+} responses. Here, only compounds with a high affinity towards $\text{PI3K}\beta$ (i.e. PI-103 and TGX-221) and the general PI3K inhibitors (wortmannin and LY-294002) reduced the Ca^{2+} signal by 15–20% (Supplementary Fig. 1). In this case, the $\text{PI3K}\alpha$ inhibitor PIK-75 was without effect. Together, these results point to high and non-redundant roles of the $\text{PI3K}\alpha$ and β isoforms in Ca^{2+} signaling directly downstream of GPVI, but not of thrombin receptors.

Control experiments were performed to exclude that the high-affinity $\text{PI3K}\alpha$ inhibitor, PIK-75, acted by residual inhibition of $\text{PI3K}\beta$. We examined the effect of PIK-75 (0.5–1 μM) on P2Y_{12} -induced platelet responses and integrin $\alpha\text{IIb}\beta_3$ activation, since these are exclusively mediated by the $\text{PI3K}\beta$ and γ isoforms (20,21). As shown in the supplementary Fig. 2, PIK-75 was completely inactive in both platelet aggregation and integrin activation in response to ADP, such in marked contrast to the established $\text{PI3K}\beta$ inhibitor, TGX-221. On the other hand, TGX-221 did not affect Akt phosphorylation at Ser473 evoked by insulin-like growth factor (IGF)-1, which is known to be mediated by only the $\text{PI3K}\alpha$ isoform (S. Kim, data not shown but see Ref. 42).

A Thrombin

Supplementary figure 1. Inhibition of platelet PI3K isoforms moderately affects thrombin receptor induced Ca^{2+} responses. Human Fura-2-loaded platelets were treated with aspirin, preincubated with ADP receptor blockers, and treated with PI3K inhibitor, as described for Fig. 2. Shown are Ca^{2+} responses upon stimulation with thrombin (10 nM). A. Representative $[\text{Ca}^{2+}]_i$ traces; B. inhibitor effects expressed as percentages of time- $[\text{Ca}^{2+}]_i$ integrals relative to the control condition. Note that at a low dose of thrombin (1 nM), PIK-75, TGX-221 or the combination of both (0.5 μM) suppressed the Ca^{2+} rise to $107 \pm 3\%$, $87 \pm 2\%$ or $101 \pm 5\%$ of control, respectively. Data are means $\pm$ S.E. ($n=3-5$); * $p < 0.05$ compared to control.

Contribution of both $\text{PI3K}\alpha$ and β isoforms to GPVI-induced signaling

In platelets, the GPVI receptor activates the PH-domain containing $\text{PLC}\gamma_2$, such in contrast to thrombin receptors which activate $\text{PLC}\beta$ isoforms. For other cells, it was hypothesized that an increase in $\text{PI}(3,4,5)\text{P}_3$ level caused by PI3K pulls $\text{PLC}\gamma_2$ to the membrane and increases its action (18). To verify this for GPVI-stimulated platelets (again with blocked autocrine responses), we determined overall PLC activity by measuring the accumulation of InsP_1 due to InsP_3 production. Platelet stimulation with convulxin led to a production of InsP_3 for up to 10 min (Fig. 3A). Wortmannin treatment reduced the InsP_3 formation and moreover confined it to 5 min. Such a shortening of InsP_3 production was not seen in thrombin-stimulated platelets. The isoform-specific inhibitors, PIK-75 or TGX-221 (targeting at $\text{PI3K}\alpha$ and β , respectively) suppressed the InsP_3 production to a similar extent as wortmannin, while the combination of the two did not have an additional effect (Fig. 3B). In contrast, inhibition of $\text{PI3K}\gamma$ (AS-252424) or $\text{PI3K}\delta$ (IC-87114) was not effective on InsP_3 amounts. Hence, these results point to a GPVI-induced prolongation of PLC activity by $\text{PI3K}\alpha/\beta$.

Supplementary figure 2. Specificity of PI3K β inhibitor TGX-221 for affecting ADP-induced platelet activation. Human PRP was preincubated with Me2SO vehicle (control), TGX-221 (0.5-1 μ M), PIK-75 (0.5-1 μ M) or wortmannin (1 μ M), prior to stimulation with ADP. A. Representative light transmission aggregation traces induced by 5 μ M ADP, and quantitative effect of inhibitors on ADP-induced platelet aggregation. B. Binding of FITC-PAC1 to platelets stimulated with 20 μ M ADP (10 min). Fluorescence histograms are shown of unstimulated platelets (filled grey), vehicle controls (grey lines) and samples containing indicated inhibitor (black overlays). Means $\pm$ S.E. (n=4); *p<0.05 compared to control.

We measured agonist-induced Ser⁴⁷³ phosphorylation of Akt as an established downstream effector of lipid PI3K (21). In platelets stimulated with convulxin (Fig. 3C) or CRP (not shown), wortmannin treatment suppressed the phosphorylation of Akt, as expected. Interestingly, also inhibition of PI3K α (PIK-75) or β (TGX-221) reduced this phosphorylation, while the combination was not more effective (Fig. 3D).

A key downstream effect of [Ca²⁺]_i elevation in platelets is the secretion of α -granules, which is measured as expression of granular P-selectin at the platelet surface. Platelet treatment with PIK-75 or TGX-221, alone or in combination, suppressed convulxin- or CRP-induced P-selectin expression with 40-50%, i.e. to the same extent as did wortmannin or LY-294002 (Fig. 4). This points to a non-additive effect of both isoform-selective compounds on GPVI-induced secretion.

A Convulxin

B

Figure 2. Inhibition of platelet PI3K α and β isoforms strongly suppresses GPVI-induced Ca²⁺ responses. Aspirin-treated, Fura-2-loaded platelets were preincubated with ADP receptor blockers (10 μ M AR-C69931MX, 40 μ M MRS-2179, 0.1 U/ml apyrase) and 1 mM CaCl₂. Platelets were then treated with one of the following substances (PI3K isoform specificity in brackets): Me2SO vehicle (control), PIK-112 (inactive analog of PIK-75), PIK-75 (α), PI-103 (α/β), YM-024 (α/δ), TGX-221 (β), AS-252424 (γ), IC-87114 (δ), LY-294002 ($\alpha-\delta$) or wortmannin ($\alpha-\delta$). Numbers give final concentrations in μ M. A. Representative [Ca²⁺]_i traces upon stimulation with convulxin (70 ng/ml). B. Effects of inhibitors on Ca²⁺ responses, expressed as percentages of time-[Ca²⁺]_i integrals relative to the control condition. Note that at a low dose of convulxin (7 ng/ml), PIK-75, TGX-221 or the combination of both (0.5 μ M) suppressed the Ca²⁺ rise to 51 $\pm$ 3%, 40 $\pm$ 6% or 31 $\pm$ 1% of control, respectively. Data are means $\pm$ S.E. (n=3-5); *p<0.05 compared to control.

Roles of PI3K α and β isoforms in GPVI-induced platelet activation and thrombus formation on collagen under flow

The pharmacological evidence so far points to a common role of the PI3K α/β isoforms in GPVI-induced Ca²⁺ signaling and downstream platelet activation events. We then performed studies to determine the importance of these isoforms in flow-dependent thrombus formation at physiological shear rates. Earlier work has shown that, during platelet interaction with collagen under flow, GPVI-induced rises in [Ca²⁺]_i are important for granule secretion, activation of integrins and exposure of PS (22,34,42,43). In whole blood, supplemented with Fluo-3-loaded platelets, [Ca²⁺]_i rises were measured of individual platelets upon adhesion to collagen under flow. Interestingly, pretreatment with either PIK-75, TGX-221 or wortmannin led to a marked suppression in single cell Ca²⁺ responses, so that the adhered platelets showed low-amplitude spiking changes in [Ca²⁺]_i (Fig. 5). Control experiments indicated that the Ca²⁺ responses under flow were not influenced by aspirin and blockage of ADP receptors (not shown), thus confirming that autocrine ADP release did not contribute to this initial platelet response. Other controls indicated that these [Ca²⁺]_i rises were completely suppressed by blocking GPVI (22).

Figure 3. Contribution of PI3K α and β isoforms to GPVI-induced InsP₃ production and Akt phosphorylation. Aspirin-treated human platelets (1×10^6 /ml) were preincubated with ADP receptor blockers (Fig. 2) and Me₂SO vehicle (control) or 1 μ M PI3K inhibitor (25 μ M LY-294002). A,B. Effect of PI3K blockers on accumulation of InsP₃ due to InsP₃ production. Platelets were stimulated for 10 min with convulxin (70 ng/ml) or thrombin (10 nM) in the presence of 1 mM LiCl. C,D. Effect of PI3K blockers on convulxin-induced Ser⁴⁷³ Akt phosphorylation. C. Representative Western blots of phospho-Akt and total Akt (60 kD). D. Data are densitometric intensities of phospho-Akt/Akt, expressed as percentage of vehicle. Means $\pm$ S.E. (n=3-5); *p<0.05 compared to PIK-112 or control.

The effects of inhibitors on thrombus formation were assessed from measurements of the deposition of platelets on the collagen surface (Fig. 6A). In addition, the exposure of PS of collagen-adhered platelets (detected with OG-annexin A5) was measured, which response is shown by a subpopulation of the platelets in direct contact with collagen due to GPVI activation (38). Both parameters were substantially diminished with wortmannin, with the PI3K α inhibitors PIK-75 and YM-024, or with the PI3K β inhibitor TGX-221. In the presence of all inhibitors, platelet deposition on collagen was substantially reduced in comparison to the vehicle control (Fig. 6B). In addition, the number of PS-exposing platelets was reduced to ~50% by PIK-75, YM-024 or TGX-221. On the other hand, inhibition of PI3K γ (AS-252424) or δ (IC-87114) was without effect. Together, these results suggest that inhibition of only PI3K α or β isoforms suppresses flow-dependent thrombus formation on collagen, by reducing GPVI-dependent Ca²⁺ responses and downstream activation processes leading to platelet aggregation and PS exposure.

Figure 4. Contribution of PI3K α and β isoforms to GPVI-induced α -granule secretion. Human washed aspirin-treated platelets (1×10^8 /ml) were preincubated with ADP receptor blockers (Fig. 2), and treated with Me2SO vehicle (control) or specific PI3K inhibitor (in μ M): PIK-75 (1.0), TGX-221 (0.5), wortmannin (0.1), LY-294002 (25). The platelets were activated for 10 min with convulxin (70 ng/ml) or CRP (10 μ g/ml). **A.** Histogram showing effect of PI3K α / β blockers on P-selectin expression (FL1 fluorescence). **B.** Quantitative effect of PI3K blockage on P-selectin expression. Data are relative to the control condition. Means $\pm$ S.E. (n=3); *p<0.05 compared to control.

Roles of murine PI3K isoforms in GPVI-induced platelet activation and thrombus formation

Platelets from mice lacking the p85 α regulatory PI3K subunit are impaired in collagen-induced aggregation, which points to diminished GPVI signaling (11). In the p85 $\alpha^{-/-}$ platelets, expression of the p110 α subunit (PI3K α) is almost undetectable, while both p85 β and p110 β are still present at low levels (11,29,44). We used blood from p85 $\alpha^{-/-}$ mice to measure collagen-dependent $[Ca^{2+}]_i$ rises in adhered platelets, thrombus formation and PS exposure under flow. In contrast to the potent and prolonged Ca^{2+} responses of p85 $\alpha^{+/+}$ platelets, p85 α -deficient platelets showed a markedly lower Ca^{2+} signal, which in 36% of the cells were spiking in appearance (Fig. 7A).

Treatment of $p85\alpha^{+/+}$ blood with TGX-221 reduced the average Ca^{2+} signal to the level seen in $p85\alpha^{-/-}$ platelets. Treatment of wildtype blood with YM-024 had a similar suppressive effect on the Ca^{2+} response ($p=0.012$). As a comparison, blood was used from $p110\gamma^{-/-}$ mice, in which case Ca^{2+} responses under flow were not different from those of corresponding wildtype platelets (I. Munnix, unpublished data 2008).

Figure 5. Role of PI3K in collagen-induced Ca^{2+} responses under flow in the absence of secondary mediators. PPACK-anticoagulated blood, containing 10% autologous Fluo-3-loaded platelets, was preincubated with Me2SO vehicle (control), PIK-75, TGX-221 or wortmannin (each 1 μ M). A. Single cell Ca^{2+} responses during flow of blood over collagen in the presence of indicated inhibitor at a shear rate of 1000 s^{-1} . Upper panels: mean overlays of $[Ca^{2+}]_i$ traces from >35 individual platelets; lower panels: traces from representative single platelets. B. Quantification of rises in $[Ca^{2+}]_i$ at 20 s (gray) or 60 s (dark gray) after initial platelet activation. Means $\pm$ S.E. ($n=27-50$); * $p<0.05$ compared to control.

Measurements of flow-dependent thrombus formation on collagen showed a reduced deposition of $p85\alpha^{-/-}$ platelets, as well as a reduced PS exposure of the cells in comparison to wildtype blood (Fig. 7B). Similarly, addition of TGX-221 to $p85\alpha^{+/+}$ blood, but not to $p85\alpha^{-/-}$ blood, significantly decreased thrombus formation and number of PS-exposing platelets. A role for PI3K α and β in murine thrombus formation was further supported by inhibitor studies using blood from wildtype mice. Treatment with PIK-75 and/or TGX-221 led to a similar reduction in platelet deposition and PS exposure as treatment with the general PI3K inhibitor, wortmannin (Fig. 7C). Together, these results demonstrated that, in mouse, the regulatory $p85\alpha$ subunit and the catalytic PI3K α and β subunits are essential in flow-dependent Ca^{2+} signaling, PS exposure and thrombus formation on collagen.

Figure 6. Role of PI3K α and β isoforms in collagen-induced thrombus formation and PS exposure under flow. PPACK-anticoagulated blood was flowed during 4 min over collagen at a shear rate of 1000 s^{-1} . Preincubation was with Me2SO vehicle (control) or one of the PI3K inhibitors: PIK-75, YM-024, TGX-221, AS-252424, IC-87144 or wortmannin (all 1 μM). A. Representative images of phase contrast (120 $\times$ 120 μm) and OG-annexin A5 fluorescence (150 $\times$ 150 μm) after 4 min flow. B,C. Surface area coverage of all deposited platelets and PS-exposing platelets. Data are expressed as percentages of control condition. Means $\pm$ S.E. (n=4); *p<0.05 compared to control.

Experiments carried out with washed mouse platelets, in the presence of autocrine stimulation inhibitors, confirmed that GPVI-induced activation relied on PI3K α/β activity, since LY-294002, PIK-75 and TGX-221 all inhibited CRP-induced aggregation (not shown). In addition, platelets from mice lacking the catalytic p110 γ or p110 δ subunits were essentially unaltered in aggregation, when compared to wildtype platelets (P. Mangin, unpublished data 2008). Together, this points to major functions of murine p110 α and p110 β PI3K isoforms and to minor roles for p110 γ and p110 δ in the aggregation response.

Figure 7. Murine p85α regulates collagen-induced Ca²⁺ responses and thrombus formation under flow. PPACK/heparin-anticoagulated blood from p85α^{+/+} or p85α^{-/-} mice, spiked with 10% Fluo-3-loaded platelets from the same mouse strain, was flowed over collagen at 1000 s⁻¹. The blood was preincubated with Me2SO vehicle or TGX-221 (1 μM). **A.** Contribution of p85α to Ca²⁺ responses under high-shear perfusion. Upper panels: averaged overlays of [Ca²⁺]_i traces from >25 platelets; lower panels: traces from representative single platelets. Bars show quantification of [Ca²⁺]_i rises at 20 s (gray) or 60 s (white) after initial platelet activation. **B.** Contribution of p85α to thrombus formation and PS exposure. Shown are representative images of phase-contrast (120×120 μm) and OG-annexin A5 fluorescence (150×150 μm) of p85α^{+/+} and p85α^{-/-} thrombi. Bars represent surface area coverage of all deposited platelets and PS-exposing platelets. **C.** Effect of pharmacological PI3K inhibition on thrombus formation and PS exposure. Wildtype blood was preincubated with Me2SO vehicle (control), PIK-75, TGX-221 or wortmannin (each 1 μM). Data are means ± S.E. (n=3-5); *p<0.05 compared to vehicle control, #p<0.05 compared to wildtype.

Roles of PI3K isoforms in GPVI-induced Rap1b activation in human and mouse platelets

In platelets, the low-molecular weight GTPase, Rap1b, is a well-characterized effector downstream of PI3K (27). However, there is no conclusive evidence how Rap1b functions directly downstream of GPVI. Platelets from mice deficient in Rap1b were used to investigate this. In the presence of autocrine stimulation inhibitors, Rap1b^{-/-} platelets showed a 36% reduction in CRP-induced aggregation in comparison to Rap1b^{+/+} wildtypes (Fig. 8A). This suggested a direct role for this GTPase in GPVI-induced platelet aggregation. Inhibition of PI3K with LY-294002 markedly reduced the aggregation of Rap1b^{+/+} platelets and, surprisingly, of Rap1b^{-/-} platelets, with reductions of 89% and 86%, respectively, in comparison to vehicle-treated controls. The observation that LY-294002 further reduced the aggregation of Rap1b^{-/-} platelets indicated that, even though Rap1b is involved in GPVI-mediated activation, it is not the only effector of PI3K in this response.

Figure 8. Contribution of Rap1b to GPVI-induced integrin α IIb β 3 activation and platelet aggregation. (A) Washed platelets (3×10^8 /ml) from Rap1b^{+/+} or Rap1b^{-/-} mice were preincubated with autocrine stimulation inhibitors (AR-C6931MX, MRS-2179 and 10 μ M indomethacin), in the presence of Me2SO vehicle or LY-294002 (25 μ M). Platelets were stimulated with 10 μ g/ml CRP in the presence of purified fibrinogen (0.5 mg/ml) and aggregation was monitored. Representative aggregation traces are shown. Bars give percentages of light transmission at 3 min after CRP addition. Data are means $\pm$ S.E. (n=3-4); *p<0.05 compared to control.

To elucidate the PI3K isoforms implicated in Rap1b activation directly downstream of GPVI, the effect of CRP was evaluated on Rap1b-GTP levels in platelets treated with autocrine stimulation inhibitors. An inhibitor of α IIb β 3 of human (40 μ g/ml Reopro) or mouse (100 μ M GPI-562) platelets served to suppress platelet aggregation, which process interferes with the assay. In both human and mouse platelets, CRP induced a potent activation of Rap1b (Fig. 9A,B). Treatment with LY-294002 strongly suppressed Rap1b activation with Rap1-GTP levels being reduced in human and mouse platelets by 72% and 69%, respectively. Markedly, treatment with TGX-221 or PIK-75 resulted in a similar degree of reduction in Rap1b activation, both in mouse or human platelets. In contrast, effects of AS-252424 treatment were negligible. These findings demonstrate a major role for PI3K α and β but not γ in Rap1b activation downstream of GPVI.

Figure 9. Contribution of PI3K isoforms to GPVI-induced activation of Rap1b. Washed human (A) and wildtype mouse (B) platelets (3×10^8 /ml) were preincubated with autocrine stimulation inhibitors (ASI) (Fig. 8) and α IIb β 3 inhibitor (human: 40 μ g/ml Reopro; mouse: 100 μ M GPI-562). Incubations contained Me₂SO vehicle or PI3K inhibitor LY-294002 (25 μ M), TGX-221 (0.5 μ M) or AS-252424 (10 μ M). Platelets were stimulated with 20 μ g/ml CRP, lysed, and used for immunoprecipitation of GTP-Rap1. A,B. Representative Western blots of active Rap1-GTP and total Rap1. Right panels: densitometric analysis of the amount of active Rap1-GTP (arbitrary units). Means $\pm$ S.E. (n=3-5); *p<0.05 compared to control.

Discussion

The present paper provides first data showing the contribution of multiple PI3K isoforms in GPVI-induced activation of (human) platelets, and the role of these isoforms in GPVI-dependent thrombus formation. Our findings significantly extend the earlier evidence that GPVI agonists increase the formation of PI(3,4,5)P₃ and its derivative PI(3,4)P₂ via PI3K activation (9,10,45). A marked finding is that both PI3K α and β isoforms are needed for full InsP₃ formation and Ca²⁺ signal generation, thus suggesting that both activities are necessary for optimal activation of the key effector enzyme of GPVI, PLC γ 2. Detailed Ca²⁺ measurements in the presence of extracellular EGTA and CaCl₂ pointed to a marked contribution of PI3K to Ca²⁺ mobilization from intracellular stores, which is a direct readout of PLC, and not to a (store-independent) effect on Ca²⁺ entry. Hence, the earlier suggested regulation of Ca²⁺ entry by PI3K or its product PI(3,4,5)P₃ (23,24) seems to be secondary to the effect of PI3K regulation of Ca²⁺ store depletion.

Several observations point to a non-redundant contribution of PI3K α and β isoforms in GPVI-induced platelet activation. These include the inhibitory effects on human platelets of a panel of compounds with high affinity to PI3K α (PIK-75, PI-103, YM-024) or to PI3K β (TGX-221 and also PI-103). Other support comes from studies with mouse platelets, which responded similarly to the isoform-specific inhibitors as did human platelets. Furthermore, we found that mouse platelets lacking p85 α were strikingly impaired in collagen-induced Ca²⁺ signaling and thrombus formation. These p85 $\alpha^{-/-}$ platelets are fully depleted in p110 α but still express residual p85 β and p110 β (11). However, treatment of these platelets with TGX-221 did not further suppress Ca²⁺ signaling and downstream responses such as PS exposure, although this compound did insignificantly reduce p85 $\alpha^{-/-}$ platelet deposition under flow. This suggested a function of the residual p110 β in ADP-dependent platelet aggregate formation.

In human platelets, inhibition of PI3K γ (AS-252424) failed to affect the GPVI-induced Ca²⁺ response, while inhibition of PI3K δ (IC-87114) caused a minor reduction, such in contrast to the marked effect of PI3K α/β inhibition. Similarly, mouse platelets which were deficient in p110 γ or p110 δ showed undiminished activation, when stimulated with GPVI agonist under conditions where autocrine stimulators were blocked. These results are supported by data from others, showing that deficiency in murine p110 γ (19) or p110 δ (26) has no more than a modest role in GPVI-induced platelet activation.

The role of PI3K α and β isoforms was particularly clear in measurements of GPVI-dependent thrombus formation, where platelets were flowed over collagen at a defined physiological shear rate. Blocking or deficiency in PI3K α/β resulted in suppression of Ca²⁺ rises and PS exposure of platelets adhered to collagen, as well as in reduced formation of aggregate formation. For both human and mouse blood, it is known that these responses fully rely on GPVI signaling via Src family kinases to LAT and PLC γ 2 (34,38,46). Hence, the present findings support a concept that full PLC γ 2-evoked activity is required for optimal platelet activation and thrombus formation under flow. Earlier, it was established that at the same flow conditions different populations of platelets assemble into aggregates or expose PS (38). While this heterogeneity in platelet responses was maintained with PI3K α/β inhibition or absence, the present results also suggest that the reduced PS exposure in inhibited platelets is a direct consequence of the reduced Ca²⁺ signal of those platelets that are adhered to collagen.

Earlier, we and others have shown that two PI3K isoforms, namely PI3K β and γ , are implicated in ADP/P2Y₁₂-induced integrin α IIb β 3 activation and subsequent thrombus stabilization (17,19,20). Recently, these observations were extended to in vivo thrombosis models, where wortmannin treatment of p110 γ deficient mice was found to cause a dramatic defect in initial thrombus growth following vascular damage (21). The present data shed a new light on these in vivo observations, since it now appears that both collagen- and ADP-dependent platelet activation processes are affected by PI3K inhibition with a likely key role of the PI3K α/β isoforms.

There is a growing body of evidence that in many cell systems individual PI3K isoforms, although involved in specific cellular functions, show functional redundancy, e.g. as noted for the additive contribution of PI3K β and γ in complement 5a-stimulated macrophages (47). So far, non-redundancy in PI3K function has been described only in particular cases. For example, in neutrophils producing inflammatory reactive oxygen species, both PI3K γ and δ isoforms contribute to PI(3,4,5)P₃ formation, but in this case the role of PI3K γ may be prior to that of

PI3K δ (31,48). In comparison to platelets, this is reminiscent of the early contribution of PI3K α/β in GPVI stimulation, and the later involvement of PI3K β/γ in responses to autocrine produced ADP.

In the classical scheme of immunoglobulin receptor-linked PLC γ activity, as proposed for lymphocytes, accumulation of PI(3,4,5)P $_3$ in the plasma membrane may function as an anchoring site for the N-terminal pleckstrin homology domain of PLC γ isoforms (18,49). This scheme can be applicable to GPVI-stimulated platelets as well. Given the high turnover of phosphoinositides in the platelet plasma membrane, we hypothesize that the stimulation and anchoring of PLC γ_2 requires a threshold elevation of PI(3,4,5)P $_3$ that can only be achieved by simultaneous activity of PI3K α and β . In other words, activation of either PI3K α or β alone may be insufficient for PLC γ_2 targeting to membrane via its PH domain. It is conceivable that this is a consequence of the need of PLC γ_2 to compete with other PH-domain containing signaling proteins such as Akt isoforms. Such a mechanism would provide an attractive, but not the only, explanation for the identified non-redundant contribution to GPVI-induced signaling of both PI3K isoforms. One possible scenario is that one isoform may produce an initial small amount of PI(3,4,5)P $_3$, which in turn promotes activation of the other isoform through PI(3,4,5)P $_3$ regulation of p85 subunits. Future studies examining temporal signaling by individual PI3K isoforms, as previously performed in neutrophils (31), will be needed to address this issue. The enhancing effect of PI3K activity on PLC γ_2 is in contrast to the essential contribution of Src family kinases to GPVI-induced activation, as this is apparent from the complete abrogation of GPVI-induced Ca $^{2+}$ rises by Src family kinase inhibition (51).

The literature contains solid evidence that platelet Rap1b is activated in a PI3K-dependent manner, in particular after occupation of Gi-coupled receptors such as the P2Y $_{12}$ receptor for ADP (27,28). By using autocrine stimulation inhibitors, where the P2Y $_{12}$ -dependent component was blocked, we could identify - for both human and mouse platelets - the existence of a direct GPVI-stimulated and PI3K-dependent component of Rap1b activation. Use of the isoform specific inhibitors revealed involvement of PI3K α/β in this activation of Rap1b. However, the prominent suppressive effect of PI3K inhibitors on α IIb β_3 activation and aggregation seen in Rap1b $^{-/-}$ platelets indicates that the PI3K isoforms contribute to GPVI-induced integrin activation also through a pathway that is independent of Rap1b. This pathway may involve PI3K α/β -dependent stimulation of PLC γ_2 , which can contribute to the Ca $^{2+}$ - and protein kinase C-mediated activation of α IIb β_3 .

PI3K α is the most frequently mutated kinase in human cancer. Hence, this isoform has raised great interest as a target for novel anti-tumor drugs, some of which are currently in early-stage clinical trials (50). Given the likely important function of GPVI in (experimental) thrombosis, the present data suggest another important application for PI3K α and PI3K β inhibitors, namely in antiplatelet therapy and arterial thrombosis. In this context, it is relevant to note that arterial thrombosis is a known companion of some cancers. The availability of selective pharmacological agents against specific isoforms may thus offer new potential approaches for prevention of these diseases.

Note: While this paper was in revision, other authors have also reported that PIK3 β plays an essential role in GPVI-induced activation of mouse platelets (53).

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Chapter 4

PKC α regulates platelet granule secretion and
thrombus formation in mice

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Abstract

Platelets are central players in the atherothrombosis of coronary artery disease. The protein kinase C (PKC) family provides important intracellular mechanisms for regulation of platelet activity, and platelets express several members of this family including classical isoforms PKC α and PKC β and novel isoforms PKC δ and PKC θ . Here we use a genetic approach to demonstrate definitively the role played by PKC α in regulating thrombus formation and platelet function. Thrombus formation in vivo was attenuated in *Prkca*^{-/-} mice, and PKC α was required for thrombus formation in vitro, although this PKC isoform did not regulate platelet adhesion to collagen. The ablation of in vitro thrombus formation in *Prkca*^{-/-} platelets was rescued by addition of ADP, consistent with the key mechanistic finding that dense-granule biogenesis and secretion depends upon PKC α expression. Furthermore, defective platelet aggregation in response to either collagen-related peptide or thrombin could be overcome by an increase in agonist concentration. Evidence of overt bleeding, including gastrointestinal and tail bleeding, was not seen in *Prkca*^{-/-} mice. In summary, the effects of PKC α ablation on thrombus formation and granule secretion may implicate PKC α as a drug target for antithrombotic therapy.

Introduction

The protein kinase C (PKC) family comprises 10 isozymes grouped into 3 classes: conventional (α , β I, β II, γ), novel (δ , ϵ , η /L, θ) and atypical (ζ , ι / λ) (1). PKCs have long been known to play major roles in a number of platelet processes (2), most importantly aggregation and secretion, where stimulation of platelets with diacyl glycerol (DAG) or phorbol ester can induce aggregation, and agonist-induced secretion can be prevented by broad spectrum pharmacological inhibition of PKC isoforms (3-6). Recently it has been shown pharmacologically that PKC isoforms may exert dual control role in thrombus formation by mediating secretion and integrin activation under flow, whilst also suppressing phosphatidylserine exposure and subsequent thrombin generation and coagulation (7).

At least four PKC isoforms (α , β , δ , θ) are expressed in human platelets (8-14) and it is becoming clear that each isoform may play different roles in platelet function. Using genetic and pharmacological approaches we have shown PKC δ to play a negative role regulating filopodia formation and platelet aggregation in response to collagen through a functional interaction with the actin regulatory protein VASP (15,16). Using biochemical approaches PKC α has been identified as an essential factor in positively regulating alpha- and dense-granule secretion in platelets (17) as well as platelet aggregation (18). We had recently shown, using biochemical and pharmacological approaches, that two tyrosine kinases Syk and Src physically and functionally interact with PKC α to regulate each other and cellular activities in platelets (19). It is now important that the function of PKC α in platelets is addressed definitively by a genetic approach.

