Studies on regulation and signaling of platelet glycoproteins GPV and GPVI

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Summary

Platelets are crucial to inhibit extensive blood loss at sites of vascular injury. However, under pathological conditions such as rupture of an atherosclerotic plaque, activated platelets form aggregates that may occlude the vessel. This can lead to heart attack and stroke. Various and complex signaling pathways in the cell are involved in the steps of platelet adhesion, activation and aggregation. Single aspects of these processes were studied in three different subprojects in this work.

The Glycoprotein (GP) Ib-V-IX complex is responsible for the first contact of platelets with the vessel wall. Subsequently, GPVI can bind to collagen of the subendothelium, which initiates a signaling cascade leading to platelet activation, aggregation, characterized by integrin activation and granule secretion and platelet procoagulant activity. The latter is characterized by exposed phosphatidylserine (PS) on the platelet surface, which enhances thrombin generation and thereby the coagulation cascade. A controlled regulation of GP receptors on the platelet surface is vital for an intact response of the cell to platelet agonists.

In the first subproject described here the regulation of GPV and GPVI on mouse platelets was investigated and it was found that both receptors are shed from the platelet surface in a metalloproteinase dependent manner. However, GPVI is shed upon mitochondrial injury, while GPV cleavage could be observed upon platelet stimulation. The metalloproteinase responsible for GPVI shedding remains unknown whereas the metallproteinase that sheds GPV was identified in this work as being ADAM17. This shows that the expression of both receptors underlies a controlled mechanism regulated through distinct metalloproteinases.

In the second subproject the role of protein kinase C (PKC) in platelet activation and procoagulant response was investigated using PKC specific inhibitors. It was found that PKC blockage reduced platelet activation but enhanced platelet procoagulant activity. This is the first time that a dual role in platelet activation and procoagulant activity is defined for PKC.

In the third project the role of the small GTPase Rac1 in platelet signaling was studied using conditional Rac1 knock out mice. It is reported here that Rac1 lies downstream of GPVI and is involved in integrin activation and cytsolic Ca^{2+} changes *in vitro* and platelet adhesion and thrombus formation *in vivo*.

This is the first time that Rac1 is demonstrated to have a pivotal role in GPVI signaling and furthermore points to a novel, unknown pathway downstream of GPVI.

Zusammenfassung

Bei Verletzung einer Gefäßwand kommen Blutplättchen in Kontakt mit den Substanzen des Subendothels; Die Plättchen werden dadurch aktiviert, sie aggregieren und verschließen die Wunde, wodurch ein hoher Blutverlust verhindert wird. Unter pathologischen Bedingungen, bei Aufbrechen eines artherosklerotischen Plaques an der Gefäßwand, können sich jedoch große Plättchenaggregate, die Thromben, formen, die das Gefäß verschließen, den Blutfluss stoppen und somit zu Schlaganfall und Herzinfarkt führen können. Die kontrollierte Regulation und Signaltransduktion von bzw. durch Plättchenoberflächenrezeptoren ist wesentlich für das Funktionieren der Zellen und die intakte Balance zwischen physiologischer Plättchen-Aktivierung und der pathologischen Bildung eines Thrombus. In der vorliegenden Arbeit wird über wichtige Aspekte dieser Signalwege, die in drei Unterprojekten untersucht worden sind, berichtet.

In dem ersten Unterprojekt wurde die Regulation von Plättchenoberflächenrezeptoren, den Glykoproteinen (GP) V und VI, bei Mäusen analysiert. Hier wird beschrieben, dass GPV und GPVI von der Plättchenoberfläche durch Metalloproteinasen geschnitten werden. Während physiologischer Stress, wie das Entkoppeln der oxidativen Phosphorylierung in den Mitochondrien, das Schneiden von GPVI durch eine unbekannte Proteinase auslöst, verursacht die Aktivierung von Plättchen mit bestimmten Agonisten das Schneiden von GPV. Die dafür verantwortliche Metalloproteinase wurde als ADAM17 identifiziert.

In dem zweiten Unterprojekt wurde die Rolle der Protein Kinase C (PKC) in