Genetic and molecular approaches have revealed a wide range of roles for PKC α in other cell types. Cell proliferation, differentiation, apoptosis, motility and adhesion are all regulated by PKC (1,20) with consequent roles for PKC α in regulation of tumour progression. Although there is evidence that in some tumours PKC α may have a suppressor role

(21,22), in the majority of cases PKC α expression and activity are higher in tumours than in normal tissues (23,24), and PKC α activity promotes a more aggressive phenotype in breast cancer cells with an increased metastatic potential (25-27). PKC α has therefore become a major target for therapeutic intervention in various cancers (28). In immune regulation PKC α mediates T cell-dependent interferon generation (29), and is also critical for T cell receptor down-regulation (30). Insulin signaling to PI3K and subsequently to glucose transport is enhanced in mice lacking PKC α (31), suggesting a negative feedback role for PKC α in metabolic processes. Finally, in the heart, PKC α regulates cardiac contractility and Ca²⁺ handling in myocytes, and deficiency of PKC α protects against heart failure induced by pressure overload, and against dilated cardiomyopathy (32).

In a recent elegant study reconstructing the signaling pathway regulating platelet integrin α IIb β 3 in a nucleated cell system, it was shown that expression levels of PKC α equivalent to those found in platelets are required for activation of the integrin through the Rap1 pathway (33). This is in agreement with previous biochemical evidence suggesting an essential role for PKC α in regulating the integrin (18). Importantly also, genome-wide association analysis of coronary artery disease revealed a cluster of SNPs in the *Prkca* gene, with a maximal p-value association of just over 10⁻³ (SNP rs12600582) (34,35). This analysis marks the gene as one requiring further phenotypic analysis. It was now essential to identify definitively, by a genetic approach, the role of PKC α in regulating platelet function and thrombus formation. Here we report that genetic ablation of PKC α in platelets reveals non-redundant roles in regulating platelet dense granule biogenesis and exocytosis, and in the control of thrombus formation in vitro and in vivo.

Experimental procedures

Materials

Collagen-related peptide (CRP) was from Prof Richard Farndale (University of Cambridge, UK). Thrombin, BSA, ADP, protein G-sepharose, fibrinogen were purchased from Sigma. Complete mini protease inhibitor tablets were from Roche Applied Science. Secondary HRP-conjugated anti-rabbit antibody were obtained from Santa Cruz Biotechnology Inc. SNAP23 (P-T95) antibody was a generous gift from Dr Paul Roche (NIH, Bethesda). JON/A (anti- α IIb β 3) and WugEg (anti-P-selectin) antibody were from Emfret Analytics. Phycocerythrin (PE)-conjugated anti-CD41 antibody, and its isotype-matched control PE-conjugated rat IgG1, were from Serotec. Isoform-specific anti-PKC mouse monoclonal antibodies were from BD Biosciences. LY333531 was supplied by A.G. Scientific (Fluorochem Ltd), U46619 was from Alexis Biochemicals, Gö6976 was from Calbiochem and CHRONO-LUME reagent came from Chrono-Log Corp. (Labmedics). The P2Y12 antagonist, AR-C69931MX, was a kind gift from AstraZeneca. The P2Y1 antagonist MRS2500 was from Tocris Bioscience.

Platelet preparation

Mice were bred and maintained in the University of Bristol animal facility in accordance with UK Home Office regulations. *Prkca*^{-/-} mice were generated as previously described (32).

Blood was drawn by cardiac puncture under terminal anaesthesia into acid citrate dextrose (20 mM citric acid/110 mM sodium citrate/5 mM glucose), 1:7 ratio v:v. Platelets were prepared as previously described (15). Briefly, blood was diluted with 250 μ l of modified Tyrodes-HEPES buffer (134 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM HEPES, 5 mM glucose, 1 mM MgCl₂, pH 7.3) and centrifuged at 180 g for 6 min at room temperature. Platelet-rich plasma (PRP) was removed and platelets were isolated by centrifugation at 550 g for 6 min in the presence of PGE₁ (140 nM) and indomethacin (10 μ M). Pelleted platelets were resuspended to the required density in modified Tyrodes-HEPES buffer and rested for 30 min at 37 °C in the presence of 10 μ M indomethacin prior to stimulation.

Platelet aggregation

Platelets were resuspended in Tyrodes-HEPES buffer at a final concentration 2×10^8 per ml. Platelets were preincubated with different inhibitors or vehicle solution (0.1% Me₂SO final concentration) for 10 minutes at 37°C, and aggregation of agonist-stimulated platelets were monitored in an optical aggregometer (Chrono-Log, Labmedics) at 37°C, with continuous stirring at 800 rpm.

Flow cytometry

Two-colour analysis of mouse platelet activation was conducted using phycoerythrin-conjugated JON/A antibody that preferentially binds to the active form of α IIb β 3 integrin and with FITC-conjugated CD62P (P-selectin) antibody. 25 μ l of washed platelets (4×10^7 /ml in Tyrode's-HEPES buffer supplemented with 1 mM CaCl₂ and 0.35% BSA) were mixed with 10 μ l of antibody and subsequently stimulated either with 5 μ g/ml CRP or 1 U/ml thrombin for 15 min at room temperature. The reaction was stopped by addition of 400 μ l ice-cold PBS and samples were analysed within 30 min. For estimation of surface expression levels of total α IIb β 3 integrin, CD41 was stained with PE-conjugated anti-CD41. Flow cytometry was performed on a FACSCalibur flow cytometer (BD Biosciences), using CellQuest version 3.1f software (BD Biosciences) and total 20,000 events per sample were collected.

Measurement of ATP secretion

ATP secretion was measured using CHRONO-LUME reagent according to the manufacturer's protocol. 5 μ l luciferase-luciferin were added directly to the cuvettes under stirring conditions (1000 rpm) and 5 μ g/ml CRP or 1 U/ml thrombin were added to activate platelets for 1 min. The luminescence intensity was measured at a luminescence setting of 0.05x.

Platelet spreading and adhesion assay

Coverslips were coated with either 0.1 mg/ml fibrinogen or 50 μ g/ml CRP overnight, followed by non-specific blocking step with 2% BSA for 1 hour. Platelets were resuspended to 2×10^7 /ml in modified Tyrodes-HEPES buffer and 500 μ l dispensed onto the coverslip mounted into a live-cell microscopy chamber. Adhesion and spreading of platelets was observed by differential interference contrast (DIC) microscopy with a wide-field micro-

scope DM IRB attached to an ORCA ER camera (63x/1.40 NA oil objective) (Leica). 5 images in different random parts of the cover slip area were taken at 15, 30 and 45 min, and processed with OpenLab 4.03 (Improvision) and Adobe Photoshop software. The surface area and number of adherent platelets was estimated using Image J software.

Immunoblotting

For stimulated samples, 2×10^8 /ml of washed platelets were activated with 5 μ g/ml CRP or 1 unit/ml thrombin in the presence of 2 mM CaCl₂ for 3 min and solubilised in Laemmli sample buffer. Proteins were resolved by electrophoresis in 9.5% (for PKC isoform estimation) or 16% (for SNAP-23 Ser95 phosphorylation estimation) SDS-polyacrylamide gels. Samples were then transferred to polyvinylidene difluoride membranes, blocked with 10% bovine serum albumin, and subjected to immunoblotting with anti-PKC α , β , δ , or θ antibodies, SNAP- antibody or phospho-SNAP-23-Ser95 antibody.

Thrombus formation under flow in vitro

Flow-induced thrombus formation was assessed basically as described before (56). Heparin/PPACK-anticoagulated mouse blood was flowed over immobilised collagen through a parallel plate perfusion chamber, at a shear rate of 1000 s^{-1} for 4 minutes. Where indicated ADP (200 μ M in saline) was co-infused at a 10% rate, immediately before the blood reached the flow chamber. For each perfusion surface, phase-contrast images from 10 random microscopic fields were collected. Surface coverage was analyzed using Image-Pro software version 4.1 (SILVERSPRING, MD, USA). The average percentage area covered by adherent platelets was measured by automated setting of masks for the ranges of grey levels corresponding to the presence of platelets and thrombi. For each image, the (blinded) observer needed to approve the mask settings. Averaged pixel fractions of the masks from 10 images were considered as a best estimate of the surface area coverage with thrombi. Aggregate volume was also estimated by confocal laser scanning microscopy, using a BioRad 2100 multiphoton system, as described before (57). Platelet aggregates that formed on collagen (4 min, shear rate 1000 s^{-1}) were post-labelled with FITC-anti-mouse CD62 mAb (1:100). Confocal stacks were recorded for measurement of the volume of individual platelet aggregates. Analysis of confocal images (grey level bit maps) and 3D reconstruction of images were with LaserPix software (Media Cybernetics).

Detection of occult fecal blood

The presence of occult fecal blood was detected by 'Hemdetect' (DIPRO med, supplied by Autogen Bioclear) on freshly obtained stool samples.

Electron microscopy

PRP was collected and spun at 590xg for 5 min. From the platelet pellet the supernatant was removed and the pellet was fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4, PB). The pellet was washed in PB and then incubated in 1% osmium tetra oxide in PB for 30 min. After washing in PB and de-ionised water the pellet was incubated in 3% uranyl acetate

in de-ionised water for 30 min. After a wash with de-ionised water the pellet was dehydrated in a graded series of ethanol (70, 80, 90, 96, 100, and 100% each step 10 min). After removal of the 100% ethanol, the pellet was incubated with pure Epon for 2 h at room temperature. Thereafter the Epon was replaced with fresh Epon and this was hardened overnight in a 60 °C oven. Ultrathin counterstained sections were imaged at a Philips CM100 equipped with a side-mount Megaview III camera (Olympus Soft Imaging Solutions).

To determine the dense and α -granule content, total numbers of granules in equivalent-sized fields of view were counted. For each genotype, 25–30 randomly chosen fields of view were examined. All microscopic images were taken at the same magnification 19000x and the numbers of cells per field of view were equivalent between wild type and *Prkca*^{-/-} preparations.

Analysis of bleeding time

Experiments were conducted on 25–35 g male and female mice. Mice were anesthetized (ketamine 75 mg/kg; medetomidine 1 mg/kg intraperitoneally) and a transverse incision was made with a scalpel at a position where the diameter of the tail was 2.25 to 2.5 mm. The tail was immersed in normal saline (37 °C) in a hand-held test tube and the time from incision to cessation of bleeding was recorded.

Intravital microscopy of thrombus formation in vivo

Intravital microscopy was performed essentially as described previously (44). Experiments were conducted on 25–30 g male *Prkca*^{-/-} mice and their littermate matched control wild types. All procedures were undertaken with United Kingdom Home Office approval in accordance with the Animals (Scientific Procedures) Act of 1986 (Project License No's: 40/2212, 40/2749 and 30/2386). Anesthesia was induced by intraperitoneal ketamine (100 mg/kg Vetalar; Pharmacia and Upjohn Ltd) and 2% xylazine (20 mg/kg; Millpledge Pharmaceuticals). The left cremaster muscle was exteriorized and spread flat over an optically clear coverslip and continuously superfused. High speed intravital microscopy experiments were performed as previously described by Falati et al. (43).

Platelets were labelled fluorescently with Alexa-488-conjugated goat anti-rat antibody (Molecular Probes, Eugene, OR, USA) and rat anti-murine CD41 antibody (BD Biosciences Pharmingen) infused via a carotid cannula. Thrombi were induced in arterioles with a diameter of 25–35 μ m by a nitrogen ablation laser (Micropoint, Photonic Instruments), which introduced the laser through the microscope objective. Brightfield and fluorescent images were captured simultaneously for 4–5 min, and multiple thrombi were generated with a distance of at least 200 μ m between them and upstream to previous injuries. The integrated intensity value for the growing thrombus was plotted against time and peak size of thrombus and time taken to reach peak was determined.

Statistics

Statistical analyses were carried on raw data using unpaired t-test and $p < 0.05$ was considered statistically significant. Values are expressed as means $\pm$ SEM. For all data "n" is number of mice tested.

Results

Major role for PKC α in inside-out regulation of α IIb β 3, but no significant role in outside-in signaling through this integrin

The PKC family of kinases has been implicated in agonist-induced α IIb β 3 integrin activation (36), and although an elegant reconstruction study showed a critical role for PKC α in regulating the integrin (33), an involvement of PKC α in this event in platelets has not yet been addressed directly and definitively. Here we report, for the first time in platelet functional and thrombosis analysis, data from a mouse gene knockout lacking PKC α . Supplementary Fig. 1 shows baseline data demonstrating lack of expression of PKC α , but normal expression of other platelet PKCs (β , δ , and θ) in *Prkca*^{-/-} platelets. Figure 1A shows that both CRP (5 μ g/ml) and thrombin (1 unit/ml) produced significantly greater α IIb β 3 activation in wild type platelets compared to *Prkca*^{-/-}. This suggested that PKC α was a major regulator of inside-out signaling to α IIb β 3, although the residual response in the absence of PKC α suggested a component independent of this kinase. By way of control, supplementary Fig. 2 shows no significant difference in surface expression of α IIb (CD41) in *Prkca*^{-/-} vs wild type, irrespective of activation state of the platelets and irrespective of agonist (CRP or thrombin).

Supplementary Figure 1. Expression of PKC isoforms in *Prkca*^{-/-} mouse platelets. Whole cell lysates from *Prkca*^{-/-} mouse platelets were analysed by western blotting with anti-PKC α , PKC β , PKC δ , and PKC θ antibodies. PVDF membranes were stripped and re-probed with anti- α -tubulin to confirm equal total protein loading. Blots shown are representative of three independent experiments.

It has been shown recently that PKC β and PKC θ play critical roles regulating outside-in signaling from α IIb β 3 (37,38). Hence in mouse platelets lacking either PKC β or PKC θ , cell spreading on fibrinogen-coated surfaces was ablated. Additionally, we had recently shown that absence of PKC δ caused platelets to spread on CRP- or collagen-coated surfaces with markedly more sustained generation of filopodia than wild type control platelets. It was important therefore to assess the role of PKC α in adhesion and spreading on either fibrinogen or CRP, and so we measured the number and surface area of adherent platelets by DIC microscopy over a 40 min period. Figure 1(B-D) shows that platelets lacking PKC α have no significant defect in their ability to spread on either fibrinogen-coated or CRP-coated surfaces, and importantly in the case of CRP there was no evident sustained development of filopodia. In addition, although Figures 1C and 1D show a trend towards decreased adhesion of platelets to both surfaces in *Prkca*^{-/-} platelets, significance was achieved only at a single time point (30 min; 90.31 $\pm$ 6.9 platelets/field of view in wild type compared to 62.18 $\pm$ 5.6 platelets/field of view in *Prkca*^{-/-} mice; mean $\pm$ SEM, $p < 0.05$) in platelets adherent to fibrinogen.

Supplementary Figure 2. Platelet surface expression of CD41 is not affected by PKC α knock-down. Washed platelets from wild type (WT) or *Prkca*^{-/-} mice were labelled with PE-anti-CD41 antibody, stimulated with CRP (5 μ g/ml) or thrombin (1 unit/ml) for 15 min and immunofluorescence intensity measured by flow cytometry. In (A), the data presented are geometric means as percentages of basal non-stimulated levels (error bar represents SEM, $n=4$). Inset panel shows geometric mean of fluorescence (arbitrary fluorescence units) of basal platelets from either wild type or *Prkca*^{-/-} mice. No difference was seen between wild type and *Prkca*^{-/-} platelet expression of CD41, and PKC α expression plays no role in regulating CD41 surface expression upon stimulation with thrombin or CRP. Data shown are mean $\pm$ SEM, $n=4$. Additionally, no difference was seen between basal and CRP- or thrombin-stimulated platelets, from either wild type or *Prkca*^{-/-} mice, in staining with an isotype-matched control antibody (PE-conjugated rat IgG1; data not shown). In (B), representative frequency histograms are shown for CD41 expression from wild type (upper panel) and *Prkca*^{-/-} (lower panel) platelets, either basal or stimulated with CRP (5 μ g/ml) or thrombin (1 unit/ml) for 15 min as indicated. Data are representative of 4 independent experiments. For color version, see page 186.

PKC α is a major regulator of dense and α -granule secretion

Figure 2A shows that *Prkca*^{-/-} platelets also exhibit a marked reduction in CRP- and thrombin-induced ATP secretion relative to wild type controls (40.9 $\pm$ 7.0 nM ATP in WT vs 8.9 $\pm$ 3.4 nM ATP in *Prkca*^{-/-} for CRP, $p < 0.05$, and 61.1 $\pm$ 14.7 nM in WT vs 10.8 $\pm$ 5.7 in *Prkca*^{-/-} for throm-

bin, $p < 0.05$). The defect in granule secretion was not restricted to dense granules however but also involved regulation of α -granules. Stimulation with CRP induced P-selectin surface expression in wild type platelets which was significantly greater than that in *Prkca*^{-/-} platelets (WT: $411.9 \pm 145.3\%$ over basal; *Prkca*^{-/-}: $130.7 \pm 14.6\%$ over basal). A similar marked knockdown of P-selectin exposure was observed after thrombin activation (Fig. 2B) ($705 \pm 235.7\%$ in WT vs $113.9 \pm 5.5\%$ in KO, $p < 0.05$), suggesting that PKC α plays a major role in platelet α -granule and dense granule release.

Figure 1. PKC α plays a major role regulating inside-out signaling to integrin $\alpha\text{IIb}\beta_3$, but no significant role in outside-in signaling or in adhesion to fibrinogen or collagen. (A) Washed platelets from wild type (WT) or *Prkca*^{-/-} mice were labelled with PE-anti $\alpha\text{IIb}\beta_3$ antibody, stimulated with CRP (5 $\mu\text{g}/\text{ml}$) or thrombin (1 units/ml) for 15 min and immunofluorescence intensity measured by flow cytometry. The data presented are geometric means as percentages of basal non-stimulated levels (error bars represent SEM, $n=3$, $*p < 0.05$). (B)-(D) Washed platelets from wild type (WT) or *Prkca*^{-/-} mice were added to CRP or fibrinogen-coated coverslips, and the levels of spreading/adherence were analysed. (B) Representative images of murine platelets 40 min after addition to CRP- or fibrinogen-coated surfaces as indicated. Images were taken under 63x oil immersion and the bars represent 5 μm . (C) & (D) Time course of WT and *Prkca*^{-/-} platelet adherence (upper panel; number of platelets estimated per field of view at 63x magnification) and spreading (as assessed by measurement of cell surface area, lower panel) on CRP-coated (C) and fibrinogen-coated (D) surface. Data shown are mean $\pm$ SEM, $n=4$, $*p < 0.05$.

It has been demonstrated that SNAP-23, a member of SNARE traffic and fusion protein complex, undergoes PKC-dependent phosphorylation during agonist-stimulated platelet degranulation, and *in vitro* PKC α was one of the isoforms able to phosphorylate recombinant SNAP-23 (39). We therefore assessed the contribution of PKC α to phosphorylation of SNAP-23 on Ser95, the site reported to be phosphorylated following thrombin stimulation (40). Supplementary Fig. 3A shows a time course of phosphorylation of SNAP-23 from wild type mouse platelets. This time course correlated well with release of ATP from those platelets stimulated with thrombin. We then showed that both CRP and thrombin activation led to a potent increase in SNAP-23 phosphorylation at Ser95, which was considerably, although not completely, inhibited by absence of PKC α (Supp. Fig. 3B). PKC α -mediated phosphorylation of SNAP-23 may therefore play a role in regulating secretion or SNARE recycling.

Supplementary Figure 3. SNAP-23 phosphorylation on Ser95 correlates with dense granule secretion and *Prkca*^{-/-} platelets. (A) Washed platelets from wild type mice were stimulated with thrombin (0.5 units/ml) and (i) secretion of ATP assessed by luminometry and (ii) phosphorylation of SNAP-23 on Ser95 assessed by immunoblotting and densitometry. Data shown are (i) increase in ATP concentration at time points after administration of thrombin, as shown, normalised to the maximum ATP release, and (ii) SNAP-23 pSer95 phosphorylation band density normalised to the maximum phosphorylation. Data are mean $\pm$ SEM (n=3). (B) Top panel: washed platelets from wild type (WT) or *Prkca*^{-/-} were stimulated with 5 μ g/ml CRP or 1 unit/ml thrombin for 3 min. Whole cells lysates were analysed for the expression of total SNAP-23 and phospho-SNAP-23-Ser95. Bottom panel: densitometric analysis of phospho-SNAP-23-Ser95 immunoblot. The immunoblots shown are representative of 3 independent experiments.

It was possible that the secretory defect seen in *Prkca*^{-/-} platelets may be as a result of a granule developmental problem. Transmission electron microscopy (TEM) analysis of platelet ultrastructure showed an abnormality in the number of dense granules in *Prkca*^{-/-} platelets, characterised by ~3.7-fold reduction compared to wild type platelets (Fig. 3). Dense granule biogenesis in megakaryocytes or peripheral distribution during proplatelet development could be the key points of regulation by PKC α , and this may additionally explain the major defect in dense granule secretion seen in *Prkca*^{-/-} platelets. TEM quantification of α -granule numbers however did not detect any substantial difference between wild type and *Prkca*^{-/-} platelets, indicating that the defect in α -granule secretion is due to an essential role played by PKC α in regulation of secretion of these granules, rather than their biogenesis.

Figure 2. Key role for PKC α in secretion of dense and α -granules. (A) Washed platelets from wild type (WT) or *Prkca*^{-/-} mice were stimulated with collagen-related peptide (CRP, 5 μ g/ml) or thrombin (0.25 units/ml) and secretion of ATP assessed by luminometry. Data shown are maximal increase in ATP concentration, and are mean $\pm$ SEM (n=3, *p<0.05). (B) Platelets from wild type (WT) or *Prkca*^{-/-} mice were labelled with FITC-C62P antibody, stimulated with CRP (5 μ g/ml) or thrombin (1 units/ml) for 15 min and fluorescence intensity measured by flow cytometry. The data presented are geometric means as percentages of basal non-stimulated levels (error bars represent SEM, n=3, *p<0.05).

Platelet aggregation deficit in $Prkca^{-/-}$ platelets is rescued by exogenous ADP

It was now important to determine whether the deficits in secretion and activation of integrin $\alpha IIb\beta_3$ would translate into functional deficits in platelet aggregation and thrombus formation. Figure 4 shows concentration-response relationships for platelet aggregation activated by CRP (A) or thrombin (B). At submaximal concentrations of either agonist, absence of PKC α has a marked effect upon the aggregation response, such that the aggregation responses to 1.25 $\mu\text{g/ml}$ CRP or 0.065 units/ml thrombin are almost ablated in $Prkca^{-/-}$ platelets. Importantly however, responses may be effectively rescued by addition of exogenous ADP (10 μM), added simultaneously with CRP or thrombin. This indicates that the important functional deficit in $Prkca^{-/-}$ platelets is the lack of dense granule secretion. The effect upon aggregation was also relative, since higher concentrations of either CRP or thrombin were able to restore aggregations to wild-type values.

Figure 3. PKC α regulates dense granule, but not α -granule, biogenesis. Washed platelets from wild type (WT) or $Prkca^{-/-}$ mice were examined by transmission electron microscopy, and the number of dense granules (black arrows) and α -granules (white arrows) was quantified as described in Methods. For (A), images of wild type (left-hand panel) or $Prkca^{-/-}$ (right-hand panel) platelets are representative of 3 independent experiments, and scale bar is 1 μm , magnification 19000x. For (B), dense granules (left-hand panel) and α -granules (right-hand panel) were counted per field of view (25-30 fields of view per preparation), and shown as mean $\pm$ SEM number of granules per μm^2 ($n=3$, * $p<0.05$).

Pharmacological inhibition of PKC β reveals redundancy with PKC α for regulation of platelet aggregation

Given that higher concentrations of agonists are capable of overcoming the aggregation deficits seen in the absence of PKC α , it was important to address whether there may be functional redundancy between PKC α and its closely related family member, PKC β . To

assess this possibility we pre-incubated platelets with the inhibitor of classical PKC isoforms Gö6976 at its maximally effective concentration (1 μ M), which completely abrogated CRP-induced aggregation both in wild type and *Prkca*^{-/-} mouse platelets (Fig. 5A). This confirmed an indispensable role for classical PKC isoforms in GPVI-mediated aggregation, and suggested a potential redundancy between PKC α and PKC β in regulating aggregation. This redundancy was demonstrated by using the PKC β -selective inhibitor, LY333531 (ruxostaurin), which at a maximally effective concentration (10 μ M) had no significant effect upon aggregation of wild type platelets to CRP, but was markedly inhibitory to this response in platelets lacking PKC α (Fig. 5B). This was paralleled for thrombin (0.5 units/ml), where 10 μ M LY333531 had no effect upon wild type platelet aggregation response, but markedly inhibited the response in *Prkca*^{-/-} platelets (data not shown). Interestingly, for weaker agonists such as the thromboxane analogue U46619, aggregation was ablated in platelets lacking PKC α , although shape change in these platelets was unaffected (Fig. 5C). This is consistent with pharmacological data that show a critical role for PKC in platelet aggregation induced through the thromboxane receptor system (41). These data therefore suggested that, for weak agonists, PKC α plays an essential role, whereas redundancy between PKC α and PKC β exists for strong agonists in regulating the platelet aggregation response.

Figure 4. ADP rescues deficient aggregation responses to CRP or thrombin in *Prkca*^{-/-} platelets. Washed platelets from wild type (WT) or *Prkca*^{-/-} mice were stimulated with various concentrations of collagen-related peptide (CRP) or thrombin with or without simultaneous addition of ADP (10 μ M) and aggregation assessed turbidimetrically. Aggregation traces shown are representative of 4 independent experiments.

Ablation of PKC α markedly attenuates thrombus formation in vitro and in vivo

Finally, it was important to determine the role played by PKC α in thrombus formation both in vitro and in vivo. Figure 6A (upper panels) shows a major role for PKC α in vitro in mediating this function, where the images reveal no reduction in primary adhesion of platelets to

the collagen-coated surface, consistent with the absence of effect upon static adhesion shown in Fig. 1, but a marked reduction in aggregated platelet thrombus formation on top of the initially adherent cells. In order to confirm, in a quantitative manner, the lack of effect of PKC α ablation on primary adhesion, Fluo-4-labeled platelets were back-added to the same blood, and used for measurements of platelet adhesion during flow over a collagen surface, as we previously described (42). Fluo-4-labelled cells flowing across the field of view were counted, and the percentage of these cells that adhere stably (adherent for >20 s) was estimated. At a shear rate of 1000 s^{-1} , $81.7 \pm 3.6\%$ of wild type and $78.1 \pm 3.0\%$ of *Prkca*^{-/-} platelets became stably adherent (mean $\pm$ SEM, $n=5$; $p>0.05$), indicating no significant difference in initial stable adhesion to collagen. Also, in order to confirm that in the absence of PKC α the 3-dimensional build up of thrombus formation was markedly impaired, reconstructed images were generated from confocal Z-stacks (as described in Methods) and platelet aggregate (thrombus) volumes estimated. The mean volume of representative platelet aggregates on the coverslips after flow at 1000 s^{-1} was $570 \pm 168 \mu\text{m}^3$ and $72.3 \pm 7.4 \mu\text{m}^3$ for wild type and *Prkca*^{-/-} blood, respectively (mean $\pm$ SEM, $n=5$; $p<0.05$). The major secretory defect seen in *Prkca*^{-/-} platelets was likely to be responsible for the impairment of thrombus formation, since addition of ADP (20 μM) to the blood in the flow chamber was capable of rescuing thrombus formation (Figs 6A (lower panels) & 6B). This indicated that the thrombotic process under flow conditions markedly relies upon the contribution to secretion made by PKC α .

Figure 5. Combined pharmacological and genetic approaches reveal redundancy between PKC α and PKC β for regulation of platelet aggregation. (A) & (B) Washed platelets from wild type (WT) or *Prkca*^{-/-} mice were pre-treated (10 min) either with the classical PKC isoform inhibitor G66976 (1 μM) (A), the PKC β -selective inhibitor LY333531 (10 μM) (B), or with DMSO as vehicle control and stimulated with collagen-related peptide (CRP, 5 $\mu\text{g}/\text{ml}$) and aggregation was monitored by turbidimetric aggregometry. Traces shown are representative of 5 independent experiments. (C) Washed platelets from wild type (WT) or *Prkca*^{-/-} mice were stimulated with U46619 (10 μM) and aggregation assessed turbidimetrically. Traces shown are representative of 4 independent experiments.

It was important finally to assess the role *in vivo* of PKC α in regulating thrombus formation. Figure 7 shows data where we apply a laser-induced endothelial injury model in

cremaster muscle arterioles, described previously (43,44). A marked difference is evident between wild type controls and mice lacking PKC α (Fig. 7), since in the absence of PKC α thrombus formation is delayed and with fewer accumulated platelets (see Supplementary Videos 1 & 2). Importantly however the ability of *Prkca*^{-/-} to undergo normal hemostasis was demonstrated by the lack of alteration in tail bleed time (Fig. 7E) and by the lack of any fecal occult blood (data not shown). This may indicate that compensatory mechanisms are sufficient to allow normal haemostasis, but are not sufficient to support normal thrombus formation, and may reflect the observation that *Prkca*^{-/-} platelets adhere normally to collagen under static and flow conditions (Figs 1 & 6), but have a marked reduction in subsequent platelet-platelet interaction (Fig. 6).

Figure 6. PKC α regulates thrombus formation in vitro. Heparin/PPACK-anticoagulated blood from WT or *Prkca*^{-/-} mice was flowed over collagen (shear rate 1000 s⁻¹). For lower panels of (A) and (B), ADP solution was co-infused at a 10% flow rate (20 μ M ADP, final concentration). (A) shows representative phase-contrast images after 4 min. Images were taken under 63 $\times$ oil immersion and the bars represent 20 μ m. (B) shows surface area coverage with thrombi (mean $\pm$ SEM, $n \geq 3$, * $p < 0.0001$).

Discussion

Platelets play a central role in mediating atherothrombosis and are therefore the target of numerous therapies aimed at reducing platelet activity, particularly in the prevention of coronary artery thrombosis in heart attacks (45). Protein kinase C is established, largely by pharmacological studies, as a major regulator of multiple platelet activities (2), and it is increasingly clear that the different isoforms of PKC expressed in platelets perform distinct functions. There is a difficulty of interpretation of data from some pharmacological studies however because of the lack of selectivity of the reagents available to target specific PKC isoforms. Here we used a genetic approach to demonstrate definitively, for the first time, the role played by PKC α in regulating platelet function and thrombus formation. Importantly the study revealed a key role for PKC α in regulating granule biogenesis and exocytosis, which was essential for thrombus formation, since ablation of thrombus formation in *Prkca*^{-/-} platelets could be rescued by addition of exogenous ADP. The findings reveal PKC α to be a potential drug target for antithrombotic therapy, since selective inhibitors would

exert a major effect upon thrombus formation whilst sparing primary platelet adhesive functions.

Figure 7. In vivo thrombus formation is impaired in the absence of PKC α , but tail bleeding time is normal. (A)-(D) Mice were either *Prkca*^{-/-} or littermate-matched wild-type controls. Platelets were labelled in vivo with Alexa 488, as described in Methods. (A) Platelets (green) composited with bright-field images (black/white) of the cremaster arteriole were viewed at $\times 40$ magnification and images were acquired using a digital CCD camera (SensiCam II, Cooke Corporation) with a 640 $\times$ 480 pixel array. (B) Traces shown are median integrated platelet fluorescence of 15 thrombi induced in 3 or more mice for each group. Fluorescent intensity of platelets in arbitrary units is presented as a function of time. (C) Data are presented as a scatter diagram, and the horizontal bar is mean of 15 thrombi induced in at least 3 mice for each group. There is a significant reduction in thrombus intensity in *Prkca*^{-/-} by comparison with wild type controls (mean *Prkca*^{+/+}, 652200; mean *Prkca*^{-/-}, 279600; $p < 0.05$). (D) Time to reach peak thrombus size also significantly differed from wild type controls, with mean data shown by the horizontal bars (mean *Prkca*^{+/+}, 68.1 s; mean *Prkca*^{-/-}, 115.2 s; $p < 0.01$). (E) Mice were anesthetized and a transverse incision made with a scalpel at a position where the diameter of the tail was 2.25 to 2.5 mm. The tail was immersed in normal saline (37 $^{\circ}$ C) in a hand-held test tube. The time from incision to cessation of bleeding was recorded, and mean times are shown as horizontal bars. No significant difference was seen comparing wild type mice (mean 140 s) with *Prkca*^{-/-} (mean 117 s; $p > 0.05$). For color version, see page 187.

It has recently been shown pharmacologically that PKC isoforms exert dual control of thrombus formation by mediating secretion and integrin activation under flow, whilst suppressing phosphatidylserine exposure and subsequent thrombin generation and coagulation (7). There is incomplete and largely indirect evidence that, of the various isoforms of PKC expressed in platelets, PKC α plays a role in regulation of integrin α IIb β 3 activation (36). The study by Han et al. (33) elegantly showed that PKC α was required for integrin α IIb β 3 activation in a reconstituted cell system rather than in the platelet. The study by Tabuchi et al. (18) used an in vitro permeabilised platelet system to investigate receptor-independent calcium-mediated activation of platelet aggregation. Although this latter study demonstrated that added purified PKC α was able to support a calcium-induced platelet aggregation response, the approach has limitations such that receptor-mediated activation is lost.

Nonetheless, these studies had provided indications that PKC α may regulate platelet integrin activation and aggregation, and it was therefore now essential to perform a genetic-based definitive study of the role played by PKC α in mediating these events.

The marked knockdown in activation of integrin $\alpha\text{IIb}\beta_3$ in response either to thrombin or CRP in platelets lacking PKC α , paralleled a deficit in the ability of these platelets to undergo aggregation at submaximal concentrations of agonist. This was consistent with the impaired ability of *Prkca*^{-/-} platelets to form a thrombus, in blood flowing over a collagen-coated surface. The effect on aggregation could however be overcome by increasing the concentration of agonist, and the reasons for this may derive from several factors. First, it is known that integrin $\alpha\text{IIb}\beta_3$ activation in response to strong agonists is in part regulated independently of PKC (3,46). Further, studies with integrin blockers have shown that not all $\alpha\text{IIb}\beta_3$ receptors are required to be activated (e.g. by the PKC-independent pathway) for a full platelet aggregation response in the platelet aggregometer (47). Thus it may be possible to achieve a maximal aggregation response with a markedly reduced expression of activated integrin. In contrast, in thrombus formation in flowing whole blood, stable platelet aggregation critically depends on limited levels of autocrine produced ADP, and hence limited and reversible integrin activation (48). Under these more physiological conditions, the number of activated integrin receptors may be low, and therefore the marked reduction in integrin activation seen in *Prkca*^{-/-} platelets may make a significant reduction in the ability of platelets to form thrombi. Additionally we provided evidence for redundancy between PKC α and PKC β in Fig. 5, where selective inhibition of PKC β only significantly attenuated aggregation responses in *Prkca*^{-/-} platelets, not wild type platelets, indicating redundant functions of these two isoforms. It is also significant to note that, in assays for occult blood in feces, no gastrointestinal bleeding was detected in either wild type (n=5) or *Prkca*^{-/-} (n=6) mice. Together with the data shown in Fig. 7, where no difference in tail bleeding time is seen in *Prkca*^{-/-} compared to wild type controls, this also implies redundancy of signaling molecules for regulation of hemostasis in vivo.

There is significant contribution to the effects of PKC α ablation on aggregation and thrombus formation by regulation of granule secretion, since these processes greatly rely upon autocrine ADP release (49). Clearly both dense and α -granule secretion are markedly disrupted in platelets lacking PKC α , and evidence that this is functionally critical is provided by the rescue experiments, shown in Fig. 4 for aggregation and Fig. 6 for in vitro thrombus formation, where addition of exogenous ADP recovers the deficits in these responses seen in *Prkca*^{-/-} platelets.

The role of unspecified PKC isoforms as a family in the regulation of secretion had been shown pharmacologically by several groups (3-6). The role of the PKC α isoform in agonist-independent secretion has been demonstrated previously in an artificial permeabilised platelet system (17). Although valuable to indicate the role of PKC α , it was now important to address the issue by a definitive genetic approach. Indeed the only study to date to address specific PKC isoform function in secretion in platelets, in a combined genetic and pharmacological approach, had shown a major discrepancy in results obtained by the two approaches for PKC δ . Pula et al. (15) had shown that genetic ablation of PKC δ had no signif-

icant effect upon dense granule secretion whereas in that study, and previous ones (9,16), the PKC δ -selective inhibitor rottlerin had been shown to enhance dense granule secretion in response to collagen and alboaggregin, operating through the GPVI receptor. The present results provide direct evidence that PKC α is a major regulator of secretion of α -granules, since P-selectin expression is reduced almost to basal levels in *Prkca*^{-/-} platelets in response either to CRP or thrombin. Importantly we show that α -granule numbers are equivalent between *Prkca*^{-/-} and wild type platelets, and therefore the secretion defect for this granule type reflects a genuine deficiency in the secretory pathway. For dense granules, this study shows an additional knock-down in numbers of granules in *Prkca*^{-/-} platelets. This suggests a role for PKC α in biogenesis of dense granules, and is therefore an area that requires further analysis. A number of different elements of the secretory machinery, including SNAP-23, SNAP-25, Munc18a and b, syntaxin 2 and Rab6, have been reported to be PKC substrates (40,50-52), and thereby their phosphorylation may influence secretion. In this study then, phosphorylation of SNAP-23 serves as a potential marker for these events, and may contribute functionally to the process of secretion. However, although SNAP-23 phosphorylation on Ser95 is markedly reduced in *Prkca*^{-/-} platelets, and this may play a role in regulating α -granule secretion, its role in dense granule secretion cannot be clarified from this study.

In summary, we have shown PKC α to play major roles in regulating platelet secretion of α -granules, biogenesis and secretion of dense granules, regulation of integrin α IIb β 3 and thrombus formation in vitro and in vivo. PKC α also regulates platelet aggregation, although the defect in *Prkca*^{-/-} platelets is relative since high concentrations of agonists overcome the deficit, and there is evident redundancy of the action of PKC α with the other expressed classical PKC isoform in platelets, PKC β . PKC α plays no significant role in regulating adhesion of platelets to collagen-coated surfaces under static or flow conditions, and does not regulate platelet spreading response or outside-in signaling through integrin α IIb β 3. It could be argued that because PKC α is the most highly expressed of the PKC isoforms in platelets, its role in platelets may be wide-ranging and effectively mask that of other PKC isoforms that are expressed to lower levels. The evidence presented here however suggests highly specific roles for PKC α , since for instance its absence has no significant effect upon platelet adhesion and spreading on collagen, but markedly suppresses thrombosis. Additionally, if it were so predominant functionally in platelets, knockout of the other PKC isoforms would be predicted not to have functional effects. This is clearly not the case since we have shown absence of PKC δ to enhance platelet responses to collagen through enhanced filopodia formation (15), and we have more recently shown absence of PKC θ to enhance α -granule secretion and integrin α IIb β 3 activation in response to GPVI agonists (53). The group of Shattil have also shown absence of PKCs β and θ to ablate outside-in signaling through integrin α IIb β 3 (37,38). These data therefore demonstrate specific functional roles for the different PKC isoforms, including for PKC α in regulating secretion in particular, such that redundancy of activity is not apparent for specific functions.

Genome-wide association analyses of major human diseases have been conducted recently, based upon technical advances in high throughput microarray analyses of SNPs. These studies are introducing major new leads in genes that may be related to disease, and

although PKC α is not in the top ranking of genes associating with coronary artery disease, a cluster of SNPs in this gene with a maximal p value just over 10^{-3} (SNP rs12600582, intronic, minor allele frequency of 0.233 in Europeans) (34,35) may indicate some significance in this disease of polygenic cause. For these reasons PKC α may represent a drug target for antithrombotic therapy, with inhibitors exerting an effect upon thrombus formation but sparing primary platelet adhesive functions. PKC α is already a target for the drug aprinocarsen, an antisense oligonucleotide therapy used in the treatment of specific neoplastic conditions (54,55), and it will now be important to assess whether this or other small molecule-based approaches may represent opportunities in the development of platelet-based antithrombotic drugs in the management of coronary artery disease and other arterial thrombotic diseases.

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Chapter 5

Functional divergence of platelet protein kinase C
isoforms in thrombus formation on collagen

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Abstract

Arterial thrombosis, a major cause of myocardial infarction and stroke, is initiated by activation of blood platelets by subendothelial collagen. The protein kinase C family centrally regulates platelet activation, and it is becoming clear that the individual PKC isoforms play distinct roles, some of which oppose each other. Here, for the first time, we address all four of the major platelet-expressed PKC isoforms, determining their comparative roles in regulating platelet adhesion to collagen and their subsequent activation under physiological flow conditions. Using mouse gene knockout and pharmacological approaches in human platelets we show that collagen-dependent α -granule secretion and thrombus formation are mediated by the conventional PKC isoforms, PKC α and PKC β , whereas the novel isoform, PKC θ , negatively regulates these events. PKC δ also negatively regulates thrombus formation but not δ -granule secretion. In addition, we demonstrate for the first time that individual PKC isoforms differentially regulate platelet calcium signaling and exposure of phosphatidylserine under flow. Whereas platelet deficient in PKC α or PKC β showed reduced calcium signaling and phosphatidylserine exposure, these responses were enhanced in the absence of PKC θ . In summary therefore, this direct comparison between individual subtypes of PKC, by standardized methodology under flow conditions reveals that the four major PKCs expressed in platelets play distinct non-redundant roles, where conventional PKCs promote and novel PKCs inhibit thrombus formation on collagen.

Introduction

Blood vessel damage or rupture of an atherosclerotic plaque exposes subendothelial collagen. Platelets rapidly adhere to collagen and are activated, inducing granule secretion and integrin $\alpha\text{IIb}\beta_3$ activation, and leading to large platelet aggregates. In addition, sustained intracellular calcium signaling induces phosphatidylserine (PS) exposure, which accelerates thrombin generation and coagulation. Together, these processes can result in formation of occlusive thrombi, leading to myocardial infarction or stroke.

The protein kinase C (PKC) family is comprised of multiple isoforms, which are responsible for a substantial part of the serine/threonine phosphorylation events in many cell types including platelets. The PKC isoforms are grouped into three classes: conventional forms (α , β I, β II, γ) that are activated by Ca^{2+} /diacylglycerol, novel forms (δ , ϵ , η , θ) activated by diacylglycerol alone, and atypical forms (ζ , ι/λ), which are diacylglycerol-independent (1). Human and mouse platelets highly express the conventional PKC isoforms α and β , and the novel isoforms δ and θ (2-6), while mouse platelets in addition express PKC ϵ (7,8). Early pharmacological studies, which could not distinguish between different isoforms, showed that key platelet activation processes, such as secretion, integrin $\alpha\text{IIb}\beta_3$ activation and aggregation, are positively regulated by PKC activity (9-13). However, there is also good evidence that PKC has a negative role in platelets, in particular by suppressing Ca^{2+} signal generation, for example by promoting Ca^{2+} extrusion (14,15), and desensitizing agonist receptors (16). This raised the intriguing question whether different conventional and novel PKC isoforms may have distinct or even opposing roles in the control of these platelet responses (17).

There is some evidence to support this hypothesis. In human and mouse platelets, PKC α has been proposed as a key kinase regulating α - and dense granule secretion (18,19) and platelet aggregate formation (20) in response to collagen and other platelet agonists (21). In mouse platelets, PKC β positively regulates outside-in α IIb β 3 signaling (22), but not inside-out integrin activation. However, the role of the novel PKC isoforms, PKC δ and PKC θ , is less straightforward, since both positive and negative signaling functions have been reported (8,23-25).

In light of this lack of a clear role for all PKC isoforms, which may result partly from differences in platelet preparation conditions, anticoagulant and other ex vivo handling of platelet samples by different laboratories, we sought to compare directly platelet responses in physiological flow settings in whole blood. We compared platelet function and thrombotic response to flow over collagen, using blood from mice deficient in each of the four PKC isoforms. The data point to markedly divergent and partly antagonistic roles of the conventional and novel PKC isoforms in collagen-induced platelet activation and thrombus formation. In addition, we show for the first time that individual PKC isoforms differentially regulate platelet calcium signaling and procoagulant activity.

Experimental procedures

Animals

Animal studies were approved by the local animal care and use committees. All mice were generated as described earlier for PKC α (26), PKC β (27), PKC δ (28) or PKC θ (29). In all mouse strains, platelet and erythrocyte counts in blood were in the normal range. Wildtype mice were used of the same background and same breeding program as the corresponding knockout mice.

Materials

H-Phe-Pro-Arg chloromethyl ketone (PPACK) was obtained from Calbiochem, as were Ro-318425, Gö6976, PKC β -inhibitor (3-(1-(3-Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione) and rottlerin. PKC θ -inhibitor (30) was a kind gift from Boehringer Ingelheim Pharmaceuticals. Annexin A5 labeled with fluorescein isothiocyanate (FITC) was from Nexins Research. Fura-2 and Fluo-4 acetoxymethyl esters, pluronic F-127 and Alexa Fluor (AF)647-labeled annexin A5 came from Molecular Probes. Apyrase (grade V), bovine serum albumin, dichloro fluorescein (DCF) and phorbol myristate acetate (PMA) were from Sigma. Fibrillar type I collagen (Horm) was from Nycomed. Convulxin was purified as described (31). FITC-labeled PAC1 mAb against activated human α IIb β 3 came from BD Biosciences. FITC-labeled anti-human-CD62 (P-selectin) mAb from Sanquin. FITC-labeled anti-mouse CD62 mAb was from Emfret Analytics, as was FITC-labeled JON/A mAb. Other materials were obtained from sources indicated before (32,33).

Blood collection and platelet preparation

Blood was taken from aspirin-free healthy volunteers, who gave full informed consent. Human platelet-rich plasma (PRP) and washed platelets were prepared by centrifugation

(34). Platelets were resuspended in Hepes buffer pH 7.45 (136 mM NaCl, 10 mM Hepes, 2.7 mM KCl, 2 mM MgCl₂, 1 mg/ml glucose, 1 mg/ml bovine serum albumin, 0.2 U/ml apyrase) at a concentration of 2×10^8 /ml.

Mouse blood was obtained by cardiac puncture under terminal anesthesia. For flow studies, blood was collected into 40 μ M PPACK, 5 U/ml heparin and 40 U/ml fragmin. For washed platelets, mouse blood was collected into 129 mM citrate, 1 U/ml heparin and 5 mM glucose (32). Washed cells were suspended in modified Tyrode's Hepes buffer pH 7.45 (134 mM NaCl, 20 mM Hepes, 12 mM NaHCO₃, 2.9 mM KCl, 1 mM MgCl₂, 0.34 mM Na₂HPO₄, 5 mM glucose). Platelets were counted with a Coulter counter and adjusted to the appropriate density.

Thrombus formation

Thrombus formation on collagen under flow was determined as described (32). Briefly, PPACK-anticoagulated mouse blood was flowed through a transparent, parallel-plate flow chamber (50 μ m deep, 3 mm wide), sealed with a collagen-coated coverslip, at a shear rate of 1000 s^{-1} . To measure thrombus volumes, platelets in blood were prelabeled with DCF (35). Activated platelets in thrombi were post-stained with FITC-annexin A₅ (0.5 μ g/ml), or with AF647-annexin A₅ (1:400) and FITC-labeled anti-P-selectin mAb (1:40). Phase-contrast and fluorescence images were recorded with a DM-IRB fluorescence microscope and an Orca ER camera (Leica) or with a Nikon Diaphot 200 microscope and an EM-CCD camera (Hamamatsu). Confocal images were captured in real-time using a Zeiss/BioRad E600FN multiphoton system (36). Phase-contrast and (confocal) fluorescence images were taken from at least 10 randomly chosen microscopic fields. Images were analyzed for area coverage, integrated fluorescence intensity or integrated fluorescence pixel density using ImagePro software (Media Cybernetics), as described before (34). For P-selectin staining, the extent of integrated fluorescence intensity depends on the number of platelets (i.e. the thrombus size) and the fluorescence per platelet. In contrast, integrated pixel density gives a measure of the amount of fluorescence per platelet. In control experiments, treatment of a pre-formed thrombus with thrombin resulted in a further increase (approximately 2.5-fold) in mean pixel density of P-selectin fluorescence (data not shown). Both integrated fluorescence intensity and pixel density are given in figures 2 and 4. In contrast, individual platelets stain strongly with annexin A₅ or remain unstained, since annexin A₅ polymerizes when binding to PS-exposing surfaces. The pixel density (i.e. staining per platelet) was therefore the same for all mouse strains, but the integrated fluorescence intensity is indicative of the number of PS-exposing platelets. Thrombus volume of DCF-labeled platelets was measured from confocal stacks of fluorescence images (35).

Measurement of single-cell Ca²⁺ responses under flow

Washed murine platelets were incubated with 8 μ M Fluo-4 acetoxymethyl ester and 0.2 mg/ml pluronic F-127 for 45 min at ambient temperature under gentle rotation (37). Dye-loaded platelets were added to PPACK-anticoagulated blood from the same mouse strain to give 10% labeled platelets. During the first min of high-shear blood flow, images were

captured from the collagen surface at 5 Hz. Pseudo-ratio F/F_0 values were converted into nanomolar concentrations of $[Ca^{2+}]_i$ using predefined calibration parameters (38).

Measurement of single platelet adhesion to collagen

Fluo-4-loaded platelets were added to whole blood as described above. Even in unactivated Fluo-4-loaded platelets there is basal fluorescence, which can be used to track the platelets under flow conditions, as described previously (37). Stably-adhered platelets were defined as labeled cells that remained in one position on a collagen fiber for > 30 seconds.

Measurement of PKC activity

PKC activity was determined by Ser phosphorylation of modified PKC pseudo-substrate RFARKGSLRQKNV (39), using a biotinylated mAb recognizing the phosphorylated form (Calbiochem). Washed platelets (1×10^8 /ml), pretreated with indicated inhibitors (10 min), were pelleted and immediately sonicated on ice, and then further processed as indicated by the manufacturer.

Platelet aggregation and flow cytometry

Washed platelets were pretreated for 10 min with Me2SO vehicle or indicated inhibitor. Platelet aggregation was measured under stirring by light transmission aggregometry at 37°C. Using flow cytometry, α -granule secretion was assessed as P-selectin exposure with FITC-labeled anti-human or anti-mouse CD62 mAb. Similarly, α IIb β 3 activation was assessed with FITC-labeled mAb against activation epitopes of this integrin (36).

Calcium responses in suspension

Fura-2-loaded platelets (1×10^8 /ml), prepared as described (33), were used to measure changes in cytosolic $[Ca^{2+}]_i$ by calibrated ratio fluorimetry. Time integrals of $[Ca^{2+}]_i$ (in nM) were determined as before.

Statistical analysis

Differences between experimental groups were tested for significance with the non-parametric Mann-Whitney U test, using SPSS. Differences in frequency distributions of features with mean values per field were determined by χ^2 test (34).

Results

Deficiency in conventional isoforms PKC α or β impairs collagen-induced thrombus formation and platelet activation under flow

To investigate the functional consequences of specific PKC isoform deficiency, we used an in vitro flow model of platelet-vessel wall interaction, in which whole blood is perfused over a collagen-coated surface at defined, arterial shear rate (32,37,40,41). Previous human studies demonstrated that general inhibition of PKC almost fully abrogates the formation of platelet aggregates, whereas adhered platelets show increased procoagulant activity, as assessed by labeled annexin A5, which detects exposed PS (17). During flow over collagen at

1000 s^{-1} , wildtype mouse platelets rapidly assembled into large aggregates and by contrast, after 4 min (Fig. 1A) or longer perfusion times, $\text{PKC}\alpha^{-/-}$ platelets were markedly impaired in aggregate formation, in agreement with our previous report (18). Formation of pseudopods and lamellipods of the adhered $\text{PKC}\alpha^{-/-}$ platelets was however normal. Quantitative analysis showed a reduction in platelet deposition by 70% (Fig. 1B), while morphometric analysis of the $\text{PKC}\alpha^{-/-}$ thrombi showed that mostly single cells or small-sized aggregates were present (Fig. 1C). The latter observation was confirmed by measurement of thrombus volume after perfusion, using platelets prelabeled with DCF. Mean volumes of 323 ± 76 and $89 \pm 18\text{ }\mu\text{m}^3$ for wild type and $\text{PKC}\alpha^{-/-}$ platelets, respectively, were achieved (mean $\pm$ S.E., $n \geq 34$, $p < 0.01$).

Figure 1. Deficiency of $\text{PKC}\alpha$ or β suppresses thrombus formation on collagen under flow. Whole blood from $\text{PKC}\alpha^{-/-}$, $\text{PKC}\beta^{-/-}$ or matched wildtype mice was flowed over a collagen surface at a shear rate of 1000 s^{-1} . A, Representative phase contrast images after 4 min of perfusion (bar $20\text{ }\mu\text{m}$, inserts $5\times$ magnified). B, Quantification of platelet deposition on collagen surface. C, Morphometric analysis of aggregates on collagen surface with indicated numbers of platelets per feature. Means $\pm$ S.E.M. ($n=3-6$), * $p < 0.05$, *** $p < 0.001$ vs. corresponding wildtypes; χ^2 test * $p < 0.05$.

Interestingly, deficiency in PKC β also resulted in diminished platelet deposition and formation of smaller size aggregates on collagen (Fig. 1), and thrombus volume was reduced to $95 \pm 24 \mu\text{m}^3$. Platelet deposition during thrombus formation involves both primary adhesion of platelets to collagen and platelet-platelet aggregation. Analysis of primary platelet adhesion to collagen however showed no effect of loss of either PKC α or β since fractions of stably adhered platelets were similar for wild type and PKC $\alpha^{-/-}$ blood ($80 \pm 4\%$ and $77 \pm 3\%$), as well as for wild type and PKC $\beta^{-/-}$ blood ($80 \pm 5\%$ and $81 \pm 4\%$; $n=4$ experiments, $p>0.5$). Hence, the impaired thrombus formation is not a consequence of defective adhesion to collagen but rather a result of reduced platelet-platelet cohesion.

Figure 2. Deficiency of PKC α or β suppresses collagen-induced activation of platelets in thrombi. Whole blood from indicated mice was flowed over collagen (see Fig. 1), and platelet thrombi were double-stained with FITC-anti-mouse CD62P mAb (green) and AF647-annexin A5 (red). A, Representative confocal images ($180 \times 180 \mu\text{m}$) after staining. B, Integrated fluorescence intensity presenting activated platelets in arbitrary units, to quantify cumulative expression of P-selectin and PS. Means $\pm$ S.E.M. ($n=3-5$); $**p<0.01$ vs. corresponding wildtypes. Numbers above each bar indicate the mean integrated pixel density of P-selectin staining, an indication of staining per platelet. For color version, see page 188.

To investigate whether the reduced thrombus formation was accompanied by altered platelet activation, thrombi formed on the collagen surface were labeled for the α -granule secretion marker, P-selectin, and for procoagulant activity, with PS-binding annexin A5. Fig. 2A shows images obtained after dual staining (staining with each probe individually gave similar results). For both $\text{PKC}\alpha^{-/-}$ and $\text{PKC}\beta^{-/-}$ thrombi, measurement of integrated fluorescence intensity demonstrated a dramatic reduction in surface expression of P-selectin and PS, when compared to wildtypes (Fig. 2B). Although this reduction was partly explained by the diminished platelet deposition, the P-selectin mean fluorescence intensity per pixel was also reduced, suggesting a lower degree of α -granule secretion per platelet. Interestingly, in contrast to general PKC inhibition studies (17), where greater numbers of platelets show PS exposure, fewer platelets exposed PS in the absence of $\text{PKC}\alpha$ or $\text{PKC}\beta$ compared to wild type controls (Fig. 2A).

Figure 3. Deficiency of $\text{PKC}\alpha$ or β suppresses collagen-induced Ca^{2+} signaling under flow, but not adhesion. Whole blood containing autologous Fluo-4-loaded platelets was flowed over collagen (see Fig. 1), while fluorescence images of adherent platelets were captured in real-time (5 Hz). **A**, Traces of pseudo-ratioed changes in $[\text{Ca}^{2+}]_i$ from representative, single platelets as well as mean traces (S.E.) from overlays of 30–40 platelets. **B**, Quantitative analysis of increased $[\text{Ca}^{2+}]_i$ at 30 s (white) and 60 s (gray) after initial response. No quantitative differences in response between wild type mice were seen. After 30 sec, averaged rises in $[\text{Ca}^{2+}]_i$ were 225 ± 26.3 nM for $\text{PKC}\alpha^{+/+}$ platelets, and 270 ± 33.9 nM for $\text{PKC}\beta^{+/+}$ platelets ($n=3$). Data are means $\pm$ S.E.M. ($n=30-40$, 3–4 experiments); *** $p < 0.001$ vs. corresponding wildtypes.

The results so far suggested that deficiency in PKC α or β impairs platelet activation by collagen. To investigate this further and mechanistically, we monitored Ca^{2+} signaling in collagen-adhered platelets during whole-blood perfusion (36,37). Analysis of the Ca^{2+} responses showed that although platelets from PKC $\alpha^{-/-}$ and PKC $\beta^{-/-}$ mice were still capable of prolonged rises in $[\text{Ca}^{2+}]_i$, after adhesion, these rises were lower in amplitude by comparison with those of corresponding wildtype platelets (Fig. 3A&B).

Together, these results indicate that deficiency in either PKC α or PKC β does not change primary platelet adhesion to collagen under flow, but results impaired secretion and aggregate formation and in lower GPVI-induced Ca^{2+} rises and PS exposure. Furthermore, PKC α and β isoforms appear to play non-redundant roles.

Deficiency in novel isoforms PKC δ and PKC θ enhances GPVI-induced murine thrombus formation and platelet activation under flow

To investigate the contribution of the major novel PKC isoforms in thrombus formation, flow experiments on collagen were carried out with blood from mice lacking PKC θ or δ . Platelets from PKC $\theta^{-/-}$ or PKC $\delta^{-/-}$ mice readily assembled into aggregates (Fig. 4A). In comparison to wild type controls, these thrombi covered more of the collagen surface, and more frequently consisted of larger-size aggregates (Fig. 4B-C). Thrombus volumes from wild type, PKC $\theta^{-/-}$, and PKC $\delta^{-/-}$ thrombi were 490 ± 136 , 1923 ± 456 and $2304 \pm 261 \mu\text{m}^3$, respectively ($n \geq 16$, $p < 0.05$). Thrombi on collagen were then stained for P-selectin and PS (Fig. 5A). The increased deposition of PKC $\theta^{-/-}$ platelets was accompanied by higher integrated fluorescence intensities of both stains (Fig. 5B) and a higher fluorescence per pixel for P-selectin staining, suggesting an overall higher activation state of the platelets. In contrast, this was not observed for PKC $\delta^{-/-}$ platelets. As for PKC $\alpha^{-/-}$ and PKC $\beta^{-/-}$, similar fractions of labeled wildtype ($87 \pm 5\%$), PKC $\theta^{-/-}$ ($80 \pm 2\%$) and PKC $\delta^{-/-}$ ($84 \pm 4\%$) platelets were found stably adherent to collagen under flow ($n = 4-5$ experiments, $p > 0.2$), suggesting that enhanced thrombus formation does not result from increased adhesion to collagen, but rather enhanced ability to form large aggregates.

Using Fluo-4-loaded platelets, collagen-adhered PKC $\theta^{-/-}$ platelets, but not PKC $\delta^{-/-}$ platelets, exhibited higher amplitude rises in $[\text{Ca}^{2+}]_i$ in comparison to the corresponding wildtype cells (Fig. 6A). The increased Ca^{2+} signal in PKC $\theta^{-/-}$ platelets was detectable at both earlier (30 s) and later (60 s) time points (Fig. 6B). Together, these results suggest that absence of PKC θ augments collagen-dependent thrombus formation under flow by increasing GPVI-induced Ca^{2+} signaling, and downstream responses such as α -granule secretion and procoagulant activity.

Figure 4. Deficiency of PKC δ or θ increases thrombus formation on collagen under flow. Blood from PKC δ ^{-/-}, PKC θ ^{-/-} or matched wildtype mice was flowed over collagen (see Fig. 1). A, Representative contrast images after 4 min of perfusion (bars 20 μ m, inserts 5 $\times$ magnified). B, Quantification of platelet deposition on collagen surface. C, Morphometric analysis of aggregates on collagen surface with indicated numbers of platelets per feature. Means $\pm$ S.E.M. (n=3-6); *p<0.01 vs. corresponding wildtypes; χ^2 test *p<0.05.

Inhibition of conventional or novel PKC isoforms differently influences collagen-induced human platelet aggregation and activation

It was important to determine whether the roles defined for the major PKC isoforms in mouse platelets could be paralleled in human platelets. We therefore studied the relative effects of isoform non-selective and isoform-selective PKC inhibitors: the bis-indolyl maleimide, Ro-318425 (non-selective PKC inhibitor); the modified bis-indolyl maleimides, Gö6976 (potency PKC α / β > ϵ) and β -inhibitor (potency PKC β > α > ϵ); the biphloroglucinol,

Figure 5. Deficiency of PKC θ but not δ increases collagen-induced activation of platelets in thrombi. Blood from PKC δ ^{-/-}, PKC θ ^{-/-} or matched wildtype mice was flowed over collagen, and platelet thrombi were double stained with FITC-anti-mouse CD62P mAb (green) and AF647-annexin A5 (red). **A**, Representative confocal images (180×180 μ m) after staining. **B**, Integrated fluorescence intensity presenting activated platelets in arbitrary units, to quantify cumulative expression of P-selectin and PS. Means $\pm$ S.E.M. (n=3-5); **p<0.01 and *p<0.05 vs. corresponding wildtypes. Numbers above each bar indicate the mean integrated pixel density of P-selectin staining, an indication of staining per platelet. For color version, see page 189.

rottlerin (potency PKC δ > α/β); and θ -inhibitor (compound A, potency PKC θ > δ > α/β). As a first approach to determine the effects of inhibitors on PKC-dependent platelet function, we assessed aggregation responses to PMA. Similar to Ro-318425, which completely abolished aggregation at 0.5 μ M, both Gö6976 and β -inhibitor abolished this response, although at higher concentrations (Supp. Fig. 1A). In contrast, rottlerin and θ -inhibitor were much less inhibitory and, in the case of rottlerin, potentiated platelet aggregation to PMA.

Similarly, Ro-318425, Gö6976 and β -inhibitor each dose-dependently suppressed PMA-induced $\alpha\text{IIb}\beta_3$ activation as well as α -granule secretion (Supp. Fig. 1B-C). In contrast, θ -inhibitor potentiated $\alpha\text{IIb}\beta_3$ activation, whilst rottlerin potentiated secretion. This indicates that inhibition of conventional PKC isoforms, but not novel isoforms, suppresses PMA-induced integrin activation and secretion.

Figure 6. Deficiency of PKC θ but not δ increases collagen-induced Ca^{2+} signaling under flow. Whole blood containing autologous Fluo-4-loaded platelets was flowed over collagen (see Fig. 1), while fluorescence images of adhered platelets were captured in real-time (5 Hz). **A**, Traces of pseudo-ratioed changes in $[\text{Ca}^{2+}]_i$ from representative, single platelets as well as mean traces (S.E.) from overlays of >38 platelets. **B**, Graph of quantitative analysis of increased $[\text{Ca}^{2+}]_i$ at 30 s (white) and 60 s (gray) after initial response. Data are means $\pm$ S.E.M. ($n=38$ –40, 4 experiments); $**p<0.01$ vs. corresponding wildtypes.

Supplementary figure 1. Inhibition of conventional or novel PKC isoforms differentially affects PMA-induced platelet activation. Washed human platelets (2×10^8 /ml) were pretreated with Me2SO vehicle (control), Gö6976 (blocking PKC α/β), β -inhibitor (PKC $\beta > \alpha$), rottlerin (PKC δ), θ -inhibitor (PKC θ), or Ro-318425 (all PKC), at a concentration of 0.5–10 μ M. **A**, Effect of inhibitors (μ M) on platelet aggregation induced by PMA (100 nM). Shown are representative aggregation traces (optical light transmission), and dose-response curves. **B–C**, Inhibitor effects on platelet activation induced by PMA (100 nM), as measured by flow cytometric analysis of α IIb β 3 activation and P-selectin expression. Effects are expressed as percentages of control condition. Means $\pm$ S.E.M. ($n=3-5$).

To assess how the inhibitors suppressed platelet PKC kinase activity, their influence on PKC exogenous substrate peptide phosphorylation was determined under the same experimental conditions (1×10^8 platelets/ml, no plasma). Whilst Ro-318425 (10 μ M) abolished kinase activity to $2.1 \pm 0.5\%$ ($n=5$) of control, Gö6976 (1 μ M) and β -inhibitor (2.5 μ M) reduced phosphorylation to $54 \pm 4\%$ and $38 \pm 6\%$, respectively. The inhibitors of novel PKC isoforms, rottlerin (10 μ M) and θ -inhibitor (2.5 μ M) reduced PKC activity to $63 \pm 8\%$ and $75 \pm 5\%$ of control, respectively. Combinations of inhibitors fully suppressed substrate peptide phosphorylation. These data are in agreement with previously published data (22), and demonstrate that the classical isoforms of PKC represent the more major component of platelet PKC activity in human platelets.

Figure 7. Inhibition of conventional or novel PKC isoforms differentially affects collagen-induced platelet activation and calcium signaling. A-D: Washed human platelets were pretreated as described for Supplementary Fig. 1, and stimulated with collagen (5 μg/ml) or convulxin (70 ng/ml). A, Effect of inhibitors (μM) on collagen-induced platelet aggregation. Shown are representative aggregation traces (optical light transmission), and dose-response curves. B, Rescue of collagen-induced aggregation by ADP (10 μM) but not ionomycin (iono; 100 nM). Shown are representative aggregation traces and mean ± S.E.M. (n = 4). C-D, Inhibitor effects on platelet activation induced by convulxin (cvx), as measured by flow cytometric analysis of αIIbβ3 activation and P-selectin expression. Effects are expressed as percentages of control condition. Means ± S.E. (n=3-5). E & F: Washed human platelets (1×10⁸/ml), loaded with Fura-2, were treated with Me2SO vehicle (control), Gö6976 (1.0 μM), β-inhibitor (2.5 μM), rottlerin (10 μM) or θ-inhibitor (1.0 μM) Ro-318425 (10 μM). E, Traces of changes in [Ca²⁺]_i were measured in response to convulxin (cvx, 70 ng/ml). F, Quantitative effect on convulxin-induced [Ca²⁺]_i-time integrals. Means ± S.E.M. (n=3-4); *p<0.05 vs. control condition.

Subsequently, we determined the influence of these compounds on human platelet activation via the collagen receptor, GPVI. Collagen-induced platelet aggregation was concentration-dependently inhibited by Ro-318425 (Fig. 7A) and near complete inhibition was achieved at 1 μM Gö6976, while β-inhibitor was effective at higher concentrations. Ro-318425 was ineffective when platelets were co-stimulated with collagen and ADP, suggesting that the inhibition of aggregation is explained by loss of granule secretion. By contrast, the calcium ionophore, ionomycin, did not rescue aggregation, suggesting that reduced

Ca^{2+} signaling is not responsible for the inhibition of aggregation (Fig. 7B). Inhibition of novel PKC isoforms with rottlerin or θ -inhibitor enhanced convulxin-induced platelet aggregation. In general, measurements of convulxin-induced $\alpha\text{IIb}\beta_3$ activation and P-selectin expression showed similar effects with these inhibitors (Fig. 7C-D). However, strikingly, θ -inhibitor but not rottlerin markedly increased these responses, in agreement with previously published gene knockout studies (8,24).

Inhibition of conventional or novel PKC isoforms differently influences GPVI-induced Ca^{2+} signaling

Finally, we measured GPVI-induced Ca^{2+} responses in washed human platelets. Gö6976 and β -inhibitor suppressed the $[\text{Ca}^{2+}]_i$ peak and $[\text{Ca}^{2+}]_i$ -time integral (Fig. 7E-F), while rottlerin was without effect, and pretreatment with θ -inhibitor markedly increased the platelet Ca^{2+} signal. This resembled the potentiatory effect seen with Ro-318425, and also noted in earlier studies with human platelets (17). Hence, the enhancement of Ca^{2+} signaling by general PKC inhibitors is mimicked only by inhibition of the novel PKC θ isoform. Under these conditions, convulxin (70 ng/ml; 5 min) did not cause shedding of GPVI. Although treatment of platelets with a high concentration of convulxin (250 ng/ml) for 1 hour induced approximately 50 % GPVI shedding, this was not affected by treatment with Ro-318425 did not affect this (Suppl. Fig. 2), suggesting that differences in Ca^{2+} signaling were not caused by regulated shedding of GPVI.

Supplementary figure 2. GPVI surface expression in washed human platelets was monitored by flow cytometry. Platelets were treated with Ro-318425 (10 μM) or vehicle control (DMSO) then stimulated with convulxin (CVX) as indicated. No significant difference was seen between DMSO- or Ro-318425-treated platelets ($n = 4$).

Relative contribution of conventional and novel PKC isoforms to secretion and Ca^{2+} signaling

Since PKC α and PKC θ regulate α -granule secretion in opposite directions, we investigated which isoform was dominant during platelet activation by treating PKC $\alpha^{+/+}$ and PKC $\alpha^{-/-}$ platelets with θ -inhibitor, and measured P-selectin expression by flow cytometry. Consistent with the above data, P-selectin expression was significantly inhibited in PKC $\alpha^{-/-}$ platelets. Interestingly, whereas θ -inhibitor slightly increased the P-selectin expression in PKC $\alpha^{+/+}$ platelets, there was little effect of θ -inhibitor on PKC $\alpha^{-/-}$ platelets (Fig. 8A). These data suggest that the conventional isoforms are essential for secretion whereas PKC θ regulates the extent of secretion.

Figure 8. Relative contributions of PKC α and PKC θ to secretion and Ca^{2+} signaling. A, Washed PKC $\alpha^{+/+}$ or $\alpha^{-/-}$ platelets, were pretreated with θ -inhibitor or vehicle control were stimulated with convulxin (70 ng/ml). α -granule secretion was assessed by flow cytometric analysis of P-selectin expression. Data are expressed as % cells expressing surface P-selectin ($n = 3$). B, Blood from PKC $\alpha^{+/+}$ or $\alpha^{-/-}$ mice containing autologous Fluo-4-loaded platelets was treated with θ -inhibitor or vehicle control then flowed over collagen, as described for Fig. 3. Representative traces are shown ($n = 3$). * $p < 0.05$ θ -inhibitor vs. untreated condition. C, Summary diagram of the roles of different PKC isoforms in platelet secretion, thrombus formation, sustained Ca^{2+} signaling and PS exposure. PKC α/β are necessary for collagen-induced granule secretion, which is negatively regulated by PKC θ . In the absence of PKC α/β there is no granule secretion and so no effect of PKC θ . PKC α/β positively regulate sustained Ca^{2+} signaling and PS exposure, whereas it is negatively regulated by PKC θ even in the absence of PKC α . PKC δ negatively regulates thrombus formation independently of granule secretion, and does not regulate Ca^{2+} signaling or PS exposure.

PKC α and PKC θ also regulate Ca^{2+} signaling under flow conditions in opposite directions. Treatment with θ -inhibitor enhanced the Ca^{2+} signal in platelets flowed over collagen in PKC $\alpha^{+/+}$ platelets, consistent with the data obtained from PKC $\theta^{-/-}$ platelets. Moreover, θ -inhibitor also enhanced the Ca^{2+} signal in PKC $\alpha^{-/-}$ platelets (Fig. 8B). These data suggest that PKC θ is a more important regulator of Ca^{2+} signaling than of granule secretion.

Discussion

Here, for the first time, we have addressed all four of the major platelet-expressed PKC isoforms, determining their comparative roles in regulating platelet activation by collagen under physiological flow conditions. The study is also the first to determine the role of individual PKC isoforms in regulating calcium responses, at the single cell level, in growing thrombi visualized in real time. Importantly the study reveals major positive roles for the conventional isoforms, PKC α and PKC β , in mediating thrombus formation. The absence of either isoform leads to marked suppression of secretion of α -granules, aggregate formation, calcium signaling and PS exposure under flow. The data therefore suggest essential but non-redundant roles for these kinases in regulating these events. In contrast, absence of the novel isoforms PKC θ and PKC δ however lead to enhanced thrombus formation on collagen. The mechanisms for these isoforms are also distinct and non-redundant, since although absence of PKC θ also leads to enhanced secretion, calcium signaling and phosphatidylserine exposure, absence of PKC δ does not potentiate any of these functions. We conclude that all four major expressed PKC isoforms play distinct non-redundant roles, where the conventional PKCs promote and the novel PKCs inhibit thrombus formation on collagen, by a variety of mechanisms (summarized in Fig. 8C).

The studies here with mice lacking PKC α or PKC β demonstrated that platelet thrombi formed on collagen were significantly smaller in comparison to wild-type thrombi. This reduced thrombus formation was associated with impaired GPVI-induced α -granule secretion. A key role for murine PKC α in exocytosis of platelet α - and dense granules has recently been demonstrated (18). Importantly, these effects were not caused by diminished primary adhesion to collagen, since time lapse studies with Fluo-4-loaded platelets showed normal adhesion under shear in case of PKC α or PKC β deficiency. Instead, our data suggest that the defect is in the ability of platelets to form aggregates on platelets that have already adhered.

In addition, in both PKC α ^{-/-} and PKC β ^{-/-} mice, the procoagulant activity of collagen-adhered platelets was diminished, consistent with the reduced Ca²⁺ signal. Earlier, it had been shown that the platelet procoagulant response is a direct consequence of GPVI-induced activation of collagen-adhered platelets (42,43). How PKC α and β regulate Ca²⁺ signaling is yet understood, although a role for conventional PKCs in store-operated calcium entry has previously been proposed on the basis of pharmacological studies in human platelets (44). Together, these data support the concept that both conventional PKC isoforms positively regulate thrombus formation by enhancing GPVI-induced platelet activation, leading to secretion and procoagulant activity. Interestingly, there is a high degree of non-redundancy in this process, indicating that PKCs α and β may each play essential, but distinct, roles.

It was important to extend the studies in mouse platelets by studies with human platelets, using the compounds Gö6976 and β -inhibitor. Dose-response curves showed an inhibitory effect of both compounds on collagen-induced platelet aggregation as well as on GPVI-induced integrin activation and P-selectin expression. In addition, these compounds diminished GPVI-induced Ca²⁺ signal generation. The reduction in aggregation is likely to be

mainly caused by loss of granule secretion, as full aggregation could be restored by costimulation with ADP. This is consistent with our previous report that coinfusion with ADP could restore thrombus formation on collagen in PKC $\alpha^{-/-}$ deficient platelets. By contrast, enhancing cytosolic Ca $^{2+}$ by costimulation with ionomycin did not rescue collagen-inducing aggregation.

For mouse platelets, the present data indicate that both isoforms are required for full GPVI-dependent activation, suggesting a non-redundancy in function, particularly for regulation of P-selectin expression and annexin V binding, which are both markedly suppressed in the absence of either PKC α or PKC β . For human platelets, the studies with Gö6976 and β -inhibitor point to a substantial reduction in platelet responses. The β -inhibitor is however much less effective than Gö6976 on convulxin-induced responses (Fig. 7A-C). This may suggest that, in human platelets, there is more redundancy of function between PKC α and PKC β than in mouse platelets. It may however be a reflection of the selectivity profile of these inhibitors, and may also reflect differences between the effects of gene deletion versus pharmacological inhibition of a kinase. In support of the concept that both PKC α and β positively contribute to human platelet activation, are the findings in the literature that purified PKC α mediates granule secretion (19), PKC α is phosphorylated upon GPVI stimulation (45), and that PKC in general and PKC β in particular is implicated in α IIb β 3 signaling (22,46).

In contrast, the current studies with mice deficient in PKC θ point to a negative role in collagen-dependent granule secretion and thrombus formation. In addition, increased GPVI-induced activation of PKC $\theta^{-/-}$ platelets was apparent from the higher Ca $^{2+}$ responses of adhered single platelets, increased numbers of procoagulant, PS-exposing cells. Consistent with these data, the θ -inhibitor increased GPVI-induced human platelet aggregation as well as α IIb β 3 activation, Ca $^{2+}$ mobilization and α -granule secretion. There are discrepancies in the literature about negative or positive roles for PKC θ in regulating platelet function (24,25,47,48), which may be a result of differences in platelet preparation conditions. We have however previously shown platelet responses to GPVI activation to be enhanced in the absence of PKC θ (24,48), and taken together these data would support the concept that PKC θ may down-regulate rather than up-regulate GPVI-mediated granule secretion and aggregate formation under physiological flow conditions. Mouse platelets express relatively high amounts of PKC θ compared to the other novel PKC isoforms, PKC δ and ϵ (7,24). Furthermore, all three isoforms are phosphorylated on tyrosine following GPVI stimulation (7,45,47,49,50).

In addition, the present data suggest that PKC θ is the principal isoform mediating the earlier recognized effect of PKC in down-regulating GPVI-induced platelet Ca $^{2+}$ signaling and procoagulant activity (17). The mechanism is currently unknown, although PKC may reduce phospholipase C activation (51) or increase Ca $^{2+}$ extrusion via the plasma membrane Ca $^{2+}$ ATPase (14,15).

The enhanced thrombus formation that we report in PKC $\theta^{-/-}$ blood is in apparent contrast to a previous report (25), in which PKC $\theta^{-/-}$ mice showed reduced thrombus formation *in vivo*, in a FeCl $_3$ -induced carotid injury model. This is likely to reflect the additional contribu-

tion of thrombin generation *in vivo*. Importantly, although PKC θ negatively regulates collagen-dependent platelet activation, it appears to have a positive role in thrombin-induced signaling (25,52). This agonist-dependent difference is similar to that proposed by Kunapuli and colleagues for PKC δ (23). The relative importance of collagen and thrombin *in vivo* appear to depend on the injury model used and the extent of injury (53). We therefore suggest that the effect seen *in vivo* is a combination of enhanced collagen-dependent platelet activation and reduced thrombin-dependent signaling.

A tendency to increased thrombus formation was also seen with PKC $\delta^{-/-}$ mice in the present study. Although some reports suggest that GPVI-induced granule secretion is increased in PKC $\delta^{-/-}$ mice (23), we have not been able to show this (present study and (8)), but rather demonstrate a potentiated aggregation response to collagen through enhanced filopodia formation in the absence of PKC δ . Consistent with this, we found with human platelets that rottlerin had little effect on collagen-induced integrin $\alpha\text{IIb}\beta_3$ activation and α -granule secretion, but enhanced platelet aggregation. Therefore, although PKC θ and PKC δ negatively regulate collagen-dependent thrombus formation, they act through distinct mechanisms.

Treatment of human platelets with broad-spectrum PKC inhibitors blocks granule secretion and formation of large aggregates, but also enhances platelet Ca^{2+} signaling and PS exposure (17). This suggests that the major regulators of granule secretion and thrombus formation are PKC α and PKC β , since inhibition of all PKC isoforms replicates the phenotype of PKC $\alpha^{-/-}$ or PKC $\beta^{-/-}$ platelets. In contrast, the major regulator of Ca^{2+} signaling and PS exposure appears to be PKC θ , since absence or pharmacological inhibition of PKC θ has a similar effect on Ca^{2+} signaling and PS exposure to broad-spectrum PKC inhibition.

We tested this directly by examining the effect of θ -inhibitor on PKC $\alpha^{-/-}$ platelets. Importantly, combined loss of PKC α and PKC θ signaling produced a phenotype that resembled PKC $\alpha^{-/-}$ platelets for secretion, and PKC $\theta^{-/-}$ platelets for Ca^{2+} signaling. Moreover, the phenotype resembled that of platelets treated with a broad-spectrum PKC inhibitor. We propose that the conventional PKC isoforms are essential for collagen-induced granule secretion, whereas PKC θ negatively regulates the extent of this secretion. This means that in the absence of conventional PKC signaling there is no granule secretion for PKC θ to regulate (Fig. 8C). In contrast, we suggest that collagen-induced sustained Ca^{2+} signals and subsequent PS exposure are positively regulated by PKC α and β and negatively regulated by PKC θ . Unlike secretion, however, the conventional isoforms are not essential for generation of the Ca^{2+} signal.

The data presented here are the first comparative analysis of platelet PKCs in thrombus formation on collagen, and the first to determine calcium responses, secretion and procoagulant activity at the single cell level in the growing thrombus, in platelets lacking specific PKC isoforms. Together, the data form a comprehensive analysis of the roles played by PKCs α , β , δ , and θ , and reveal important distinctions in mechanism and function of these kinases. The data will provide a platform for future exploitation of these different family members in modulation of platelet function and thrombus formation.

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Chapter 6

Roles of platelet *STIM1* and *Orai1* in glycoprotein VI-
and thrombin-dependent procoagulant activity and
thrombus formation

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Abstract

In platelets, STIM1 has been recognized as the key regulatory protein in store-operated Ca^{2+} entry (SOCE) with Orai1 as principal Ca^{2+} entry channel. Both proteins contribute to collagen-dependent arterial thrombosis in mice *in vivo*. Unclear is whether STIM2 is involved. A key platelet response relying on Ca^{2+} entry is the surface exposure of phosphatidylserine (PS), which accomplishes platelet procoagulant activity. We studied this response in mouse platelets deficient in STIM1, STIM2 or Orai1. Upon high-shear flow of blood over collagen, Stim1^{-/-} and Orai1^{-/-} platelets had greatly impaired GPVI-dependent Ca^{2+} signals, and they were deficient in PS exposure and thrombus formation. In contrast, Stim2^{-/-} platelets reacted normally. Upon blood flow in the presence of thrombin generation and coagulation, Ca^{2+} signals of Stim1^{-/-} and Orai1^{-/-} platelets were partly reduced, while the PS exposure and formation of fibrin-rich thrombi were normalized. Washed Stim1^{-/-} and Orai1^{-/-} platelets were deficient in GPVI-induced PS exposure and prothrombinase activity, but not when thrombin was present as co-agonist. Markedly, SKF96365, a blocker of (receptor-operated) Ca^{2+} entry, inhibited Ca^{2+} and procoagulant responses even in Stim1^{-/-} and Orai1^{-/-} platelets. These data show for the first time that: (i) STIM1 and Orai1 jointly contribute to GPVI-induced SOCE, procoagulant activity and thrombus formation, (ii) a compensating Ca^{2+} entry pathway is effective in the additional presence of thrombin; (iii) platelets contain two mechanisms of Ca^{2+} entry and PS exposure, only one relying on STIM1-Orai1 interaction.

Introduction

In platelets, elevation in cytosolic $[\text{Ca}^{2+}]_i$ is imperative to almost all functional responses. Moderate and transient rises in $[\text{Ca}^{2+}]_i$ mediate shape change, integrin $\alpha\text{IIb}\beta_3$ activation, thromboxane formation and secretion of granule contents, while high and prolonged $[\text{Ca}^{2+}]_i$ rises are required for the procoagulant response (1). The latter is achieved by a Ca^{2+} -activated scramblase mechanism disturbing the normal phospholipid asymmetry in the plasma membrane, with as a result the exposure of phosphatidylserine (PS) at the outer membrane surface (2,3). Exposed PS provides high-affinity binding sites for key coagulation factors and, thereby, facilitates the assembly of tenase and prothrombinase complexes, which are responsible for the formation of factor Xa and thrombin, respectively (2). Since thrombin is one of the most potent platelet agonists, the procoagulant platelet response triggers a potent positive feedback loop of platelet and coagulation activation. Recent *in vivo* studies have indicated that PS exposure and ensuing thrombin generation are key regulatory events in murine arterial thrombus formation (4,5).

Whereas stored platelets may expose procoagulant PS in a Ca^{2+} -independent way, PS exposure in activated platelets relies on a high and prolonged rise in cytosolic $[\text{Ca}^{2+}]_i$ (6). Platelet stimulation with single G-protein-coupled agonists, like thrombin and ADP, results in limited PS exposure (7,8), but stimulation of the tyrosine kinase-linked collagen receptor glycoprotein VI (GPVI), with ligands such as collagen-related peptide (CRP) or convulxin, results in appreciable procoagulant activity (9,10). Combined stimulation of the collagen and thrombin receptors though results in high PS exposure, likely because these agonists use different signaling pathways for mobilizing cytosolic Ca^{2+} (1). While thrombin transient-

ly activates Gq α and phospholipase C β 2/ β 3 isoforms, activation of GPVI causes a more persistent activation of the phospholipase C γ 2 isoform (11). For PS exposure, entry of extracellular Ca²⁺ is required, complementing the Ca²⁺-mobilizing effect of phospholipase C stimulation, in order to reach sufficiently high [Ca²⁺]_i (9,12,13).

In platelets like other cells, Ca²⁺ entry can be triggered by receptor stimulation as well as by Ca²⁺ mobilization from stores via the processes of receptor-operated Ca²⁺ entry (ROCE) and store-operated Ca²⁺ entry (SOCE), respectively (14). For long, not only the responsible Ca²⁺ entry channels, but also the coupling mechanisms of receptor activation and Ca²⁺ store depletion to channel opening have remained elusive. In earlier work with platelets, roles of the TRPC1 and TRPC6 channel proteins in Ca²⁺ entry have been proposed (15,16). Recent studies however have shown the importance of the Orai class of plasma membrane Ca²⁺ channels. The channel Orai1 (also called CRACM1) oligomerizes and opens, following depletion of the Ca²⁺ stores, by interacting with Ca²⁺ sensing stromal-interacting molecule 1 (STIM1), which is a transmembrane protein located in the endoplasmic reticulum (17-19). The homologous protein STIM2 can have a similar regulatory role in Ca²⁺ entry (20). Both Orai1 and STIM1 have been implicated in the physiological activation of T cells and mast cells (21,22). Recent studies using genetically modified mice have established that STIM1 and Orai1 account for the large majority of SOCE in platelets. The importance of this SOCE pathway appeared from the finding that platelet deficiency in either Orai1 or STIM1 protects against collagen-dependent arterial thrombus formation and brain infarction in vivo (23,24). In confirmation, others have provided evidence that a functional R93W mutation in Orai1 leads to impaired GPVI-induced platelet activation (25).

In the present paper, we investigated whether the STIM isoforms and Orai1 provide the main Ca²⁺ entry mechanism responsible for PS exposure and procoagulant activity in platelets stimulated by the collagen and thrombin receptors. The studies were carried out using mice with Stim1^{-/-}, Stim2^{-/-}, or Orai1^{-/-} platelets.

Experimental procedures

Mouse Strains

Animal studies were approved by the local animal care and use committees. Mice homozygously deficient in Stim1 or Orai1 were generated from embryonic stem cell clones and germline transmission, as described (23,24). Since these animals suffered from early lethality and growth retardation, bone marrow chimeras were created which had normal viability. Female, 5-6 weeks old C57BL/6 mice were irradiated with a single dose of 10 Gy, and injected intravenously with bone marrow cells from donor Stim1^{-/-}, Orai1^{-/-} or wild type mice (4 × 10⁶ cells/animal). The recipient mice received acidified water containing 2 g/l neomycin sulfate for 6 weeks after transplantation. Blood was taken from the chimeras after >6 weeks. Mice homozygously deficient in Stim2 had a mixed genetic background and were compared with wild types of the same background (26). Blood cell counts of all mice were in the normal range. Purified platelets from (bone marrow-transplanted) mice were subjected to Western blotting to confirm knockout of STIM proteins. Deficiency in Orai1 transcripts was confirmed by RT-PCR analysis (24).

Materials

H-Phe-Pro-Arg chloromethyl ketone (PPACK) was obtained from Calbiochem. Annexin A5 labeled with fluorescein isothiocyanate (FITC), Fura-2 and Fluo-4 acetoxymethyl esters and pluronic F-127 were from Invitrogen. Thrombin substrate, Z-Gly-Gly-Arg aminomethyl coumarin (Z-GGR-AMC), was from Bachem; fibrillar type I collagen (Horm) from Nycomed; recombinant human tissue factor from Dade Behring. Apyrase (grade V), bovine serum albumin (BSA), heparin, SKF96365 and thrombin were from Sigma. The GPVI agonist, CRP was synthesized and cross-linked by Tana Laboratories; the agonist convulxin was purified as described (10). FITC-labeled anti-mouse P-selectin mAb was from Emfret Analytics. Bovine coagulation factors and other materials were from sources indicated before (27).

Blood collection and platelet preparation

Mouse blood was obtained via orbital puncture under anesthesia. For perfusion studies in the absence of coagulation, blood was collected into a mixture of 40 μ M PPACK, 5 U/ml heparin and 40 U/ml fragmin. For later perfusions with coagulation, blood was collected into 12.9 mM trisodium citrate. Citrate-anticoagulated blood was also used to prepare platelet-rich plasma (PRP), normalized with autologous platelet-poor plasma (PPP) to a platelet count of 1×10^8 /ml. Washed platelets were prepared from PRP by supplementation of ACD solution (1:25). After centrifugation, platelets were suspended in Hepes buffer pH 7.45 (136 mM NaCl, 5 mM Hepes, 2.7 mM KCl, 2 mM $MgCl_2$, 0.42 mM Na_2HPO_4 , 5 mM glucose and 0.1% BSA). Cells were counted with a Coulter counter.

Activation of suspended platelets

Washed mouse platelets were loaded with the ratiometric Ca^{2+} probe, Fura-2, as described (23). Platelets suspended in the presence or absence of 1 mM $CaCl_2$ were preincubated with SKF96365 (100 μ M), and activated with collagen and/or thrombin receptor agonists, as indicated. Changes in fluorescence was measured with a PerkinElmer LS 55 fluorometer. Excitation was alternated between 340 and 380 nm, and emission was measured at 509 nm. Calibration parameters of nanomolar $[Ca^{2+}]_i$ were obtained by lysis with 1% Triton X-100 and addition of a surplus of EGTA. For flow cytometry, unloaded washed platelets (1×10^8 /ml) were activated in the presence of $CaCl_2$ with indicated agonists for 10 min; stirring was absent to prevent platelet aggregation (28). Surface expression of PS was detected in the presence of $CaCl_2$ (2 mM) with FITC-annexin A5 (0.5 μ g/ml).

Thrombus formation on collagen

Glass coverslips were coated with fibrillar type I collagen and blocked with BSA-containing Hepes buffer pH 7.45. The coverslips were mounted in a transparent, 50 μ m-deep parallel-plate poly(methyl) methacrylate flow chamber (29). Mouse blood was perfused through the flow device at defined shear rate under physiological, millimolar concentrations of divalent cations. For experiments in the absence of coagulation, PPACK/heparin-treated blood was flowed over collagen at $1000\ s^{-1}$ during 4 min. For experiments in the presence of coagulation, citrate-anticoagulated blood was recalcified directly before entering the flow chamber,

using a two-pump system (28). Briefly, 1 ml syringes were filled with citrate-anticoagulated blood or isotonic $\text{CaCl}_2/\text{MgCl}_2$ solution (110 mM NaCl, 13.3 mM CaCl_2 and 6.7 mM MgCl_2). The syringes were connected to the flow chamber via a v-shaped inlet, designed to give optimal fluid mixing. By co-infusing both fluids into the chamber at equal flow rate (final shear rate, 1000 s^{-1}), coagulation was started by collagen- and factor XII-dependent activation (30). The thrombi on coverslips were post-labeled by perfusion with FITC-annexin A5 (0.5 $\mu\text{g}/\text{ml}$) in Hepes buffer pH 7.45, containing 2 mM CaCl_2 and 1 U/ml heparin. Brightfield and fluorescence images were recorded from at least 10 randomly chosen microscopic fields (31). Images were analyzed with ImagePro software (Media Cybernetics). The procoagulant index of thrombi was determined as the ratio of surface coverage of PS-exposing platelets (FITC-annexin A5) to the coverage of total platelets (32).

Single platelet Ca^{2+} fluxes under flow

Mouse platelets were loaded with Fluo-4 (33), and added to PPACK/fragmin- or citrate-anticoagulated blood from the same genotype (10% labeled platelets). During blood flow over collagen, 16-bit digital fluorescence images were recorded at high-speed (5 Hz) using an EM-CCD camera (30). Regions-of-interest representing single adhered platelets were analyzed off-line for changes in fluorescence (F) (33). Pseudo-ratio F/Fo values were converted into nanomolar concentrations of $[\text{Ca}^{2+}]_i$ using predefined calibration parameters (34). For quantitative purposes, traces from individual cells were superimposed, so that $[\text{Ca}^{2+}]_i$ rises started at the same frame number.

Prothrombinase activity

Prothrombinase-stimulating activity was determined at linear assay conditions, as previously assessed for human platelets (35). Washed mouse platelets were diluted in Hepes buffer pH 7.45 containing 3 mM CaCl_2 to a count of $4 \times 10^5/\text{ml}$, and incubated with 0.5 μM prothrombin, 2 nM factor Va and 1 nM factor Xa (37 °C). Samples were taken after exactly 3 min, and transferred to vials containing thrombin substrate, S2238 (0.5 mM), for chromogenic measurement of the thrombin formed.

Thrombin generation

Thrombin generation was measured in citrate-anticoagulated PRP (27). The PRP, pooled from 3 animals with the same (chimeric) genotype, was diluted with autologous PPP to a count of 1.5×10^8 platelets/ml. Samples were activated with convulxin (100 ng/ml), ionomycin (20 μM) or vehicle for 15 min. Aliquots (4 vol.) were then transferred to a polystyrene 96-wells plate (Immulon 2HB, Dynex Technologies), already containing 1 vol. of buffer A (20 mM Hepes, 140 mM NaCl, 0.5% BSA and 6 pM tissue factor). Coagulation was started by adding 1 vol. of buffer B (2.5 mM Z-GGR-AMC, 20 mM Hepes, 140 mM NaCl, 100 mM CaCl_2 and 6% BSA). First-derivative curves were converted into nanomolar thrombin concentrations using a calibrator for human α -thrombin (27). All analyses were in duplicate.

Statistics

Differences between groups were tested for significance with the non-parametric Mann-Whitney U test. Paired data were compared with a Student t-test. The statistical package for social sciences (SPSS 15.0) was used.

Results

Deficiency in platelet STIM1 or Orai1 impedes GPVI-mediated PS exposure and thrombus formation

For the experiments, chimeric mice were generated by transplantation of irradiated wild type animals with *Stim1*^{-/-}, *Orai1*^{-/-} or wild type bone marrow cells. Platelets from these mice, isolated at least 4 weeks after transplantation, were checked for absence of STIM1 protein or Orai1 transcript (supplemental Fig. 1A, B). The platelets from chimeric *Stim1*^{-/-} and *Orai1*^{-/-} mice were greatly impaired in Ca²⁺ rises evoked by the GPVI agonist, CRP (a triple helical collagen peptide), or by thrombin, most markedly when extracellular CaCl₂ was present (supplemental Fig. 1C), which is in full agreement with earlier data (23,24). In addition, *Stim1*^{-/-} platelets showed reduced Ca²⁺ rises in the absence of CaCl₂ and Ca²⁺ entry, supporting the concept that STIM1 controls the filling state of Ca²⁺ stores (23).

Supplemental figure 1. Defective GPVI- and thrombin-receptor induced Ca²⁺ signaling in platelets deficient in STIM1 or Orai1. A, Expression profiles of STIM1 protein in platelets from wild type (WT) and chimeric *Stim1*^{-/-} or *Orai1*^{-/-} mice. Western blots are given after probing with anti-STIM1 mAb; blots were reprobed with anti-tubulin mAb as control. B, RT-PCR indicating absence of *Orai1* transcript but presence of *Stim1* and *Stim2* transcripts in platelets from chimeric *Orai1*^{-/-} mice. C, impaired Ca²⁺ responses of *Stim1*^{-/-} and *Orai1*^{-/-} platelets especially in the presence of CaCl₂. Fura-2-loaded platelets were stimulated with 10 µg/ml CRP (upper panels) or 0.9 nM thrombin (Thr, lower panels) in the presence or absence of 1 mM CaCl₂, as indicated. Shown are representative traces of rises in [Ca²⁺]_i (n = 3-5).

Blood from these chimeric mice was anticoagulated with PPACK/heparin, and perfused at high-shear flow rate over a collagen surface. By using these thrombin inhibitors, blood coagulation was ablated, while high, physiological concentrations of free Ca^{2+} and Mg^{2+} were maintained. In this test of collagen-induced thrombus formation, platelets in contact with the collagen fibers become activated via GPVI, and respond by Ca^{2+} elevation, PS exposure and secretion of autocrine mediators, with as a result platelet aggregate formation (4,32,36). Strikingly, with blood from chimeric $\text{Stim1}^{-/-}$ and $\text{Orai1}^{-/-}$ mice, no or only small aggregates were formed, while PS exposure was almost completely absent, as apparent from the lack of staining with FITC-labeled annexin A5 (Fig. 1A). This contrasted to the large aggregates and many PS-exposing platelets observed with blood from corresponding wild type mice. Analysis of microscopic images pointed to a markedly reduced deposition of $\text{Stim1}^{-/-}$ (~70%) and $\text{Orai1}^{-/-}$ (~46%) platelets in comparison to wild type. The procoagulant index, i.e. the relative formation of PS-exposing platelets compared to all adhered platelets (32), was also greatly reduced in either knockout (Fig. 1B).

Mouse platelets were loaded with Fluo-4 and back-added to blood of the same genotype in order to measure Ca^{2+} rises in the cells during blood flow over collagen. Wild type platelets showed high Ca^{2+} responses shortly after adhesion (Fig. 2A). However, adhered $\text{Stim1}^{-/-}$ platelets gave only minute Ca^{2+} spikes, in a manner resembling the low responses of $\text{Fcer1g}^{-/-}$ platelets, known to lack GPVI signaling activity (33). Many of the adhered $\text{Orai1}^{-/-}$ platelets gave similar, minute Ca^{2+} spikes (~50%), but the remaining $\text{Orai1}^{-/-}$ cells showed short series of medium amplitude spikes (Fig. 2A). Quantitative analysis demonstrated a nearly complete ablation of the average Ca^{2+} signal in $\text{Stim1}^{-/-}$ platelets and a greatly reduced signal in $\text{Orai1}^{-/-}$ platelets (Fig. 2B). Together, these results point to important roles of both STIM1 and Orai1 in collagen-dependent Ca^{2+} signaling, PS exposure and thrombus formation under non-coagulant conditions.

Deficiency in STIM1 or Orai1 does not abolish PS exposure and thrombus formation under coagulant conditions

GPVI-induced platelet activation has been shown to control collagen-dependent thrombus formation also under conditions favoring coagulation (28). To determine the role of STIM1 and Orai1 in this setting, citrate-anticoagulated blood from chimeric animals was co-infused with $\text{CaCl}_2/\text{MgCl}_2$ to achieve again millimolar free Ca^{2+} and Mg^{2+} concentrations. Perfusion of the recalcified blood over collagen leads to onset of coagulation via collagen-dependent activation of factor XII (30). With wild type blood, massive and dense thrombi (clots) were formed within 4 min of perfusion, which were covered with PS-exposing platelets and connected by fibrin fibers (Fig. 3A). Surprisingly, dense fibrin-containing thrombi were also formed with $\text{Stim1}^{-/-}$ or $\text{Orai1}^{-/-}$ blood. These thrombi were also surrounded by PS-exposing platelets, but to a lesser degree in comparison to wild type blood. However, the procoagulant index was similarly high for images recorded from wild type, $\text{Stim1}^{-/-}$ and $\text{Orai1}^{-/-}$ thrombi (Fig. 3B). These results thus indicate that neither STIM1 nor Orai1 is essential for collagen-dependent thrombus formation and PS exposure under coagulant conditions.

Figure 1. Deficiency in STIM1 or Orai1 impedes GPVI-dependent thrombus formation and PS exposure under flow. PPACK/heparin-anticoagulated blood of C57BL/6 mice transplanted with bone marrow from wild type, *Stim1*^{-/-} or *Orai1*^{-/-} animals was flowed over collagen at a shear rate of 1000 s⁻¹. A, representative contrast images after 4 min, captured at low (upper panels) or high (middle panels) magnification. Lower panels, fluorescence images after staining with FITC-annexin A5. Percentages in italic indicate area covered with platelets (bars, 50 μm). B, procoagulant index representing relative number of PS-exposing platelets. Data are percentage fractions of adhered platelets exposing PS. Means ± S.E. (n = 6-8); *, *p* < 0.05 and **, *p* < 0.01 versus wild type.

Fluo-4-loaded platelets were then used to determine rises in Ca²⁺ under the same conditions of flow and coagulation. Wild type platelets, adhered to collagen, showed prolonged, high Ca²⁺ responses (Fig. 4A), which were higher in level than those measured in anti-coagulated blood (Fig. 2B). With the thrombin inhibitor hirudin present, the mean Ca²⁺ rises reduced from about 400 to 250 nM, which confirmed the contribution of thrombin to

the Ca^{2+} signal. Interestingly, in recalcified blood, also collagen-adhered $\text{Stim1}^{-/-}$ and $\text{Orai1}^{-/-}$ platelets showed prolonged Ca^{2+} responses, but these remained lower in magnitude than those of wild type platelets (Fig. 4B). The effect of STIM1 knockout was again more pronounced than that of Orai1 deficiency. Hence, under these conditions of in situ formation of thrombin, platelet Ca^{2+} responses seem to be sufficiently high for PS exposure even in the absence of the STIM1-Orai1 SOCE pathway.

Figure 2. Deficiency in STIM1 or Orai1 impedes GPVI-dependent Ca^{2+} responses of collagen-adhered platelets under flow. PPACK/heparin-anticoagulated blood of wild type or chimeric $\text{Stim1}^{-/-}$ or $\text{Orai1}^{-/-}$ mice was supplemented with 10% Fluo-4-loaded platelets of the same genotype. Blood samples were flowed over collagen at 1000 s^{-1} , and fluorescence images from the collagen surface were recorded at 5 Hz. A, single-cell rises in Ca^{2+} of 2 representative platelets per genotype. Arrows indicate time point of adhesion. B, quantitative analysis of Ca^{2+} responses at 30 and 60 s after initial Ca^{2+} rises. Means $\pm$ S.E. ($n = 35\text{--}45$ cells). ***, $p < 0.001$ compared to wild type.

Deficiency in STIM1 or Orai1 does not affect GPVI-induced PS exposure and prothrombinase activity with thrombin present

To investigate this further, washed platelets were stimulated with collagen (GPVI)- and/or thrombin-receptor agonist, and staining of the cells was observed with the PS probe, FITC-annexin A5. Extracellular CaCl_2 was present to allow Ca^{2+} entry. The GPVI agonist convulxin was used for these studies (12), which is similarly effective as CRP in wild type and Orai1-deficient platelets (25). Flow cytometric analysis showed that convulxin stimulation of wild type platelets resulted in about 20% PS-exposing platelets (Fig. 5A). This fraction increased to 73% with thrombin as co-agonist, whereas thrombin alone had no more than little effect. Stimulation of platelets from chimeric $\text{Stim1}^{-/-}$ and $\text{Orai1}^{-/-}$ mice with convulxin resulted in impaired PS exposure, comparable to stimulation with thrombin alone. However, combined stimulation with convulxin and thrombin resulted in 53–58% PS positive platelets, which was similar to that of wild type platelets. Control experiments indicated that all platelets responded nearly completely, when stimulated with the Ca^{2+} ionophore, ionomycin. As an

alternative approach, we studied the capacity of platelets to support prothrombinase activity, i.e., the PS-dependent cleavage of prothrombin by factors Xa and Va (12). In wild type platelets, but not *Stim1*^{-/-} or *Orai1*^{-/-} platelets, convulxin caused a moderate increase in prothrombinase activity (Fig. 5B). The combination of convulxin and thrombin induced a five-fold increased prothrombinase activity with wild type platelets and a slightly lower increase with knockout platelets, but this was not significantly different from wild type. Stimulation with ionomycin/CaCl₂ caused high, maximal prothrombinase activity in all groups.

Figure 3. Deficiency in STIM1 or Orai1 permits GPVI-dependent thrombus formation and PS exposure in the presence of coagulation. Citrate-anticoagulated blood of indicated mice was recalcified with CaCl₂/MgCl₂ and flowed over collagen (4 min). Thrombi with platelets and fibrin (arrows) were post-stained with FITC-annexin A₅. A, representative phase contrast and fluorescence images after 4 min (bars, 50 μm). Percentages in italic indicate area covered with (fluorescent) platelets. B, procoagulant index of relative number of PS-exposing platelets. Means ± S.E. (n = 5-7); difference between groups not significant.

The procoagulant response of platelets was also studied in natural plasma environment, by activating PRP with tissue factor/CaCl₂ and measuring generation of thrombin (4). Similar thrombin generation curves were obtained with PRP from wild type, *Stim1*^{-/-} and *Orai1*^{-/-} mice (Fig. 6A). In all groups, pre-stimulation with ionomycin caused a 3-4 fold increase in thrombin generation, indicating that the knockout platelets were normally capable to support thrombin generation (Fig. 6B). Pre-stimulation with convulxin resulted in a significant increase in thrombin peak height in all groups, but in this case STIM1- or Orai1-deficient PRP was less effective than wild type PRP in comparison to pre-stimulation with ionomycin. The time-to-peak was less shortened in convulxin-stimulated knockout PRP in comparison to wild type (Fig. 6C). Taken together, these results show that, in the presence of thrombin (either externally added or formed in situ), GPVI-dependent PS exposure and procoagulant activity are only partly affected by deficiency in platelet STIM1 or Orai1.

Figure 4. Deficiency in STIM1 or Orai1 partly reduces Ca^{2+} signaling in the presence of coagulation. Citrate-anticoagulated blood of indicated mice was supplemented with 10% Fluo-4-loaded platelets of the same genotype, and recalcified. During blood flow over collagen, fluorescence images from the collagen surface were recorded at 5 Hz. A, single-cell rises in $[Ca^{2+}]_i$ of 2 representative platelets per genotype. Arrows indicate time point of adhesion. B, quantitative analysis of Ca^{2+} responses at 30 and 60 s after initial $[Ca^{2+}]_i$ rises. Means $\pm$ S.E. (n = 15-29 cells). **, p < 0.01 compared to wild type.

Roles of Ca^{2+} entry channels other than Orai1

The modest roles of STIM1 and Orai1 in GPVI-dependent platelet procoagulant activity in the presence of thrombin suggests the presence of a compensating Ca^{2+} entry mechanism, that for instance could be triggered in a receptor-operated fashion. In other cell types, it has been shown that also STIM2 regulates Ca^{2+} entry by controlling cytoplasmic Ca^{2+} levels (37). Since mouse platelets highly express this isoform (supplemental Fig. 1B), we studied the possibility that STIM2-mediated Ca^{2+} regulation controls the extent of PS exposure. Mice

homozygously deficient in STIM2 are viable and have normal platelet counts (26). Stimulation of Fura-2-loaded Stim2^{-/-} platelets showed unaltered Ca²⁺ responses upon stimulation of GPVI (Fig. 7A, B) or thrombin-receptors (not shown). Flow perfusion experiments over collagen, performed with blood from the Stim2^{-/-} mice, indicated the formation of large thrombi and the presence of many PS exposing platelets (Fig. 7C). Furthermore, detailed image analysis pointed to an unchanged procoagulant index of Stim2^{-/-} thrombi in comparison to Stim2^{+/+} thrombi (Fig. 7D).

Figure 5. Deficiency in STIM1 or Orai1 permits GPVI-dependent procoagulant activity in the presence of thrombin. Platelets in buffer containing 2 mM CaCl₂ (1×10^8 /ml) were stimulated for 10 min with convulxin (100 ng/ml), thrombin (4 nM), or ionomycin (20 μ M), as indicated. A, flow cytometric analysis of binding of FITC-annexin A5 to platelets (n = 5). B, determination of prothrombinase activity of platelet suspensions. Activity is expressed as pmol thrombin/min/ 10^6 platelets (n = 7-8). Means $\pm$ S.E.. *, $p < 0.05$ compared to wild type.

The putative role of other Ca²⁺ entry channels was investigated by using the established Ca²⁺ entry blocker, SKF96365 (38). To compare with earlier established Ca²⁺ traces (supplemental Fig. 1), Fura-2-loaded platelets were activated with GPVI agonist CRP, thrombin or the combination of both. In wild type platelets, pretreatment with SKF96365

inhibited the Ca^{2+} responses to all agonists with 60-70%, only if CaCl_2 was present in the incubation medium (Fig. 8A). On the other hand, when no CaCl_2 was added and Ca^{2+} entry was prevented, SKF96365 did not have a significant effect (Fig. 8B). Pretreatment with SKF96365 had a similar, 70% inhibitory effect on the Ca^{2+} response evoked by convulxin, again only in the presence of CaCl_2 . Strikingly, in $\text{Stim1}^{-/-}$ and $\text{Orai1}^{-/-}$ platelets, SKF96365 still suppressed both the CRP- and thrombin-induced Ca^{2+} responses by 40-60%, again if extracellular CaCl_2 was present (Fig. 8B). For all genotypes, the inhibition by SKF96365 was significant in the presence of CaCl_2 ($p < 0.001$), but not without CaCl_2 ($p = 0.05-0.38$). The $\text{Stim1}^{-/-}$ and $\text{Orai1}^{-/-}$ platelets responded similarly, but the former had a lower residual Ca^{2+} signal in the presence of SKF96365, which is explained by the reduced Ca^{2+} store content in these cells. Comparison of time traces of knockout platelets shows that the SKF96365-inhibitable Ca^{2+} signal is a relatively slow component, operating after approximately 10-60 s (Fig. 8A).

Figure 6. Deficiency in STIM1 or Orai1 reduces GPVI-dependent thrombin generation. Citrate-anticoagulated PRP (1×10^8 platelets/ml) of indicated mice was pre-incubated with vehicle solvent (control), convulxin (100 ng/ml) or ionomycin (20 μM). Thrombin generation was triggered with tissue factor/ CaCl_2 . A, representative thrombin generation curves per genotype. B, quantification of thrombin peak height. C, quantification of time-to-peak. Means $\pm$ S.E. ($n = 4-6$). #, $p < 0.05$ compared to wild type (ionomycin), *, $p < 0.05$ compared to control.

Further experiments demonstrated that the SKF96365-inhibitable Ca^{2+} entry pathway was important for procoagulant activity, as it suppressed the GPVI/thrombin-mediated PS exposure in both wild type ($-73 \pm 8\%$) and $\text{Orai1}^{-/-}$ platelets ($-34 \pm 6\%$, $p < 0.05$). Together, these data point to the involvement of a second Ca^{2+} entry pathway in platelet PS exposure that is different from the Orai1 channels.

Figure 7. Unchanged GPVI-dependent Ca²⁺ responses and thrombus formation of STIM2-deficient platelets. A, representative Ca²⁺ rises of Fura-2-loaded platelets from wild type and Stim2^{-/-} mice, induced by CRP (10 µg/ml) plus CaCl₂ (1 mM); B, quantification of maximal Ca²⁺ rises. C, D, thrombus formation after flow of PPACK/heparin-anticoagulated blood over collagen at high shear rate (4 min). C, representative contrast images captured at low (upper panels) or high (middle panels) magnification. Lower panels, fluorescence images after staining with FITC-annexin A5 (bars, 50 µm). D, procoagulant index of relative number of PS-exposing platelets. Means ± S.E. (*n* = 4-6); difference between groups not significant.

Discussion

This study examines the contribution of recently discovered Ca²⁺ entry pathways to the regulation of platelet procoagulant activity and thrombus formation. A first key finding is that deficiency in the Ca²⁺ sensor STIM1 or the Ca²⁺-selective channel Orai1 greatly reduces GPVI-dependent PS exposure and thrombus formation under conditions of flow and absence of coagulation. This corresponds to a major reduction in the Ca²⁺ responses of collagen-adhered Stim1^{-/-} and Orai1^{-/-} platelets. Thus, it can be concluded that the reduced GPVI-induced Ca²⁺ signaling leads to impaired PS exposure and platelet aggregation. Interestingly, platelets from Stim2^{-/-} mice are normally active in thrombus formation and PS exposure under the same flow conditions, indicating that the STIM2 homologue is not implicated in these platelet responses.

Figure 8. Residual receptor-induced Ca²⁺ entry in STIM1- and Orai1-deficient platelets. Calcium responses were measured of Fura-2-loaded platelets from wild type (WT), chimeric *Stim1*^{-/-} or *Orai1*^{-/-} mice, induced by CRP (10 µg/ml) and/or thrombin (Thr, 0.9 nM) and CaCl₂ (1 mM), as indicated. A, representative traces of SKF96365 (SKF, 100 µM) effect on Ca²⁺ rises. Arrows indicate addition of SKF and agonist(s), respectively. B, quantitative effect of SKF on Ca²⁺ rises. Total bars give rises without SKF, black bars rises with SKF, gray parts represent inhibition by SKF (means ± S.E., n = 3-4).

Detailed single cell analysis yet points to discernible differences between the genotypes, in that a sub-population of the collagen-adhered *Orai1*^{-/-} platelets, but not of the *Stim1*^{-/-} platelets, displays residual spiking in Ca²⁺. This residual Ca²⁺ signal will be a consequence of the appreciable GPVI-induced Ca²⁺ mobilization from internal stores of *Orai1*-deficient platelet (25), such in contrast to the markedly reduced Ca²⁺ store mobilization of STIM1-deficient platelets (24). This difference between the genotypes was confirmed in CRP- and thrombin-induced activation studies with Fura-2-loaded platelets. The present findings agree with the recent evidence that a dysfunctional R93W mutation of the *Orai1* channel leads to partial impairment of GPVI-induced PS exposure (25). Collectively, our data underline the importance of both SOCE regulating proteins in shear-dependent platelet activation by collagen under conditions where the contribution of thrombin is absent or limited.

The second key finding is that, under conditions of flow and coagulation, deficiency in platelet STIM1 or *Orai1* does not abolish the capability to PS exposure and formation of a fibrin-containing thrombus. Under these conditions, collagen-adhered *Stim1*^{-/-} and *Orai1*^{-/-} platelets still show prolonged rises in Ca²⁺, although these are lower in magnitude in com-

parison to wild type platelets. Although the number of PS exposing $\text{Stim1}^{-/-}$ and $\text{Orai1}^{-/-}$ platelets is still reduced, the procoagulant index is similar for all genotypes. This suggests that the GPVI-induced SOCE pathway mediated by STIM1 and Orai1 becomes redundant in the regulation of thrombus formation in cases where thrombin acts as a co-agonist. This conclusion is supported by the data showing that stimulation of isolated $\text{Stim1}^{-/-}$ or $\text{Orai1}^{-/-}$ platelets with a combination of GPVI and thrombin receptor agonists result in near unchanged PS exposure and prothrombinase activity. Furthermore, these platelets are partly inhibited in tissue factor-triggered thrombin generation, when stimulated with GPVI ligand. It has been shown that polyphosphates released from platelets significantly contribute to thrombin generation in PRP by activating factor XII (40). The above findings suggest that the polyphosphate contribution is still effective in the absence of STIM1 or Orai1.

The standard protocol for assessment of SOCE is measurement of entry of extracellular Ca^{2+} following Ca^{2+} store depletion, e.g. by the reticular Ca^{2+} -ATPase inhibitor thapsigargin. This Ca^{2+} entry is almost totally blocked in $\text{Stim1}^{-/-}$ and $\text{Orai1}^{-/-}$ platelets, implying that the STIM1-Orai1 interaction forms the principal SOCE mechanism (23,24). A similar conclusion has been drawn for other secretory cells, e.g. T cells and mast cells (39). However, it now appears that both $\text{Stim1}^{-/-}$ and $\text{Orai1}^{-/-}$ platelets show significant Ca^{2+} responses and PS exposure, when co-stimulated via GPVI and thrombin receptors. This is suggestive for the presence of another, compensatory mechanism of Ca^{2+} entry, acting in a (thrombin) receptor-operated fashion.

This hypothesis is supported by our finding that the imidazole antagonist SKF96365, which is a well-studied inhibitor of Ca^{2+} entry in platelets (38), is still capable to suppress the Ca^{2+} and procoagulant responses of platelets deficient in STIM1 or Orai1. Unfortunately, this compound can not be used in measurements of thrombus formation, since it rapidly inactivates in the presence of blood plasma (data not shown). In Fura-2-loaded $\text{Stim1}^{-/-}$ and $\text{Orai1}^{-/-}$ platelets, SKF96365 still inhibits 40-60% of the Ca^{2+} signal independently of the agonist (GPVI ligand and/or thrombin). The slow, non-Orai1 Ca^{2+} entry most likely does not involve the TRPC1 channel (15), since $\text{Trpc1}^{-/-}$ platelets have a fully intact Ca^{2+} signaling machinery (40). Other candidate target proteins of SKF96365 are the non-SOCE channels, TRPC6 and Orai3, both of which act in a receptor-operated way, and are expressed in platelets (16,18). Further studies are required to identify this channel.

A pending question is whether the SOCE mechanism per se is implicated in the transmembrane scrambling of phospholipids, which is the underlying event of PS exposure. Such a role of SOCE has been proposed by others (41). However, the current findings do not support this, since $\text{Stim1}^{-/-}$ and $\text{Orai1}^{-/-}$ platelets (both devoid of SOCE) almost normally expose PS upon combined collagen- and thrombin-receptor stimulation. This is in agreement with the finding that platelets from Scott syndrome patients, which have a defect in PS exposure, show unchanged Ca^{2+} signals in response to collagen/thrombin (42). Together, this strongly argues against a role of SOCE or other Ca^{2+} channels as phospholipid scramblase proteins.

The present findings support the idea that the Orai1 channel is an attractive target for pharmacological treatment of thrombosis. Previous results have demonstrated that the

deficiency of Orai1 in mouse platelets protects from arterial thrombus formation, particularly in thrombosis models that are collagen-dependent (23,24). Since collagen-independent thrombosis models mostly rely on tissue factor exposure and thrombin generation (5), it is plausible that specific targeting of Orai1 (or STIM1) restricts platelet procoagulant activity and thrombus formation only under conditions, where the role of tissue factor is limited. Hence, targeting of Orai1 may be more effective to prevent thrombosis in arteries, where (tissue factor-dependent) thrombin accumulation is confined by the high-shear flow conditions. In contrast, blockage of Orai1 may not impair bleeding from wounds where tissue factor is abundantly exposed. Indeed, mice deficient in Orai1 have only a mild prolongation of the tail bleeding time (25). Whether inhibition of the non-Orai1 (non-SOCE) channels targeted by SKF96365 also provides antithrombotic protection, still needs to be investigated.

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Chapter 7

Regulation of integrin $\alpha\text{IIb}\beta 3$ closure in phosphatidyl-
serine-exposing platelets

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Abstract

Thrombi formed under flow conditions contain microdomains of phosphatidylserine (PS)-exposing platelets, separated from aggregated platelets that express activated integrin $\alpha\text{IIb}\beta_3$. In platelets stimulated with ionomycin or convulxin/thrombin, we aimed to elucidate the mechanism responsible for inactivation or closure of integrin $\alpha\text{IIb}\beta_3$ in PS-exposing platelets. Western blotting indicated that the closure of $\alpha\text{IIb}\beta_3$ coincided with the cleavage of Src protein kinase. However, inhibition of the Ca^{2+} -dependent protease calpain completely antagonized Src cleavage, whereas $\alpha\text{IIb}\beta_3$ closure was only partly reduced. A role of formation of the mitochondrial permeability transition pore (mPTP) was investigated by using the inhibitor cyclosporin A or platelet from mice lacking the mPTP forming protein, cyclophilin D. In either case, convulxin/thrombin-induced Ca^{2+} rises were no more than moderately affected, whereas integrin closure and PS exposure were more strongly suppressed. Conclusively, this study suggests a model of a calpain-dependent and a mitochondrial (mPTP)-dependent route of integrin closure in PS-exposing platelets.

Introduction

Platelet aggregation, in which platelets become connected via active integrin $\alpha\text{IIb}\beta_3$ and fibrinogen bridges, is fundamental to intravascular thrombus formation (1,2). However, thrombi also contain microdomains of procoagulant platelets, characterized by exposure of phosphatidylserine (PS), which bind coagulation factors and generate thrombin (3,4). In earlier work, the process of PS exposure was shown to rely on a sustained, high intracellular Ca^{2+} concentration (3,5). PS-exposing platelets assume a balloon-like shape and gradually detach from the thrombus (1,6). Initial experiments suggested that this detachment is linked to inactivation or closure of their $\alpha\text{IIb}\beta_3$ integrins (1,7).

Glycoprotein VI (GPVI) is the major signaling receptor for collagen on platelets (8). Upon cross-linking of GPVI, the immunoreceptor tyrosine-based activation motif of the FcR γ -chain undergoes dual tyrosine phosphorylation by members of the Src family kinases, which leads to phosphorylation and activation of the protein tyrosine kinase Syk. This kinase mediates assembly of the LAT signalosome by phosphorylating several docking, adaptor and effector molecules, among which is the key effector enzyme, phospholipase $\text{C}\gamma_2$ (PLC γ_2) (9,10). Activation of PLC γ_2 , further enhanced by phosphoinositide 3-kinases, results in the formation of two second messengers, i.e. 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (11,12).

Platelet aggregation relies on the activation of integrin $\alpha\text{IIb}\beta_3$, which provides high-affinity binding sites for fibrinogen (13). Activation of $\alpha\text{IIb}\beta_3$ can be achieved by many platelet agonists via a complex of signaling cascades that is gradually being revealed and includes the small molecule G-protein Rap1b (14-16). One accepted model for the reversible transition from the resting to the activated state of $\alpha\text{IIb}\beta_3$ is by cytoskeletal-mediated unclasping of the αIIb and β_3 chains (17,18). Among the proteins involved, linked to the actin cytoskeleton, are filamin A, kindlin-3 and talin-1 (7,19,20).

The process of integrin closure in PS-exposing platelets likely involves more factors than simply a reversion of the activation process (21), but this has not yet been investigated

in detail. Especially the requirement of sustained and high rises in Ca^{2+} for agonist-induced PS exposure suggests that different signaling processes are involved in the regulation of Ca^{2+} -dependent integrin closure. There is conflicting evidence for a role of the Ca^{2+} -dependent thiol protease, calpain, in the modulation of $\alpha\text{IIb}\beta_3$ function. Early studies reported that calpain exerts a positive effect on $\alpha\text{IIb}\beta_3$ by cleaving talin, enabling talin to bind to the β_3 subunit and provoke unclasp (22). However, other papers considered that calpain activation is a late, $\alpha\text{IIb}\beta_3$ -dependent process, mediating proteolysis of signaling and cytoskeletal proteins to achieve full platelet activation (23,24). Another report suggested that activated calpain cleaves the cytoplasmic tail of the β_3 chain, which would counteract the high-affinity conformational state of $\alpha\text{IIb}\beta_3$ (6). Hence, the precise role of calpain in regulating $\alpha\text{IIb}\beta_3$ function is still controversial.

Surface exposure of PS, due to scrambling of phospholipids in the plasma membrane, is provoked by strong Ca^{2+} -mobilizing conditions, such as achieved by Ca^{2+} ionophores like ionomycin or by specific combinations of platelet agonists, such as convulxin (a GPVI agonist) and thrombin (3,25). A recent study also reports a Ca^{2+} -dependent role of mitochondria in the induction of PS exposure (26). Evidence for this came from the observation that platelets from mice lacking cyclophilin D (CypD), which is a component of the mitochondrial permeability transition pore (mPTP), are impaired in agonist-induced PS exposure and platelet procoagulant activity (27,28). Whether mPTP formation contributes to the process of integrin closure in PS-exposing platelets is however unknown. Here, we studied the signaling processes involved in the closure of $\alpha\text{IIb}\beta_3$ in activated, PS-exposing platelets. In particular, we examined the possible roles of Src-family kinases, mPTP formation and calpain-dependent protein cleavage.

Experimental procedures

Materials

H-Phe-Pro-Arg chloromethyl ketone (PPACK) and anti-Src mAb (327) were obtained from Calbiochem. Fibrillar type I collagen (Horm) was from Nycomed. Apyrase (grade V), bovine serum albumin (BSA), heparin, and human thrombin were from Sigma. Convulxin was purified to homogeneity from the venom of *Crotalus durissus terrificus* (Latoxan), as described (29). Fluorescein isothiocyanate (FITC)-labeled annexin A5 was from Nexins Research. Alexa Fluor-647 (AF647)-labeled annexin A5, Fura-2 and Fluo-4 acetoxymethyl esters, and pluronic F-127 were from Invitrogen. FITC-labeled PAC1 monoclonal antibody (mAb) against activated human $\alpha\text{IIb}\beta_3$ came from BD Biosciences. Phycoerythrin (PE)-labeled anti-mouse mAb JON/A was from Emfret Analytics. Anti-phosphotyrosine mAb 4G10 was from Upstate Biotechnology. Src kinase inhibitors PP2 and Src inhibitor I, Syk inhibitor II, PTP inhibitor I, calpeptin, and cyclosporin A were from Sigma. The PKC θ inhibitor (30) was a kind gift from Boehringer Ingelheim Pharmaceuticals. Other materials were obtained from sources indicated before (31,32).

Animals

Animal studies were approved by the local animal care and use committees. CypD-deficient mice, which were homozygous for a targeted deletion of the Ppif gene (27), and wildtype CypD^{+/+} mice were generated, as described previously (27), and maintained on an inbred SV129 background. Age- and sex-matched mice were used for the experiments.

Blood collection and platelet preparation

Blood was obtained from healthy volunteers, who had not taken aspirin or related drugs for at least 2 weeks, and gave full informed consent. For whole blood flow experiments, the blood was collected into 1/10 volume saline, containing 40 μ M PPACK and 40 U/ml fragmin, as described (1,33). Where indicated, blood was also collected into 1/6 volume ACD solution (85 mM sodium citrate, 78 mM citric acid, and 11 mM D-glucose). Washed platelets were prepared by centrifugation of platelet-rich plasma (PRP) (5), and resuspended into Hepes buffer, pH 7.45 (136 mM NaCl, 10 mM Hepes, 2.7 mM KCl, 2 mM MgCl₂, 0.1% glucose and 0.1% BSA). Platelet count was determined with a Coulter counter and adjusted to 1×10^8 platelets/ml.

Mouse blood was obtained via orbital puncture under anesthesia. The blood was collected into 12.9 mM trisodium citrate, 1 U/ml heparin, and 5 mM glucose. Washed mouse platelets were prepared from PRP and resuspended in modified Hepes buffer, pH 7.45 (136 mM NaCl, 5 mM Hepes, 2.7 mM KCl, 2 mM MgCl₂, 0.42 mM Na₂HPO₄, 0.1% glucose, and 0.1% BSA), as described (31).

Platelet flow cytometry and aggregation

Washed human or mouse platelets (1×10^8 platelets/ml) were preincubated for 10 minutes with Me₂SO vehicle or inhibitor, as indicated in the text. After 5 and 30 minutes of activation with different agonists in the presence of 2 mM CaCl₂ (37 °C), platelet samples were analyzed with a FACScan flow cytometer, equipped with an argon and a red diode laser (Becton Dickinson). Surface expression of PS was detected with AF647-labeled annexin A₅ (1:100), while expression of active integrin α IIb β ₃ was detected with FITC-labeled PAC1 mAb (1:10) for human platelets, or with PE-labeled JON/A mAb (1:20) for mouse platelets. Aggregation of platelets after activation under stirring conditions was determined by single-cell platelet counting using a Coulter counter (34).

Western blotting and immunohistochemistry

Platelet samples were lysed in ice-cold NP-40-based lysis buffer pH 7.4 (300 mM NaCl, 20 mM Tris-HCl, 2 mM EGTA, 2 mM EDTA, 2 mM Na₃VO₄, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 μ g/ml pepstatin, 2% NP-40). Protein amounts in the lysates were quantified with a BioRad DC protein kit. Lysates with equal protein amounts, resolved in 4x Laemmli sample buffer, were analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), basically as described elsewhere (35). Membranes were immunoblotted using an ECL system (Amersham Biosciences) with

primary anti-phosphotyrosine 4G10 mAb or anti-Src mAb, and then stained with horseradish peroxidase-coupled secondary Ab.

Intracellular Ca^{2+} measurements

For Ca^{2+} measurements of human platelets in suspension, PRP (2×10^8 platelets/ml) was loaded with 2.5 μM Fura-2 acetoxymethyl ester at 37 °C for 45 minutes (36). Loaded platelets were isolated, resuspended into Hepes buffer pH 7.45, and preincubated with indicated inhibitors or Me2SO vehicle for 10 minutes. Calcium responses were recorded under stirring with a spectrofluorometer (SLM/Aminco) at alternate excitation wavelengths of 340 and 380 nm (37 °C). Ratio values of fluorescence at 340/380 nm were converted into nanomolar concentrations of $[\text{Ca}^{2+}]_i$, as described (36). Rises in $[\text{Ca}^{2+}]_i$ were expressed as 5 minutes time integrals (37).

For single cell Ca^{2+} measurements in flowing whole mouse blood, washed mouse platelets were incubated with 8 μM Fluo-4 acetoxymethyl ester and 0.2 mg/ml pluronic F-127 for 45 minutes at ambient room temperature under gentle rotation (38). The dye-loaded platelets were added to PPACK-anticoagulated blood from the same mouse strain to give 10% labeled platelets. During the first minute of whole blood perfusion over collagen at a shear rate of 1000 s^{-1} , fluorescence images were captured from the collagen surface at a frequency of 5 Hz. Changes in fluorescence (F) were analyzed in regions-of-interest corresponding to single platelets. Pseudo-ratio F/Fo values were converted into nanomolar concentrations of $[\text{Ca}^{2+}]_i$ using predefined calibration parameters (39).

Statistical analysis

Differences between experimental groups were tested for significance with the non-parametric Mann-Whitney U test. The statistical package for social sciences (SPSS 17.0) was used.

Results

Platelet PS exposure and integrin $\alpha\text{IIb}\beta_3$ closure coincide with Src kinase cleavage

To reinvestigate the relation between PS exposure and integrin $\alpha\text{IIb}\beta_3$ activation during thrombus formation, both processes were examined in platelet thrombi that were generated by flowing PPACK-anticoagulated whole blood over collagen at intermediately high shear rate of 1000 s^{-1} . As shown in Fig. 1A, platelet aggregates that were formed on the collagen surface were surrounded by single, bleb-forming platelets, only weakly attached to the aggregates. Dual staining for activated integrin $\alpha\text{IIb}\beta_3$ (using FITC-PAC1 mAb) and exposure of PS (using AF647-annexin A5) confirmed the presence of two separate platelet populations, e.g. bleb-shaped, PS-exposing platelets surrounding aggregated platelets with activated integrins. This is in agreement with earlier observations that thrombi are heterogeneous in composition with discrete patches of procoagulant and aggregated platelets (1). Considering that in platelet activation integrin $\alpha\text{IIb}\beta_3$ activation is a fast response and PS exposure is relatively slow, these results suggest that the integrins of PS-exposing platelets are secondarily inactivated.

In suspensions of human platelets, the ability was studied of activated integrin $\alpha\text{IIb}\beta_3$ to close in time. Two-color flow cytometry was used to examine the binding of FITC-PAC1 mAb (detecting activated $\alpha\text{IIb}\beta_3$) as well as AF647-annexin A5 (detecting PS exposure). After 5 minutes of activation with convulxin/thrombin in the presence of CaCl_2 , about 70% of all platelets bound PAC1 mAb, a fraction which reduced to 25% at a later time point of 30 minutes after activation (Fig. 1B). On the other hand, the fraction of annexin A5-binding platelets increased between 5 and 30 minutes from 53% to 78%. Combined analysis of the two colors showed that, after 30 minutes, the vast majority annexin A5-binding platelets did not bind PAC1 mAb, while after 5 minutes there was a small sub-population of platelets binding both probes (data not shown). These results thus suggested that convulxin/thrombin stimulation results in a gradual accumulation of PS-exposing platelets, in which the initially activated integrins close in time.

Figure 1. Integrin $\alpha\text{IIb}\beta_3$ closure in PS-exposing platelets is accompanied by Src kinase cleavage. A, Ex vivo flow experiments were performed with PPACK-anticoagulated human blood perfused over a collagen surface at 1000 s^{-1} for 4 minutes. Shown is a representative phase contrast image and a two-color fluorescence image, after staining for activated integrin $\alpha\text{IIb}\beta_3$ (FITC-PAC1 mAb) and PS exposure (AF647-annexin A5); bar, 15 μm . B, Flow cytometric analysis of washed human platelets stimulated for 5–30 minutes with convulxin (CVX, 100 ng/ml) plus thrombin (Thr, 4 nM) in the presence of 2 mM CaCl_2 . Shown are platelet fractions with activated $\alpha\text{IIb}\beta_3$ (FITC-PAC1 positive) or exposed PS (FITC-annexin A5-positive). Mean $\pm$ S.E. ($n=8-12$); **, $p<0.02$; ***, $p<0.001$. C, Single platelet counts after 5 minutes stimulation of platelets with CVX (100 ng/ml), Thr (4 nM), or ionomycin (20 μM), in the presence of 2 mM CaCl_2 . Mean $\pm$ S.E. ($n=3$); *, $p<0.05$. D, Representative western blots from platelets stimulated for 5–30 minutes with CVX (100 ng/ml) and Thr (4 nM) or ionomycin (20 μM). Activations were in the presence of 2 mM CaCl_2 or a 2 mM EGTA, as indicated. Blots were immunostained with 4G10 mAb (upper), and reprobed with anti-Src Ab (lower panel). Arrows indicate position of Src family proteins. For color version, see page 190.

To find out whether the gradual integrin closure impairs the ability of platelets to form aggregates, we measured platelet counts in suspension at fixed time points after activation. Previously, this method of single platelet counting was found to be a sensitive way of detecting platelet (micro)aggregates (34). After 30 minutes of activation with either convulxin or thrombin, single platelet counts were reduced to <15% of the original count, compatible with persistent, strong platelet aggregation (Fig. 1C). In contrast, after 30 minutes activation with convulxin/thrombin, platelet count reduced to only 38% of the initial count, pointing to an increased number of platelets that was not captured into aggregates. This was confirmed by stimulation with the Ca^{2+} ionophore ionomycin, causing PS exposure in >90% of the platelets, which resulted in only a minor reduction in original platelet count, indicating an absence of platelet aggregation.

To determine the intracellular signaling events of PS-exposing platelets, the protein tyrosine phosphorylation state was examined by staining western blots with 4G10 mAb. Platelets were stimulated during 5-30 minutes with convulxin/thrombin or ionomycin in the presence of CaCl_2 or EGTA. With CaCl_2 present in the extracellular medium the influx of Ca^{2+} is known to enhance PS exposure (40). As shown in Fig. 1D, the tyrosine phosphorylation pattern markedly changed in time, under activated conditions where CaCl_2 was present. Most striking was a shift of the 60 kDa band present in unstimulated platelets to a band of around 55 kDa after 30 minutes of stimulation with convulxin/thrombin (partly) or ionomycin (completely). Reprobing of the blots with anti-Src mAb indicated that Src kinase was present in the shifted tyrosine-phosphorylated band. Considering that the abundantly expressed Src is tyrosine-phosphorylated in resting platelets (41), these results suggested that Src kinase is cleaved after prolonged platelet activation with convulxin/thrombin or ionomycin, i.e. under activation conditions where platelets expose PS and integrin $\alpha\text{IIb}\beta_3$ is closed.

Inhibition of Src kinase does not affect integrin closure or PS exposure in activated platelets

Whether Src activity is required for the process of integrin closure in PS-exposing platelets was studied with several kinase inhibitors, applied at maximally effective concentrations. We used the compound PP2 (40 μM), which is a general Src family kinase inhibitor, and Src inhibitor I (10 μM), which only inhibits Src itself (42,43). Integrin closure and PS exposure were simultaneously examined by two color flow cytometry. In the interval between 5 and 30 minutes after platelet activation with convulxin/thrombin, integrin closure and PS exposure were reduced by PP2, but not by Src inhibitor I (Fig. 2A). This may be due to the fact that PP2 suppresses platelet activation via GPVI and $\text{PLC}\gamma_2$, which pathway relies on the Src-family kinase members, Fyn and Lyn (44). Src kinase itself is not involved in this pathway. This was investigated in more detail.

Figure 2. Src kinase inhibition does not affect integrin $\alpha IIb\beta 3$ closure in PS-exposing platelets. **A**, Increase in PS exposure (left) or integrin closure (right) of human platelets stimulated with convulxin (CVX, 100 ng/ml) and thrombin (Thr, 4 nM) in the presence of 2 mM CaCl₂ for 5 to 30 minutes. Platelets were preincubated with vehicle (0.2% Me₂SO), PP2 (40 μ M), Src inhibitor I (10 μ M), Syk inhibitor II (1 μ M) or PTP inhibitor I (100 μ M), as indicated. Samples were analyzed by dual color flow cytometry for binding of FITC-PAC1 mAb and AF647-annexin A₅. Bars indicate changes between 5 and 30 minutes; numbers above bars indicate absolute percentages of platelets with PS exposure or integrin activation after 30 minutes. Means $\pm$ S.E. (n=3). **B**, Ca²⁺ responses in Fura-2-loaded platelets stimulated with CVX (100 ng/ml) and Thr (4 nM) in the presence of 2 mM CaCl₂. Inhibitors were present as described above. Shown are 5 minutes time integrals of nM rises in [Ca²⁺]_i, expressed as percentages of control (574 $\pm$ 31 μ M/min). Mean $\pm$ S.E. (n=3); *, p<0.05. **C**, Effects of inhibitors on protein tyrosine phosphorylation after 5-30 minutes stimulation with CVX (100 ng/ml) plus Thr (4 nM) or ionomycin (20 μ M) in the presence of 2 mM CaCl₂. Western blots were immunostained with 4G10 mAb. Blot shown is representative for 3 independent experiments. Arrow indicates position of Src family proteins.

First, platelets were preincubated with Syk inhibitor II, which compound blocks Syk kinase (9), and stimulated with convulxin/thrombin. This inhibitor allowed integrins to open, but abolished integrin closure as well as PS exposure in time (Fig. 2A). Second, the GPVI pathway was blocked with protein tyrosine phosphatase (PTP) inhibitor I, which suppresses the phosphatases SHP1 (45) and PTP1B (46). In the presence of PTP inhibitor I, convulxin/thrombin stimulation did not activate platelet integrins (which thus could not close), and greatly suppressed PS exposure in time. Effects of these inhibitors were also measured on convulxin/thrombin-induced rises in intracellular Ca²⁺ level. Consistent with suppression of the GPVI/PLC γ 2 pathway, Ca²⁺ responses were markedly decreased in the presence of PP2, Syk inhibitor II or PTP inhibitor I, but not with Src inhibitor I (Fig. 2B). Western blot analysis indicated that both PP2 and PTP inhibitor I markedly prevented the tyrosine phosphorylated band shift (Fig. 2C). Together, these results point to a close association of convulxin-

in/thrombin-induced integrin closure and PS exposure, relying on GPVI stimulation and high rises in Ca^{2+} and associated with Src degradation. However, the failure of Src inhibitor I to suppress integrin closure suggests that activation of Src kinase itself is not crucial for this event.

Figure 3. Inhibition of calpain suppresses integrin $\alpha\text{IIb}\beta_3$ closure and prevents Src kinase cleavage. A, Washed human platelets were stimulated with convulxin (CVX, 100 ng/ml) and thrombin (Thr, 4 nM) in the presence of 2 mM CaCl_2 . Platelets were preincubated with vehicle (0.2% Me₂SO), calpeptin (200 μM), or MDL28170 (200 μM). Time-dependent (5–30 minutes) changes in binding of AF647-annexin A5 (left panel) and FITC-PAC1 mAb (right panel) were measured by dual color flow cytometry. Numbers above bars represent absolute percentages of platelets with PS exposure or integrin activation, after 30 minutes. B, Intracellular rises in $[\text{Ca}^{2+}]$, measured in suspensions of Fura-2-loaded platelets stimulated with CVX (100 ng/ml) and Thr (4 nM) in the presence of 2 mM CaCl_2 . Preincubation with vehicle or calpeptin (200 μM) was as indicated. C, Western blot and immunostaining with 4G10 mAb of platelet lysates after preincubation with vehicle or calpeptin (200 μM), and time-dependent co-stimulation with CVX (100 ng/ml) plus Thr (4 nM) or ionomycin (20 μM) in the presence of 2 mM CaCl_2 . Means $\pm$ S.E. (n=3); *, $p < 0.05$, **, $p < 0.02$ compared to vehicle. Arrow indicates position of Src family proteins.

Calpain activity contributes to integrin $\alpha\text{IIb}\beta_3$ closure

Given that the Ca^{2+} -dependent protease calpain can degrade the actin cytoskeleton in PS-exposing platelets (47), calpain activation might play a role in integrin closure and Src degradation. To study this hypothesis, the calpain inhibitors calpeptin and MDL28170 were used at a maximally effective concentration of 200 μM (48,49). Previously, calpain inhibition has been shown to prevent degradation of the cytoskeleton-associated protein talin (48). Two-color flow cytometry demonstrated that, in convulxin/thrombin-stimulated platelets, pretreatment with calpeptin or MDL28170 partly decreased integrin closure in the time

interval of 5–30 minutes, while PS exposure was not significantly changed (Fig. 3A). Measurements with Fura-2-loaded platelets indicated that calpeptin did not alter the convulxin/thrombin-induced Ca^{2+} rises (Fig. 3B). In contrast, calpeptin did abolish the Src kinase shift on tyrosine phosphorylation blots (Fig. 3C). Together, this denotes a major role of calpain in degradation of Src kinase, but only a partial role in integrin closure.

Subtle role of mitochondrial permeability transition pore (mPTP) formation in integrin $\alpha\text{IIb}\beta_3$ closure

Mitochondria act as Ca^{2+} -buffering organelles (50), but this role is disrupted upon mPTP formation (51). Previous work from other laboratories has shown that PS exposure in mouse platelets deficient in cyclophilin D (CypD) is abolished due to their inability to mPTP formation (27,28). To investigate the role of mPTP formation in integrin closure, we used the established mPTP inhibitor, cyclosporin A. In human platelets pretreated with cyclosporin A, stimulation with convulxin/thrombin greatly reduced early (5 minutes) PS exposure from 53% to 4% and late (30 minutes) PS exposure from 79% to 9%, (Fig. 4A). Strikingly, this pretreatment also prevented integrin closure in this time interval, but it did not affect integrin activation, as 85% of the platelets showed activated integrins after 30 minutes. Other experiments showed that cyclosporin A did not influence convulxin/thrombin-induced Ca^{2+} rises measured in platelet suspension (Fig. 4B). Western blots showed that cyclosporin A did prevent the band shift of tyrosine phosphorylated Src kinase (Fig. 4C).

The role of mPTP formation was also examined in pilot studies with platelets from mice homozygously deficient in the protein CypD, which is an essential component of the mPTP. Two-color flow cytometry showed that stimulation of CypD^{+/+} or CypD^{-/-} platelets with ionomycin for 5 minutes resulted in maximal PS exposure ($94 \pm 1.7\%$ vs. $91 \pm 2.7\%$) and in low integrin activation ($27 \pm 6.4\%$ vs. $29 \pm 5.7\%$). This response remained unchanged during the following 25 minutes. Stimulation with convulxin/thrombin (5 minutes) led to more integrin activation and less PS exposure in CypD^{-/-} platelets in comparison to wildtypes (Fig. 5A). The knockout platelets had a similar activation profile after 30 minutes. Pretreatment with cyclosporin A suppressed integrin closure and PS exposure in CypD^{+/+} platelets but not CypD^{-/-} platelets, thus confirming the specificity of cyclosporin A as an inhibitor of mPTP formation. Interestingly, pretreatment with an inhibitor of protein kinase C θ (PKC θ) tended to decrease integrin closure in CypD^{+/+} platelets, but not in CypD^{-/-} platelets. Calpain inhibition with calpeptin gave inconclusive effects. Measurements of Ca^{2+} rises in Fluo-4-loaded platelets during flow demonstrated a moderate, but significant lowering in CypD^{-/-} compared to CypD^{+/+} platelets, especially at early time points (Fig. 5B). Together, these results point to a (subtle) role of mPTP formation in integrin closure along with PS exposure in human and mouse platelets.

Figure 4. Inhibition of mPTP formation suppresses integrin $\alpha\text{IIb}\beta_3$ closure and prevents Src kinase cleavage. Washed human platelets were co-stimulated with convulxin (CVX, 100 ng/ml) and thrombin (Thr, 4 nM) in the presence of 2 mM CaCl_2 . Platelets were preincubated with vehicle (0.2% Me₂SO) or cyclosporin A (4 μM). **A**, Time-dependent changes in binding of AF647-annexin A5 (left panel) and FITC-PAC1 mAb (right panel) was measured by dual color flow cytometry. Numbers above bars indicate absolute percentages of platelets with PS exposure or integrin activation after 30 minutes. **B**, Rises in $[\text{Ca}^{2+}]_i$ were measured in suspensions of Fura-2-loaded platelets co-stimulated with CVX (100 ng/ml) and Thr (4 nM) in the presence of 2 mM CaCl_2 . Preincubation with vehicle or cyclosporin A (4 μM) was as indicated. **C**, Western blot and immunostaining with 4G10 mAb of platelet lysates after preincubation with vehicle or cyclosporin A (4 μM), and time-dependent co-stimulation with CVX (100 ng/ml) plus Thr (4 nM) or ionomycin (20 μM) in the presence of 2 mM CaCl_2 . Means $\pm$ S.E. ($n=3$); **, $p<0.02$ compared to vehicle. Arrow indicates position of Src family proteins.

Discussion

In this work, we provide new insight into the mechanism of integrin $\alpha\text{IIb}\beta_3$ closure in agonist-induced PS-exposure. In platelets stimulated with convulxin/thrombin, we could establish that initial activation of this integrin is followed by closure at later time points. Two-color flow cytometry indicated that most of the platelets that expose PS turn off their integrins. Defective adhesion of such platelets was confirmed by single platelet count analysis, showing that the count of single platelets in suspension increased after long term activation with convulxin/thrombin along with PS exposure.

Figure 5. Effects of CypD deficiency on integrin α IIb β 3 closure, PS exposure and Ca²⁺ signaling. Washed mouse platelets were stimulated for 5–30 minutes with convulxin (100 ng/ml) plus thrombin (4 nM) in the presence of 2 mM CaCl₂. A, Time-dependent binding of AF647-annexin A5 (upper panel) and PE-JON/A (lower panel) of CypD^{+/+} and CypD^{-/-} platelets, measured by flow cytometry. Preincubation was with vehicle (0.2% Me₂SO), calpeptin (200 μ M), cyclosporin A (4 μ M) or PKC θ inhibitor (1 μ M), as indicated. Means $\pm$ S.D. (n=2). B, Ca²⁺ responses during perfusion over collagen of Fluo-4-loaded platelets from CypD^{+/+} and CypD^{-/-} mice. Shown are representative Ca²⁺ traces (upper panel) and average traces from 30–50 cells (lower panel), *, p<0.05 compared to wildtype.

Mechanistically, our data indicate that integrin closure in PS-exposing platelets coincided with cleavage of the protein kinase Src in a Ca²⁺-dependent way. Markedly, Src cleavage along with integrin closure was abolished in the presence of the Src-family kinase inhibitor, PP₂, suggesting a requirement in integrin closure. However, Ca²⁺ measurements suggested that this inhibitory effect was due to PP₂-inhibited activation of the GPVI/PLC γ 2 pathway by Src-family kinase members. In contrast, a specific inhibitor of Src did not significantly affect PS exposure and integrin closure. Hence, while Src is cleaved in PS-exposing platelets, this cleavage is not responsible for integrin closure. Since Src-dependent signaling is essential in integrin α IIb β 3-induced outside-in signaling (52), these data yet reveal a novel negative feedback pathway in PS-exposing platelets, where abolition of Src functioning via calpain-dependent cleavage blocks integrin-dependent outside-in signaling.

Our findings also modify an earlier proposed scenario that calpain-mediated cleavage of cytoskeletal related proteins is a regular post-aggregation event (24). We show that platelet treatment with the calpain inhibitor calpeptin abrogates Src cleavage, but no more than partly affects integrin closure and PS exposure in response to convulxin/thrombin. In other words, inhibition of calpain under conditions of sustained high intracellular Ca²⁺ does

not abolish integrin closure, nor PS exposure. Based on these results another calpain-independent pathway of integrin closure appears to occur in PS-exposing platelets.

In various cell types, mitochondria serve as Ca^{2+} -buffering systems. In platelets, loss of the mitochondrial transmembrane potential and formation of the mPTP appear to contribute to the Ca^{2+} -dependent activation process (53,54). Similarly, platelets from CypD^{-/-} mice, which cannot form a mPTP, have been reported to be impaired in PS exposure in response to strong agonists (28). In our hands, we find a partial reduction in both integrin closure and PS exposure in mouse platelets lacking CypD, accompanied by a moderate reduction in Ca^{2+} signaling. In human platelets stimulated with convulxin/thrombin, we find that cyclosporin A treatment causes a substantial reduction in integrin closure and PS exposure, although Ca^{2+} signaling remains unaffected. These findings do not agree with those of Dachary-Prigent et al., who propose that mPTP formation does not play a role in PS exposure (26). Leaving open the possibilities of subtle differences in mechanism between mouse and human platelets and CypD-independent effects of cyclosporin A, our results jointly point to a partial role of mPTP formation that could occur independently of Ca^{2+} mobilization. Whether PKC θ indeed modulates this function of mitochondria - such as suggested by inhibitor experiments - needs to be confirmed.

Taken together, this study reveals partial contributions of calpain and of mPTP formation in agonist-induced integrin closure in PS-exposing platelets.

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Chapter 8

General discussion

In this thesis, we have investigated various *roads leading to thrombus formation*, and by finding new concepts that regulate this process, we contributed to *demystifying* the roles of earlier suggested *platelet signaling pathways*. Our investigations involved multiple aspects of thrombus formation, from platelet receptors, via the signaling roles of specific protein and lipid kinases, to the molecular components of intracellular Ca^{2+} regulation. In this respect, we define thrombus formation as the overall result of platelet activation, aggregation, and procoagulant activity during whole blood flow over a thrombogenic surface in vitro or in vivo.

Collagen-dependent thrombus formation as a multiple adhesive receptor process

Platelets display several surface receptors for binding to collagen, the function of which varies with the prevalent shear stress conditions and the type and fibrillation of the collagen substrate (1,2). By using several combinations of triple-helical peptide substrates, mimicking distinct adhesive domains of collagen, whereby platelets bind to integrin $\alpha_2\beta_1$, glycoprotein VI (GPVI) or GPIb-V-IX/von Willebrand factor (vWF), we established that platelet binding to multiple collagen receptors is required for full thrombus formation in particular at high shear, arterial shear rate. In chapter 2, we show that two collagen-mimetic peptide sequences, GFOGER and $(\text{GPO})_n$ – binding to $\alpha_2\beta_1$ and GPVI, respectively –, in combination with immobilized vWF causes full thrombus formation. The combination of these three substrate sequences induces similar platelet responses as native collagen type I fibers. Furthermore, with the triple-helical peptide $(\text{GPO})_n$ -GFOGER- $(\text{GPO})_n$, we find a similar pattern of heterogeneity of aggregated platelets versus single phosphatidylserine (PS) exposing platelets as was detected earlier upon whole blood perfusion over native collagen fibers (3). As indicated in Figure 1, full-blown formation of stable thrombi under flow requires the combination of vWF and collagen-mimetic peptides with binding sites for both $\alpha_2\beta_1$ and GPVI. Hence, those triple-helical $\text{GXX}'\text{GER}(\text{GPO})_n$ sequences that bind with highest affinity to $\alpha_2\beta_1$ are also the most active ones in supporting thrombus formation and platelet procoagulant activity at given shear conditions (Figure 1). This synergy between a signaling receptor for collagen, GPVI, and (high-shear) adhesion receptors, i.e. $\alpha_2\beta_1$ and GPIb-V-IX/vWF, has also been noted by others (4). By consequence, our findings support and extend the earlier defined principle of interplay between these three receptors in stable platelet adhesion and activation at arterial shear rate (5-7). In this respect, we like to note that the earlier data were obtained by immunological blocking of $\alpha_2\beta_1$, GPVI, or GPIb, or by using mice lacking one of the receptors, i.e. conditions where side effects due to non-specific antibody inhibition or to down-regulation of other receptor-mediated responses cannot be excluded.

Figure 1. Immobilized collagen-mimetic peptides in thrombus formation at high-shear blood flow. Summary of the results from chapter 2 on thrombus formation induced by various immobilized triple-helical collagen-mimetic peptides, binding to platelet integrin $\alpha_2\beta_1$ (GXX'GER) or GPVI [(GPO)_n with (GPP)_n as negative control]. Upper part compares the different affinities of GXX'GER hexapeptides for activated integrin $\alpha_2\beta_1$ according to ref. [7]. The indicated peptides were immobilized alone or together with vWF. Stable thrombus formation with procoagulant, PS-exposing platelets – resembling the thrombi formed on native type I collagen – was obtained with the combinations of GFOGER(GPO)_n or GAO/SGER(GPO)_n plus vWF. In the absence of vWF, only GFOGER(GPO)_n with high affinity for $\alpha_2\beta_1$ was limitedly active in supporting thrombus formation. Immobilization of (GPO)_n peptide or vWF alone did not result in platelet adhesion.

An unpredicted finding was that the $\alpha_2\beta_1$ -binding triple-helical peptide, (GPP)_n-GFOGER-(GPP)_n, lacking the GPVI-activating (GPO)_n sequence, did weakly induce instable platelet aggregation and PS exposure in both high- and low-shear flow settings (Figure 1). Detailed analysis by the Farndale group indicated that such triple-helical (GPP)_n peptides have an extremely low affinity for GPVI (8), thus proposing that the GFOGER sequence is responsible for the platelet-activating effect. We also found that blocking of GPVI suppressed the platelet responses, suggesting that GFOGER(GPP)_n surfaces have a low GPVI-dependent activity.

These results are reminiscent to those from others, showing that static adhesion of platelets to triple helical GFOGER hexapeptide (9) provokes tyrosine phosphorylation of various signaling proteins of the GPVI-Fc receptor γ -chain (FcR γ) cascade, including Src, Syk, SLP76 and phospholipase C γ (PLC γ) (10). These authors suggested that integrin $\alpha_2\beta_1$ engagement via outside-in signaling leads to a similar activation pathway as does GPVI ligation. Another, recent paper showed that $\alpha_2\beta_1$ -mediated adhesion of platelets to

GFOGER peptides causes activation of the pathway of phosphatidylinositol 3-kinase β (PI3K β) and Akt (11). Because $\alpha 2\beta 1$ by itself is unable to stimulate PI3K β (11) and, as shown in chapter 3 of this thesis, GPVI/PLC γ induces prominent PI3K β activation and downstream signaling, these findings are supportive for a role of $\alpha 2\beta 1$ engagement with GFOGER in the activation of GPVI, possibly via a trans-receptor signaling mechanism. Support for this idea comes from the finding that both receptors appear to interact during collagen activation of platelets (6). However, future research is needed to establish how precisely such receptor cross-talk and trans-receptor signaling can take place in the platelet membrane.

Roles of PI3K and PKC isoforms in thrombus formation and procoagulant activity

Ligand binding to collagen and other platelet receptors leads to complex networks of signaling processes that eventually result in functional responses, such as integrin $\alpha IIb\beta 3$ activation, secretion, platelet aggregation, and procoagulant activity. Because they are placed at crucial nodes in these networks, two families of signaling proteins have shown to be of great importance. These are the families of phosphoinositide 3-kinase (PI3K) and protein kinase C (PKC), both of which are studied in detail in this thesis. Like other crucial signaling proteins, more than one isoform of either family is present in platelets. In order to study the roles of individual PI3K and PKC isoforms, and possible redundancy therein, we considered it important to follow both pharmacological and genetic approaches. The reason for this is that pharmacological treatment of human cells with so-called isoform-specific inhibitors has the risk of limited sensitivity and specificity of the inhibitors, whereas in studies with kinase deficient mice is it difficult to rule out that other relevant (family member) proteins are up- or down-regulated in a compensatory way.

Table 1 gives an overview of key roles of the different class 1 PI3K isoforms in platelet responses, in particular those we have been able to establish in this thesis. Considering GPVI-dependent thrombus formation under flow conditions, both pharmacological inhibition of PI3K α/β and genetic deficiency of a key regulatory subunit for both isoforms results in lesser platelet adhesion to collagen fibers, reduced platelet aggregation, suppression of platelet Ca^{2+} fluxes, and lower PS exposure. On the other hand, inhibition of PI3K γ did not affect GPVI-induced Ca^{2+} responses, while inhibition of PI3K δ caused only a slight reduction. This fully supports the data from others that genetic deficiency in murine p110 γ (12) or p110 δ (13) has no more than a modest role in GPVI-induced platelet activation. Retrospectively, these data indicate that the PI3K inhibitors used were sufficiently specific for the concerning PI3K isoforms.

Mechanistically, the experiments indicate that both PI3K α and β isoforms are needed for full PLC γ activity and inositol 1,4,5-trisphosphate (IP $_3$) formation. This pointed to a non-redundant role of both isoforms in activation of this key effector enzyme of GPVI. This is also supported by the contribution of these PI3K isoforms to Ca^{2+} mobilization from intracellular stores, which is a direct consequence of PLC activation, rather than to subsequent Ca^{2+} entry from outside the platelets. Hence, we reasoned that the earlier suggested stimulation of Ca^{2+} entry by PI3K or its product, phosphatidylinositol 3,4,5-trisphosphate (14,15), is secondary to the effect of PI3K on Ca^{2+} store depletion.

Table 1. Established functions of PI3K isoforms in platelet activation processes and thrombus formation. Overview of chapter 3 regarding modulating roles of various PI3K isoforms on GPVI-induced platelet responses (directly or indirectly via P2Y₁₂). Functions of isoforms were studied using mice deficient in p85 α (regulatory subunit of class IA isoforms). Pharmacological inhibitors used were PIK-75 (blocking PI3K α), TGX-221 (blocking PI3K β), IC-87114 (blocking PI3K δ) and AS-252424 (blocking PI3K γ). Findings not in this thesis but from the literature are indicated. See also text.

Platelet responses	Class 1A			Class 1B
	PI3K α	PI3K β	PI3K δ	PI3K γ
Akt activation	+	+	ND	ND
IP ₃ production	+	+	0	0
Ca ²⁺ rise	+	+	+	0
$\alpha_{IIb}\beta_3$ activation	+	+	0	+ ¹
Secretion (α/δ -granules)	+	+	ND	0 ²
Thrombus formation	+	+	0	0
Procoagulant activity	+	+	0	0

¹ Cossemans J, Blood 2006;108:3045-52.² Lian L, Blood 2005;106:110-7. Abbreviations: +, stimulation; 0, no effect; ND, not determined.

The small GTPase Rap1b is known to be activated in a PI3K-dependent manner, particularly after occupation of Gi-coupled receptors, such as the platelet P2Y₁₂ receptor for ADP (16,17). We were interested to determine whether GPVI is directly implicated in platelet Rap1b activation, in experiments where the contribution of P2Y₁₂ receptors was pharmacologically blocked. Such a GPVI-dependent effect may be attributed to the PI3K α and β isoforms. Interestingly, also in Rap1b^{-/-} mouse platelets, PI3K inhibition still had a prominent suppressive effect on GPVI-induced integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation. This pointed to a role of both PI3K isoforms downstream of GPVI, in part through a Rap1b-independent pathway. This pathway may again involve PI3K α/β -dependent stimulation of PLC γ , in a way to enhance the Ca²⁺- and PKC-mediated activation of integrin $\alpha_{IIb}\beta_3$.

Concerning the PKC family of signaling proteins, isoform-specific inhibitors are less well characterized than those of PI3K. Again, we used pharmacological and genetic approaches to study the roles of the four major platelet-expressed PKC isoforms (α , β , δ , and θ) in regulating GPVI-induced platelet activation, thrombus formation and procoagulant activity (Table 2). A key finding in collaboration with the Poole laboratory in Bristol (United Kingdom), was a major suppression of thrombus formation with PKC α -deficient platelets, whereas platelet adhesion and spreading on collagen was unchanged. The data also reveal

an important role for PKC α in platelet granule biogenesis and exocytosis (Chapter 4). The impaired secretion could explain the suppressed thrombus formation of PKC $\alpha^{-/-}$ platelets, since secretion could be rescued by the addition of exogenous ADP. Collectively, it was concluded that, during thrombus formation, secretion of granules, integrin $\alpha\text{IIb}\beta_3$ activation, and platelet-platelet interactions are all positively regulated by PKC α . Recent findings of the Bristol lab added to this by demonstrating that protein kinase D2 is a key component in the regulation of dense granule secretion, downstream of GPVI-induced PKC α activation (18).

Table 2. Established functions of PKC isoforms in platelet activation processes and thrombus formation. Overview of chapters 4-5 regarding modulating roles of various PKC isoforms on GPVI-induced platelet responses (directly or indirectly via P2Y12). Functions of isoforms were studied using mice deficient in PKC α , β , δ , or θ . Pharmacological inhibitors used were Gö6976 (blocking PKC α/β), β -inhibitor (blocking PKC $\beta>\alpha$), rottlerin (blocking PKC δ) and θ -inhibitor (blocking PKC θ). Findings not in this thesis but from the literature are indicated. See also text.

Platelet responses	cPKC		nPKC	
	PKC α	PKC β	PKC δ	PKC θ
Filopod formation	0	+ ¹	− ²	+ ³
Ca ²⁺ rise	+	+	0	−
$\alpha\text{IIb}\beta_3$ activation	+	+	0	−
Secretion (α -granules)	+	+	0	− ⁴
Secretion (δ -granules)	+	ND	−	0 ⁴
Aggregation	+	+	−	−
Thrombus formation	+	+	−	−
Procoagulant activity	+	+	0	−

¹Buensuceso CS, J. Biol Chem 2005;278:44369-76. ²Pula G, Blood 2006;108:4035-4. ³Soriani A, J. Thromb. Haemost 2006;4:648-55. ⁴Hall KJ, Plos One 2008;3:e3277. *Abbreviations:* +, stimulation; −, inhibition; 0, no effect; ND, not determined.

Mouse platelets lacking PKC β also formed significantly smaller thrombi on collagen under flow conditions in comparison to wildtype platelets. Platelets from both PKC α - and β -deficient mice furthermore showed a reduced collagen-induced Ca²⁺ mobilization and diminished procoagulant activity. These findings were supported by pharmacological experiments with human platelets pretreated with the compounds Gö6976 and PKC β inhibitor. Both compounds, with confirmed inhibitory effects on PKC α/β , suppressed collagen-

induced platelet aggregation as well as GPVI-induced integrin activation, P-selectin expression, and Ca^{2+} signal generation.

On the other hand, various pieces of evidence point to a negative role of PKC θ in thrombus formation. Platelets from mice deficient in PKC θ showed increased collagen-dependent granule secretion, Ca^{2+} signaling and procoagulant activity, cumulating in enhanced thrombus formation (Table 2). Consistent with this, a pharmacological PKC θ inhibitor increased GPVI-induced human platelet aggregation as well as $\alpha\text{IIb}\beta_3$ activation, Ca^{2+} mobilization and α -granule secretion (Chapter 5). A similar, although less prominent increase in thrombus formation was seen with blood from PKC $\delta^{-/-}$ mice. One report suggests that GPVI-induced granule secretion is increased rather than decreased in PKC $\delta^{-/-}$ mice (19), but we have not been able to confirm this. Data from the Bristol group also point to a potentiated aggregation response with collagen through enhanced filopod formation in PKC $\delta^{-/-}$ platelets (20). Also for PKC δ , we obtained confirming pharmacological evidence, since the compound rottlerin appeared to enhance human platelet aggregation. Although rottlerin may not act as an inhibitor for PKC δ only, as reported in some papers, its gain-of-function effect on platelets is striking and compatible with the increased thrombus formation in PKC $\delta^{-/-}$ mice. All together, we come to a negative regulatory role of both PKC θ and δ in collagen-dependent thrombus formation, although both enzymes differ in their precise action mechanism. Given the most striking phenotype of PKC $\theta^{-/-}$ platelets, it appears that this isoform is responsible for the established negative effect of general PKC inhibition on GPVI-induced platelet Ca^{2+} signaling and procoagulant activity (21), whereas PKC α is the main positive regulator of secretion and integrin activation.

While in our hands both the pharmacological approach (human platelets) and genetic approach (mouse platelets) point to a negative role for PKC θ , different findings have been reported by the Kunapuli group (22). These authors found reduced thrombus formation in PKC $\theta^{-/-}$ mice in an *in vivo* model of FeCl_3 -induced carotid artery injury. This discrepancy may be due to a contribution of thrombin, for it was shown that PKC θ positively contributes to thrombin-induced signaling in platelets (22,23). Indeed, next to collagen, also thrombin appears to control the thrombotic process in FeCl_3 -induced carotid injury (24). A next challenge is to establish the contribution of PKC θ in (patho)physiological thrombus formation in the human system.

In conclusion, our data indicate that the two conventional PKC isoforms α and β are non-redundant and essential for collagen-induced granule secretion, a sustained Ca^{2+} response, and subsequent PS exposure, while especially the novel isoform PKC θ negatively regulates these processes. By implication, when conventional PKC signaling is restricted, there is no granule secretion for PKC θ to regulate. In future work, it is important to find the molecular protein substrates by which these different PKC isoforms regulate platelet functions.

Determinants of Ca^{2+} signaling, PS exposure, and integrin closure in thrombus formation

The results of chapter 6 support the literature identifying essential roles for the Ca^{2+} sensor protein Stim1 (in the reticular membrane), and the Ca^{2+} -selective ion channel Orai1 (in the platelet plasma membrane), in the process of collagen-dependent thrombus formation and PS exposure (Figure 2). These results, thus, provide a mechanistic explanation why arterial thrombus formation *in vivo* is impaired in mice with dysfunctional Stim1 (25) or Orai1 (26,27). By comparing platelets deficient in either Stim1 or Orai1, and finding remarkably similar effects of either knockout, our findings also support the concept of direct interactions of both proteins. The store-operated Ca^{2+} entry (SOCE) pathway, mediated by Stim1 and Orai1, controls platelet function. In other words, the biochemical interaction between Stim1 and Orai1, such as established in other cell types (28), translates to similar functional roles in platelet physiology.

The data of chapter 6 also show that both Stim1 and Orai1 tend to become redundant under conditions where high levels of thrombin are generated and a solid thrombus is formed, rich in PS-exposing platelets and fibrin clots. These findings point to the presence of other signaling mechanisms in thrombus formation, operating alongside of SOCE, under conditions of high thrombin generation and, likely, high thrombin-induced platelet activation.

There is indeed evidence for the presence of another Ca^{2+} entry pathway in platelets that acts in a thrombin receptor-operated fashion. For instance, the imidazole antagonist, SKF96365, which is a well-studied inhibitor of Ca^{2+} entry in platelets (29), still suppresses the Ca^{2+} and procoagulant responses of mouse platelets deficient in Stim1 or Orai1 (Chapter 6). Literature shows that thrombin-induced Ca^{2+} signaling and PS exposure in human platelets does not only rely on SOCE, but also on a mechanism of receptor-operated Ca^{2+} entry (ROCE) (30). The alternative Ca^{2+} entry route most likely does not involve the Trpc1 channel, as was proposed once by Rosado (31). Platelets from Trpc1^{-/-} mice appear to have a completely intact Ca^{2+} signaling machinery and a normal activation repertoire (32). More likely targets of SKF96365 are the non-SOCE channels, Trpc6 and Orai3, both of which are expressed in platelets and act in a receptor-operated way (33,34). In mice, a role of Trpc6 has recently been established in thrombin-induced ROCE, although the same study suggested that this platelet protein does not have a major role in hemostasis and thrombosis (35). Another, earlier proposal is that thrombin modulates the Ca^{2+} rises in platelets by transient activation of the Na^+/H^+ exchanger (36).

Figure 2. Calcium-modulating proteins in GPVI- and thrombin-dependent thrombus formation and procoagulant activity. Conclusive scheme from chapter 6 on the roles of Orai1 and STIM1 in platelet cytosolic Ca^{2+} rises, thrombus formation and procoagulant activity. Upper platelet: GPVI-induced platelet stimulation occurs via the LAT signalosome, involving the protein kinase Syk and Src-family kinases (SFK), resulting in activation of phospholipase $\text{C}\gamma 2$ ($\text{PLC}\gamma$) and IP_3 -mediated Ca^{2+} mobilization. Calcium entry via the SOCE channel Orai1, in interaction with Ca^{2+} -sensor STIM1, is essential for thrombus formation under high-shear flow and for induction of platelet PS exposure. PS exposing platelets provide a procoagulant surface for activation of factor X (complex of factors VIIIa, IXa and X), and activation of prothrombin (complex of factors II, Va and Xa). Lower platelet: In the presence of high thrombin concentrations, Orai1 and STIM1 become redundant in GPVI-induced thrombus formation and procoagulant activity. Thrombin-receptor $\text{PAR}_{1/4}$ stimulation via Gq and $\text{PLC}\beta$ causes extra Ca^{2+} signal generation, which may include Ca^{2+} entry via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) or the TrpC6 non-SOCE channel. Other abbreviations: DAG, diacylglycerol; PIP_2 , phosphatidylinositol 4,5-bisphosphate.

Since SOCE-devoid platelets are still capable to expose PS upon combined collagen- and thrombin-receptor stimulation, we rule out a direct role of SOCE in the mechanism of transmembrane scrambling of phospholipids, i.e. the process responsible for PS exposure. This is in agreement with studies in which platelets from Scott syndrome patients, who lack the transmembrane protein TMEM16F (37), have a clear defect in PS exposure, but show unchanged Ca^{2+} signals in response to collagen and thrombin (38).

Inhibition of $\text{PL3K}\alpha$ or β in collagen-adhered platelets led to a reduced Ca^{2+} mobilization and a lower SOCE and PS exposure during whole-blood perfusion experiments (Chapter 3). Both pharmacological evidence (39) and data with mouse platelets (40) indicated that $\text{PKC}\alpha$ can indirectly enhance SOCE by regulating the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. In this light, the

isoform PKC θ seems to be an exceptional kinase, because it was found to modify Ca²⁺ signaling and procoagulant activity in a non-SOCE fashion (41). In our experiments, we found that PKC θ modulates not only GPVI-induced Ca²⁺ signaling and subsequent PS exposure but also the closure of integrin α IIb β 3 (Chapter 7).

Figure 3 schematizes the central regulatory function of cytosolic Ca²⁺, particularly with regard to the platelet responses studied in this thesis. Persistent Ca²⁺ elevation is a main controlling factor of the closure of activated integrins in PS-exposing platelets, stimulated with collagen and thrombin receptor-activating agonists. The integrin closure coincides with cleavage of the tyrosine kinase Src, a protein that is attached to the integrin β 3 subunit. However, the cleavage of Src appeared to be an epiphenomenon, because Src kinase blockers were without any effect on α IIb β 3 closure. Earlier data show that Src is cleaved as a physiologic post-aggregation event, due to action of the Ca²⁺-dependent protease calpain (42). Furthermore, calpain also mediates cleavage of the integrin β 3 subunit (43,44). Surprisingly, we found that calpain inhibitors were unable to completely suppress integrin closure. Accordingly, calpain-mediated remodeling of the actin cytoskeleton in platelets is not the main or only mechanism of integrin closure.

In many cell types, the mitochondria serve as a Ca²⁺ buffering system for avoiding cytosolic Ca²⁺ overload (45). Thus, also in platelets, mitochondria may help to dampen the high rises in cytosolic Ca²⁺ that are evoked by strong agonists. Formation of the mitochondrial permeability transition pore (mPTP) abolishes the Ca²⁺-dampening effect, because this allows leakage of the buffered Ca²⁺ out of the mitochondria (Figure 3). Compatible with this hypothesis, we found that inhibition of mPTP formation (cyclosporin A) or inability to mPTP formation (mice lacking the mPTP component cyclophilin D) resulted in impaired PS exposure and integrin closure. Findings in support of this, although made in different settings, have been published by at least two other groups (46,47).

Although the role of mitochondria in the signaling machinery is only poorly understood, several pieces of evidence in the literature indicate that these organelles contribute to Ca²⁺ flux regulation and subsequent responses. In neuronal cells, mitochondrial release of Ca²⁺ due to mPTP formation was found to contribute to cytosolic Ca²⁺ overload and cellular stress responses (48). On the long-term, mitochondrial-derived Ca²⁺ signals appear to regulate PKC activity (49). Given that mitochondria are a major source of reactive oxygen species (50), they may also regulate Ca²⁺ fluxes and PKC activation via the release of hydrogen peroxide (51). Taken together, we envision a scheme that, in platelets stimulated with strong agonists, formation of the mPTP leads to a secondary rise in cytosolic Ca²⁺ levels, which potentiates PS exposure at the one hand, and activation of the Ca²⁺-dependent protease calpain and cleavage of Src and β 3 at the other hand. Both processes together, perhaps with an assistant role of PKC θ , may lead to integrin closure (Figure 3). Whether PS exposure per se also contributes to integrin closure has not been investigated.

Figure 3. Proposed mechanism of integrin $\alpha\text{IIb}\beta_3$ closure in procoagulant platelets. As concluded in chapter 7, various routes can lead to integrin closure in PS-exposing platelets stimulated by collagen and thrombin receptors. Agonist-induced Ca^{2+} rises activate the protease calpain, which is responsible for c-Src kinase cleavage and in part for integrin closure. Mitochondrial-derived Ca^{2+} release via the mitochondrial permeability transition pore (mPTP) supports the general rise in Ca^{2+} leading to PS exposure, which process by itself may also contribute to integrin closure. An additional role of PKC θ is proposed as well. Abbreviations: see Figure 2.

Clinical implications

Basic medical science aims to disclose the molecular mechanisms of disease and treatment of disease in order to improve human well-being. Several of the platelet signaling proteins studied in this thesis may serve as molecular targets for the treatment of thrombosis or impaired hemostasis.

Although mostly investigated in genetically modified mice, the Orai1 channel has been postulated as a new pharmacological target to reduce platelet activation at sites of arterial thrombosis (25,26). Recent work from our group has shown that SOCE blockers targeted at Orai1 can also suppress thrombus formation of human platelets *in vitro*, but improvement of the sensitivity and effectiveness of the current compounds appears to be needed before investigations can start towards the prevention of human thrombotic events (52).

Isoforms of both PKC and PI3K are among the most frequently upregulated kinases in human tumor tissues (53). Recent insight into the precise functions of PI3K (54) and PKC (55) isoforms has increased the interest for these kinases as therapeutic targets not only for anticancer strategies, but also for antithrombotic treatment. In fact, all current antiplatelet drugs (aspirin, P2Y₁₂ inhibitors, integrin $\alpha\text{IIb}\beta_3$ antagonists) have as side effects impairment of normal hemostasis and a risk of bleeding complications (56). An ideal antithrom-

botic therapy would selectively suppress the role of platelets in pathological thrombus formation but not affect hemostasis. Experimental thrombosis studies with mice and other laboratory animals have indicated that this might be the case for inhibitors of PI3K β and PI3K γ (57,58). The compound, AZD6482, a reversible isoform-selective inhibitor of PI3K β has been tested in phase 1 trials and was found to be acceptably safe with a satisfying tolerability profile (59). However, the clinical research with this compound has now been discontinued for commercial reasons. Recent literature demonstrates opposing roles of PI3K α , β and δ isoforms in the migration of transformed epithelial cells (60), which also illustrates the possibilities in disease treatment of drugs directed against only one of these isoforms.

An interesting protein of the PKC family is PKC θ , which has extensively been studied in relation to inflammatory diseases, such as asthma, arthritis, inflammatory bowel disease, multiple sclerosis, and allograft rejection (61). The present findings indicate that, when using a PKC θ inhibitor as an anti-inflammatory drug, one has to be aware of possible prothrombotic side-effects due to increased platelet activation. On the other hand, in cancer, PKC α is already a used target for the drug aprinocarsen (an antisense oligonucleotide) in the treatment of specific neoplastic conditions (62,63). The findings in this thesis suggest that this drug may cause platelet inhibition and suppress thrombus formation. Since arterial thrombosis is a known companion of several cancers and platelets can actively support tumor growth, it is conceivable that the curing effect of aprinocarsen in part occurs via platelet inhibition. However, this needs to be investigated in more detail.

Given the partial thrombo-protective effect of current antiplatelet medication and the significant risk of bleeding complications, it will still be important to test new (high affinity) inhibitors, such as those mentioned above, as future platelet-based antithrombotic drugs. Unfortunately, at these days, phase 2 and 3 clinical trials for new, selective therapeutics are often not performed, because the new drugs need to perform better than standard used drugs or combinations of drugs, resulting in a negative cost-effectiveness ratio. Interestingly, the roads leading to inflammation or cancer sometimes also lead to thrombus formation. Hence, a promising escape from this pitfall is to direct the medical research for antithrombotics towards testing of the drugs directed against platelet signaling molecules, such as those discussed above, that are in current use for anticancer or anti-inflammatory therapy. Such research is highly needed, in particular for patients who suffer from bleeding complications during the current antiplatelet therapy and vice versa for patients with cancer who suffer from thrombotic complications.

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Summary

Platelets are the principal blood cell components implicated in thrombosis and hemostasis. Following vascular injury or activation, platelets form a thrombus at the damaged site, causing the cessation of bleeding. In the pathophysiological process of thrombosis at the site of a ruptured atherosclerotic plaque, uncontrolled thrombus formation can lead to vessel occlusion and result in heart attack or stroke. In platelets, many intracellular signaling pathways have been identified that contribute to platelet activation processes, like integrin activation, secretion, platelet aggregation and procoagulant activity (phosphatidylserine exposure). Unraveling which of these signaling roads lead to thrombus formation was endeavored in this thesis.

Chapter 1 gives a general overview of relevant mechanisms of platelet receptor activation and intracellular signaling pathways, leading to integrin activation, secretion and procoagulant activity. The precise role of the signaling collagen receptor, glycoprotein VI, in collagen-induced thrombus formation is extensively described, but that of the adhesive collagen receptor, integrin $\alpha 2\beta 1$, is less clear. In the signal transduction pathway induced by glycoprotein VI, phosphoinositide 3-kinase (PI3K) plays a key role in integrin $\alpha IIb\beta 3$ activation and platelet aggregation. However, which of the several PI3K isoforms is responsible for these responses remains to be clarified. This also holds for participation of the small GTPase Rap1b, which operates downstream of PI3K. The main phospholipid substrate of PI3K, phosphatidylinositol 4,5-bisphosphate, also a substrate for phospholipase C, cleaving this into inositol trisphosphate and diacylglycerol. The latter activates members of the family of protein kinase C (PKC), which are implicated in almost all platelet responses. However, which of the various expressed PKC isoforms contributes to which platelet responses during thrombus formation, needs to be investigated. As Ca^{2+} is an important second messenger in the different stages of platelet activation, possible regulators of cytosolic Ca^{2+} mobilization are discussed as well.

To address the question whether collagen-mimetic peptides support flow-dependent thrombus formation, we used in chapter 2 triple-helical peptides consisting of hexapeptide sequences of the recognition site for integrin $\alpha 2\beta 1$, i.e. peptides with high-affinity (GF/LOGER), intermediate affinity (GLM/SGER), or low-affinity (GAO/SGER) binding. The hexapeptide sequence was incorporated in a backbone of repeated (GPP)_n sequences as a neutral control, or of (GPO)_n sequences to provide additional binding sites for glycoprotein VI. These triple-helical peptides were immobilized to glass in the presence or absence of von Willebrand factor to allow platelet interaction with glycoprotein Ib-V-IX. Whole blood perfusion at high shear rate showed that immobilized peptides containing both the high-affinity $\alpha 2\beta 1$ -binding motif GFOGER and the (GPO)_n motif supported platelet aggregation and procoagulant activity, even in the absence of von Willebrand factor. With peptides containing only one of these motifs, co-immobilized von Willebrand factor was needed for thrombus formation. Peptides with intermediate affinity or low-affinity $\alpha 2\beta 1$ -binding motifs formed procoagulant thrombi only if both (GPO)_n and von Willebrand factor were present. At a low-shear rate, immobilized peptides with high- or low-affinity $\alpha 2\beta 1$ -binding motifs mediated formation of thrombi with procoagulant platelets only in combination with (GPO)_n. Taken together, chapter 2 indicates that collagen-mimetic, triple-helical peptides

effectively and efficiently can take over the functions of native collagens in mediating platelet adhesion and activation via the receptors glycoprotein VI and $\alpha 2\beta 1$. Herein, glycoprotein VI exerts a signaling rather than adhesive function, whereas $\alpha 2\beta 1$ and von Willebrand factor determine the adhesion of platelets under flow conditions.

In chapter 3, we identified $PI3K\alpha$ and β as the main $PI3K$ isoforms for full phospholipase C activation, inositol trisphosphate formation and further signaling evoked by glycoprotein VI. We could not confirm a role of these isoforms in Ca^{2+} entry from the extracellular medium. In platelets from mice lacking $p85\alpha$ (i.e. a main regulatory subunit of $PI3K\alpha/\beta$), collagen-induced Ca^{2+} signaling and thrombus formation were severely impaired. Applying a pharmacological approach with high affinity inhibitors of $PI3K\alpha$ (PIK-75) or $PI3K\beta$ (TGX-221), a functional non-redundancy of these isoforms in glycoprotein VI-induced platelet activation was found, since the inhibitors together did not have an additional effect on Akt phosphorylation, secretion, Ca^{2+} signaling, procoagulant activity, and thrombus formation. Furthermore, chapter 3 provides evidence of a direct role for glycoprotein VI in Rap1b activation in both human and mouse platelets. This direct contribution could be attributed to activation of the $PI3K\alpha$ and β isoforms. Because $PI3K$ inhibitors still suppressed integrin $\alpha IIb\beta 3$ activation and platelet aggregation in Rap1b^{-/-} platelets, we concluded that there also exists a $PI3K$ -mediated, Rap1b-independent pathway of integrin activation. This pathway may involve $PI3K\alpha/\beta$ -dependent stimulation of phospholipase $Cy2$, which possibly contributes to Ca^{2+} - and PKC-mediated activation of integrin $\alpha IIb\beta 3$. Together, these findings provide a new mechanistic explanation for the antithrombotic effect of $PI3K$ inhibition and make $PI3K\alpha/\beta$ interesting targets for anti-platelet therapy.

By applying both a genetic and pharmacological approach, in chapters 4 and 5 we identified the roles of the different PKC isoforms, i.e. the conventional ($PKC\alpha$ and β) and novel ($PKC\delta$ and θ) isoforms, in the process of thrombus formation. Chapter 4 reveals a key role for $PKC\alpha$ in regulating granule biogenesis and exocytosis. In addition, it is shown that glycoprotein VI- and thrombin-induced secretion of α -granules, integrin $\alpha IIb\beta 3$ activation, and platelet aggregation are positively regulated by $PKC\alpha$. On the other hand, as presented in chapter 4, $PKC\alpha$ does not affect platelet adhesion and spreading on collagen. The inhibitory effects of $PKC\alpha$ ablation on granule secretion and thrombus formation favor the thought of using $PKC\alpha$ as a drug target for antithrombotic therapy. In chapter 5, the comparison of mouse platelets lacking different isoforms of PKC, by measuring thrombus formation under whole blood flow conditions, reveals distinct, non-redundant roles of the isoforms. It is concluded that the conventional PKCs ($PKC\alpha$ and β) promote, and the novel PKCs ($PKC\delta$ and θ) inhibit thrombus formation on collagen. Considering Ca^{2+} signaling and procoagulant activity, these responses were both diminished in platelets from $PKC\alpha^{-/-}$ and $PKC\beta^{-/-}$ mice. These findings are supported by pharmacological studies with human platelets, using Gö6976 and a $PKC\beta$ inhibitor, respectively. On the contrary, platelets from mice deficient in $PKC\theta$, and to a lesser extent $PKC\delta$, were increased in collagen-dependent granule secretion and thrombus formation. This was supported by enhanced Ca^{2+} responses and increased phosphatidylserine exposure of $PKC\theta^{-/-}$ platelets. Comparable results were obtained with human platelets pretreated with a $PKC\theta$ inhibitor or with the $PKC\delta$ inhibitor

rottlerin. Regarding chapters 4 and 5, we propose that the conventional PKC α/β isoforms are essential for collagen-induced granule secretion, phosphatidylserine exposure, and thrombus formation, while especially PKC θ negatively regulates these processes.

We investigated in chapter 6 whether the Ca²⁺-sensing proteins STIM1 and 2 and the Ca²⁺-selective channel Orai1 are key Ca²⁺ regulatory components in platelet phosphatidylserine exposure and thrombus formation upon stimulation of the collagen and thrombin receptors. The studies were carried out using chimeric mice with platelets deficient in STIM, STIM2, or Orai1. It was demonstrated that deficiency in either STIM1 (but not STIM2) or Orai1 greatly reduced glycoprotein VI-induced Ca²⁺ signaling, phosphatidylserine exposure, and thrombus formation under flow conditions in the absence of coagulation. In the presence of high thrombin concentrations, these platelet responses tended to normalize. These findings jointly suggest that the glycoprotein VI-induced Ca²⁺ entry pathway mediated by STIM1 and Orai1 becomes redundant in the regulation of thrombus formation under conditions where thrombin acts as co-agonist.

In chapter 7, the signaling processes were studied by which phosphatidylserine-exposing platelets inactivate or close integrin $\alpha\text{IIb}\beta_3$. In particular, we examined the possible roles in integrin closure of elevated Ca²⁺, Src family kinases, mitochondrial permeability transition pore formation, and calpain-dependent protein cleavage. The data showed that both Ca²⁺ elevation (with likely a role of mitochondrial dysfunction) and calpain activity are involved in the control of integrin closure, although also the process of phosphatidylserine exposure itself may contribute to integrin closure. Key findings of this thesis as well as their possible clinical significance, in relation to the current literature, are discussed in chapter 8. It is argued that thrombus formation is delicately regulated by various cooperating signaling pathways with sometimes redundant, sometimes non-redundant roles of specific isoforms of PI3K, PKC and Ca²⁺-regulating proteins in platelets. Overall, the studies in this thesis help to demystify the current uncertainty of precise signaling pathways towards thrombus formation. In anti-thrombotic treatment the present antiplatelet drugs are only limitedly effective and have as side effect an increased risk of bleeding. To develop personalized target-specific drugs, which have lower side effects, more precise knowledge of the process of thrombus formation and platelet signaling is essential. The present insight, described in this thesis, hence may contribute to future improvements in the treatment of arterial thrombosis.

Samenvatting

Als kleinste cellulaire bloedcomponent hebben bloedplaatjes een centrale rol in de processen van trombose en hemostase. Wanneer een bloedvat beschadigd wordt, vormen geactiveerde bloedplaatjes aldaar een trombus waardoor de bloeding gestelpt wordt. De pathofysiologische tegenhanger van hemostase is trombose. Hierbij ontstaat ongecontroleerde trombusvorming, bijvoorbeeld ten gevolge van een ruptuur van een atherosclerotische plaque, mogelijk leidend tot bloedvatafsluiting resulterend in een hartinfarct of een beroerte. In bloedplaatjes zijn tot dusver vele intracellulaire signaleringspaden geïdentificeerd, die bijdragen aan de verschillende activatieprocessen van deze cellen, waaronder integrine-activatie, secretie, plaatjesaggregatie en stollingsbevorderende activiteit (oppervlakte-expositie van fosfatidylserine, PS). Het doel van deze thesis is meer inzicht te krijgen in het belang van die signaleringspaden leidend tot trombusvorming.

Hoofdstuk 1 geeft een algemeen overzicht van de relevante mechanismen van receptoractivatie en intracellulaire signaleringspaden in bloedplaatjes, betrokken bij integrine-activatie, secretie en procoagulante activiteit. De exacte rol van de signalerende collageenreceptor, glycoproteïne VI, in het proces van collageen-geïnduceerde trombusvorming was eerder al beschreven. Die van de adhesieve collageenreceptor integrine $\alpha 2\beta 1$ daarentegen is minder duidelijk. In het signaaltransductiepad geïnduceerd door glycoproteïne VI spelen eiwitten van de familie van fosfoïnositide 3-kinase (PI3K) een centrale rol bij de activatie van integrine $\alpha IIb\beta 3$ en de daaropvolgende bloedplaatjesaggregatie. Maar welke van de verschillende PI3K isovormen verantwoordelijk zijn voor deze activatieresponsen dient nog onderzocht te worden. Dit geldt ook voor de bijdrage van het onder PI3K functionerende regeleiwit, de GTPase Rap1b. Het belangrijkste fosfolipidensubstraat van PI3K, fosfatidylinositol 4,5-bisfosfaat, is ook een substraat voor fosfolipase C, die dit fosfolipide knipt in diacylglycerol en inositol-trifosfaat. Diacylglycerol activeert eiwitten van de proteïne kinase C (PKC) familie, die betrokken zijn bij zowat alle activatieresponsen in bloedplaatjes. Maar welke van de verschillende PKC isovormen betrokken zijn bij die verschillende responsen tijdens trombusvorming, dient nog uitgezocht te worden. Aangezien het ion Ca^{2+} bekend staat als een belangrijke intracellulaire boodschapper bij de verschillende fasen van bloedplaatjesactivatie, worden in dit hoofdstuk ook de mogelijke regulatoren van cytosolaire Ca^{2+} -mobilisatie besproken.

Voor het oplossen van de vraag of collageen-nabootsende peptiden trombusvorming in stromend bloed ondersteunen, zijn in hoofdstuk 2 enige triple-helix peptiden gebruikt bestaande uit hexapeptidesequenties waaraan integrine $\alpha 2\beta 1$ kan binden, dat wil zeggen peptiden met een hoge (GF/LOGER), intermediaire (GLM/SGER) of lage (GAO/SGER) affiniteit voor dit integrine. Deze hexapeptidesequenties zijn ingebouwd in een structuur van herhaalde (GPP)_n-sequenties als controle-eiwit, of van (GPO)_n-sequenties om een additionele bindingsplaats voor glycoproteïne VI te creëren. De triple-helix peptiden werden op glas geïmmobiliseerd in de aan- of afwezigheid van von Willebrand factor (vWF), waardoor al dan niet interactie van bloedplaatjes met glycoproteïne Ib-V-IX mogelijk werd. Bloeddoorstroming bij hoge afschuifsnelheden over deze eitwitoppervlakken toonde aan dat geïmmobiliseerde peptiden, die zowel het hoge-affiniteit $\alpha 2\beta 1$ -bindingsmotief GFOGER als de sequentie (GPO)_n bevatten, de aggregatie en procoagulante activiteit van

bloedplaatjes kunnen opwekken, zelfs in de afwezigheid van vWF. Peptiden die enkel een van beide motieven bevatten dienden gecoïmmobiliseerd te worden met vWF om een trombus te kunnen vormen. Peptiden met intermediaire of lage affiniteitsbinding voor $\alpha 2\beta 1$ stimuleerden de vorming van procoagulante trombi alleen in de aanwezigheid van (GPO)_n-sequenties en vWF. Bij lage afschuifsnelheden stimuleerden geïmmobiliseerde peptiden met hoge- of lage-affiniteit $\alpha 2\beta 1$ -bindende motief, enkel in combinatie met (GPO)_n, de vorming van trombi met procoagulante bloedplaatjes. Samengevat toont hoofdstuk 2 aan dat collageen-nabootsende, triple-helix peptiden effectief en efficiënt de functie kunnen overnemen van collageen in bloedplaatjesadhesie en -activatie via glycoproteïne VI en integrine $\alpha 2\beta 1$. Hieruit blijkt dat glycoproteïne VI eerder een signalerende dan een adhesieve functie heeft, terwijl integrine $\alpha 2\beta 1$ en de vWF-receptor glycoproteïne Ib-V-IX zorgen voor adhesie van bloedplaatjes onder stromingsomstandigheden.

In hoofdstuk 3 zijn PI3K α en β geïdentificeerd als de belangrijkste PI3K isovormen noodzakelijk voor activatie van fosfolipase C, vorming van inositol-trifosfaat en daaropvolgende signalering opgewekt door glycoproteïne VI. Een mogelijke bijdrage van deze isovormen in de Ca²⁺-instroom vanuit het extracellulaire milieu kon niet worden bevestigd. In bloedplaatjes van muizen zonder het eiwit p85 α (de regulatoire component van PI3K α/β) bleken de collageen-geïnduceerde Ca²⁺-signaling en trombusvorming ernstig verstoord. In een farmacologische benadering met selectieve, hoge affiniteits-remmers van PI3K α (PIK-75) of PI3K β (TGX-221) kon worden aangetoond dat de gelijktijdige werking van beide isovormen nodig is voor bloedplaatjesactivatie door glycoproteïne VI. Bewijs hiervoor was ondermeer dat de combinatie van deze remmers geen additioneel effect had op de processen van Akt fosforylatie, Ca²⁺-signaling, plaatjessecretie, procoagulante activiteit en trombusvorming. Bovendien laat hoofdstuk 3 zien dat er een directe rol is van glycoproteïne VI in de activatie van Rap1b, zowel bij humane plaatjes als bij muizenplaatjes. Deze bijdrage loopt via de isovormen PI3K α en β . Omdat ook in Rap1b^{-/-} muizen remming van PI3K nog steeds van invloed was op de $\alpha IIb\beta 3$ -activatie en bloedplaatjesaggregatie, kan geconcludeerd worden dat er een alternatieve Rap1b-onafhankelijke signaleringsroute voor integrine-activatie bestaat middels PI3K. Deze route zou kunnen verlopen door middel van PI3K α/β -afhankelijke stimulatie van fosfolipase Cy2, met als gevolg een Ca²⁺/PKC-gemedieerde activatie van integrine $\alpha IIb\beta 3$. Recapitulerend geven bovenstaande bevindingen een nieuwe mechanistische verklaring voor het antitrombotische effect van PI3K α/β remming, waarmee deze eiwitten mogelijk een effectief doelwit zijn voor anti-bloedplaatjestherapie.

Door genetische en farmacologische benaderingen werden in de hoofdstukken 4 en 5 de functies van de verschillende isovormen van PKC geïdentificeerd in het proces van trombusvorming. Dit betreft de conventionele isovormen PKC α en β , en de 'nieuwe' isovormen PKC δ en θ . Hoofdstuk 4 onthult een sleutelrol voor de isovorm PKC α in de biogenese van opslag-granula en in de secretie van de inhoud van deze granula. Aangetoond werd dat onder invloed van glycoproteïne VI en trombine de secretie van α -granula, evenals de integrine $\alpha IIb\beta 3$ -activatie en plaatjesaggregatie positief gereguleerd worden door PKC α . Evenwel heeft PKC α geen invloed op bloedplaatjesadhesie en -spreiding over een collageenop-

pervlak. De sterk stimulerende bijdrage van PKC α op bloedplaatjesactivatie en trombusvorming ondersteunen het idee om te onderzoeken of PKC α een goed medicatiedoelwit is voor anti-trombotische therapie. In hoofdstuk 5 zijn muizenbloedplaatjes met een defecte expressie van één van de verschillende PKC isovormen met elkaar vergeleken door meting van de trombusvorming in stromend bloed. Op grond van de resultaten kan worden vastgesteld dat de conventionele en nieuwe isovormen functioneel niet uitwisselbaar zijn. De conventionele PKCs (PKC α en β) bevorderen de trombusvorming op collageen, terwijl de nieuwe PKCs (PKC δ and θ) dit proces onderdrukken. Voorts waren de Ca²⁺-signalering en procoagulante activiteit gereduceerd in bloedplaatjes van muizen deficiënt in PKC α of PKC β . Deze bevindingen werden ondersteund door farmacologische studies met humane bloedplaatjes en remmers van deze isovormen (Gö6976 en PKC β remmer). Anderzijds werd een toename geconstateerd van de granula-secretie en de trombusvorming bij muizen met een deficiëntie in PKC θ of, in mindere mate, PKC δ . In overeenstemming hiermee waren de Ca²⁺-responsen en procoagulante activiteit toegenomen bij PKC θ ^{-/-}. Vergelijkbare resultaten werden verkregen met humane bloedplaatjes behandeld met een PKC θ remmer of met de PKC δ remmer rottlerin. Op basis van de bevindingen in hoofdstukken 4 en 5 is gepostuleerd dat de conventionele PKC α/β isovormen essentieel zijn voor collageengeïnduceerde granula-secretie, procoagulante activiteit (PS-expositie) en trombusvorming, terwijl vooral PKC θ deze processen negatief reguleert.

In hoofdstuk 6 is onderzocht of de Ca²⁺-gevoelige eiwitten STIM1 en STIM2 en het Ca²⁺ selectieve plasmamembraan-kanaal Orai1 een sleutelrol kunnen vervullen bij PS-expositie en trombusvorming bij bloedplaatjesstimulatie met collageen en trombine. Deze studies werden uitgevoerd met chimere muizen met bloedplaatjes die deficiënt waren in STIM1, STIM2 of Orai1. Aangetoond is dat deficiëntie in ofwel STIM1 (doch niet STIM2) ofwel Orai1 leidt tot een sterk verminderde glycoproteïne VI-geïnduceerde Ca²⁺-signalering en trombusvorming in stromend bloed onder condities waarbij trombine geïnactiveerd is. Echter bij hoge trombinevorming (en bloedstolling) bleken deze responsen van bloedplaatjes terug normaal. Deze bevindingen wijzen op een compensatoire rol van trombine als 'backup' van de glycoproteïne VI-geïnduceerde Ca²⁺-instroom via Orai1/STIM1 bij de trombusvorming.

Hoofdstuk 7 beschrijft onderzoek naar signaleringsprocessen die verantwoordelijk zijn voor de sluiting van geactiveerde integrine $\alpha\text{IIb}\beta_3$ in PS-exposerende bloedplaatjes. In het bijzonder werd de rol van een verhoogde Ca²⁺ concentratie bestudeerd als mogelijke regulator voor integrine-sluiting, maar daarnaast ook de mogelijke bijdragen van Src proteïne kinasen, vorming van de mitochondriële permeabiliteitstransitieporie en van het Ca²⁺-afhankelijk cellulaire protease calpain. De data tonen aan, dat zowel een blijvende verhoging van intracellulair Ca²⁺ (met vermoedelijk een rol van mitochondriële permeabiliteit) als de protease-activiteit van calpain betrokken zijn bij de controle van integrine-sluiting. Daarnaast is niet uitgesloten dat ook het proces van PS-expositie zelf bijdraagt aan het sluiten van de integrines.

De belangrijkste bevindingen van dit proefschrift en de mogelijke klinische relevantie daarvan worden in relatie tot de recente literatuur besproken in hoofdstuk 8. Beargumenteerd wordt dat trombusvorming een delicaat gereguleerd proces is, waarbij verschillende

signaleringspaden al dan niet samenwerken of overlappen, en waarbij de verschillende isovormen van PI3K, PKC en STIM/Orai soms vervangbaar, soms complementair en soms tegengesteld zijn in werking. De huidige anti-trombotische medicatie gericht op bloedplaatjes is maar ten dele effectief, en heeft als belangrijke complicatie een verhoogd risico op bloedingen. Om een betere gepersonaliseerde medicatie tegen bloedplaatjes te ontwikkelen met minder bijwerkingen, is een goede kennis essentieel van het complexe proces van trombusvorming en de bloedplaatjesactivatie. De nieuwe inzichten beschreven in dit proefschrift kunnen mogelijk helpen om te komen tot een verbeterde behandeling van arteriële trombose.

Dankwoord

Promoveren is net als trombusvorming en het leven zelf, een resultaat van een complex samenspel van keuzes en de gevolgen daarvan, verwezenlijkingen op basis van de hierin gestoken energie en tijd, en de steun en hulp van zo velen die je op dit ervaringspad kruisen. Daarom wil ik allen die op een of andere wijze een bijdrage hebben geleverd aan de totstandkoming van dit proefschrift hiervoor bedanken.

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Het plaatjeslab

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Curriculum Vitae

Karen Gilio was born on July 14th 1983 in Maaseik, Belgium. In 1995, she started secondary school at the Heilig-Hart College in Maasmechelen, where she received her Mathematics-Science degree in 2001. Then, she studied Biomedical Sciences at the University of Hasselt in Belgium. She obtained her Master's degree in Clinical and Molecular Sciences with magna cum laude on 7th July 2006. Subsequently, she started as a PhD student at the Cardiovascular Research Institute Maastricht (CARIM) at the Department of Biochemistry (Maastricht University, the Netherlands). Under the supervision of Prof. Dr. J.W.M. Heemskerk, she performed cellular research in the field of thrombosis and haemostasis (this thesis) and contributed to several teaching activities. During this four-year period she participated in congresses in Wittenberg-Lutherstadt (Germany), Geneva (Switzerland), London (United Kingdom), Boston (United States of America), and Nuremberg (Germany), to present her research. Part of her research was performed in the laboratories of Prof. Dr. S.P. Watson (Birmingham, United Kingdom), Prof. Dr. A.W. Poole (Bristol, United Kingdom), and Prof. Dr. B. Nieswandt (Wurzburg, Germany). To become a Medical Doctor, she started in September 2010 the four-year during Master in Medicine and Clinical Science at Maastricht University.

Karen Gilio werd geboren op 14 juli 1983 te Maaseik, in België. In 1995 begon ze haar middelbare school opleiding aan het Heilig-Hart College in Maasmechelen. Ze behaalde haar diploma in Wetenschappen-Wiskunde in 2001. Vervolgens startte ze met de opleiding Biomedische Wetenschappen aan de Universiteit van Hasselt (België), waarvoor zij met grote onderscheiding haar Master diploma behaalde op 7 juli 2006. Aansluitend begon ze als PhD-student bij het Departement Biochemie van het Cardiovascular Research Institute Maastricht (CARIM) aan de Universiteit van Maastricht (Nederland). Onder begeleiding van Prof. Dr. J.W.M. Heemskerk, verrichtte ze cellulair onderzoek binnen het domein van trombose en hemostase (dit proefschrift), alsook toonde ze een actieve bijdrage aan verschillende onderwijsactiviteiten. Gedurende deze vierjarige periode presenteerde ze haar onderzoek op congressen in Wittenberg-Lutherstadt (Duitsland), Genève (Zwitserland), Londen (Groot-Brittannië), Boston (Verenigde Staten) en Nuremberg (Duitsland). Een gedeelte van haar onderzoek verrichtte zij in de laboratoria van Prof. Dr. S.P. Watson (Birmingham, Groot-Brittannië), Prof. Dr. A.W. Poole (Bristol, Groot-Brittannië) en Prof. Dr. B. Nieswandt (Würzburg, Duitsland). In september 2010 startte ze met de vierjarige Master opleiding tot Arts en Klinisch Onderzoeker aan de Universiteit van Maastricht.

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Abstracts

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Oral presentations and awards

1. Student award 2005 of the Faculty of Medicine for 'best poster presentation' on the biomedical research topic "Diamond-based biosensors", University of Hasselt, Belgium.
2. Travel award 2007 from Tebu Bio Innovative Lab Services & Reagents, the Netherlands to visit the XX1st Congress of the International Society on Thrombosis and Haemostasis (ISTH), Geneva, Switzerland.
3. Oral presentation: Opposite role of platelet PKC isoforms α , δ and θ in flow-induced thrombus formation. 9th UK - 1st Netherlands Platelet Meeting, 2007 - London, UK.
4. Young investigator award 2009 of the International Society on Thrombosis and Haemostasis, XXIIth Congress ISTH, Boston, USA.
5. Oral presentation: Divergent roles of conventional and novel PKC isoforms in platelet activation towards thrombus formation. XXIIth Congress of the International Society on Thrombosis and Haemostasis (ISTH), 2009 - Boston, USA.

Addendum

Color figures chapters 4, 5, and 7

Supplementary Figure 2. Platelet surface expression of CD41 is not affected by PKC α knock-down. Washed platelets from wild type (WT) or *Prkca*^{-/-} mice were labelled with PE-anti-CD41 antibody, stimulated with CRP (5 μ g/ml) or thrombin (1 unit/ml) for 15 min and immunofluorescence intensity measured by flow cytometry. In (A), the data presented are geometric means as percentages of basal non-stimulated levels (error bar represents SEM, n=4). Inset panel shows geometric mean of fluorescence (arbitrary fluorescence units) of basal platelets from either wild type or *Prkca*^{-/-} mice. No difference was seen between wild type and *Prkca*^{-/-} platelet expression of CD41, and PKC α expression plays no role in regulating CD41 surface expression upon stimulation with thrombin or CRP. Data shown are mean $\pm$ SEM, n=4. Additionally, no difference was seen between basal and CRP- or thrombin-stimulated platelets, from either wild type or *Prkca*^{-/-} mice, in staining with an isotype-matched control antibody (PE-conjugated rat IgG1; data not shown). In (B), representative frequency histograms are shown for CD41 expression from wild type (upper panel) and *Prkca*^{-/-} (lower panel) platelets, either basal or stimulated with CRP (5 μ g/ml) or thrombin (1 unit/ml) for 15 min as indicated. Data are representative of 4 independent experiments.

Figure 7. In vivo thrombus formation is impaired in the absence of PKC α , but tail bleeding time is normal. (A)-(D) Mice were either *Prkca*^{-/-} or littermate-matched wild-type controls. Platelets were labelled in vivo with Alexa 488, as described in Methods. (A) Platelets (green) composited with bright-field images (black/white) of the cremaster arteriole were viewed at x40 magnification and images were acquired using a digital CCD camera (SensiCam II, Cooke Corporation) with a 640 x 480 pixel array. (B) Traces shown are median integrated platelet fluorescence of 15 thrombi induced in 3 or more mice for each group. Fluorescent intensity of platelets in arbitrary units is presented as a function of time. (C) Data are presented as a scatter diagram, and the horizontal bar is mean of 15 thrombi induced in at least 3 mice for each group. There is a significant reduction in thrombus intensity in *Prkca*^{-/-} by comparison with wild type controls (mean *Prkca*^{+/+}, 652200; mean *Prkca*^{-/-}, 279600; p < 0.05). (D) Time to reach peak thrombus size also significantly differed from wild type controls, with mean data shown by the horizontal bars (mean *Prkca*^{+/+}, 68.1 s; mean *Prkca*^{-/-}, 115.2 s; p < 0.01). (E) Mice were anesthetized and a transverse incision made with a scalpel at a position where the diameter of the tail was 2.25 to 2.5 mm. The tail was immersed in normal saline (37 °C) in a hand-held test tube. The time from incision to cessation of bleeding was recorded, and mean times are shown as horizontal bars. No significant difference was seen comparing wild type mice (mean 140 s) with *Prkca*^{-/-} (mean 117 s; p > 0.05).

Figure 2. Deficiency of PKC α or β suppresses collagen-induced activation of platelets in thrombi. Whole blood from indicated mice was flowed over collagen (see Fig. 1), and platelet thrombi were double-stained with FITC-anti-mouse CD62P mAb (green) and AF647-annexin A5 (red). A, Representative confocal images (180x180 μ m) after staining. B, Integrated fluorescence intensity presenting activated platelets in arbitrary units, to quantify cumulative expression of P-selectin and PS. Means $\pm$ S.E.M. (n=3-5); **p<0.01 vs. corresponding wildtypes. Numbers above each bar indicate the mean integrated pixel density of P-selectin staining, an indication of staining per platelet.

Figure 5. Deficiency of PKC θ but not δ increases collagen-induced activation of platelets in thrombi. Blood from PKC δ ^{-/-}, PKC θ ^{-/-} or matched wildtype mice was flowed over collagen, and platelet thrombi were double stained with FITC-anti-mouse CD62P mAb (green) and AF647-annexin A5 (red). **A**, Representative confocal images (180x180 μ m) after staining. **B**, Integrated fluorescence intensity presenting activated platelets in arbitrary units, to quantify cumulative expression of P-selectin and PS. Means $\pm$ S.E.M. (n=3-5); **p<0.01 and *p<0.05 vs. corresponding wildtypes. Numbers above each bar indicate the mean integrated pixel density of P-selectin staining, an indication of staining per platelet.

Figure 1. Integrin α IIb β 3 closure in PS-exposing platelets is accompanied by Src kinase cleavage. A, Ex vivo flow experiments were performed with PPACK-anticoagulated human blood perfused over a collagen surface at 1000 s^{-1} for 4 minutes. Shown is a representative phase contrast image and a two-color fluorescence image, after staining for activated integrin α IIb β 3 (FITC-PAC1 mAb) and PS exposure (AF647-annexin A5); bar, 15 μm . B, Flow cytometric analysis of washed human platelets stimulated for 5–30 minutes with convulxin (CVX, 100 ng/ml) plus thrombin (Thr, 4 nM) in the presence of 2 mM CaCl_2 . Shown are platelet fractions with activated α IIb β 3 (FITC-PAC1 positive) or exposed PS (FITC-annexin A5-positive). Mean $\pm$ S.E. ($n=8-12$); **, $p<0.02$; ***, $p<0.001$. C, Single platelet counts after 5 minutes stimulation of platelets with CVX (100 ng/ml), Thr (4 nM), or ionomycin (20 μM), in the presence of 2 mM CaCl_2 . Mean $\pm$ S.E. ($n=3$); *, $p<0.05$. D, Representative western blots from platelets stimulated for 5–30 minutes with CVX (100 ng/ml) and Thr (4 nM) or ionomycin (20 μM). Activations were in the presence of 2 mM CaCl_2 or a 2 mM EGTA, as indicated. Blots were immunostained with 4G10 mAb (upper), and reprobbed with anti-Src Ab (lower panel). Arrows indicate position of Src family proteins.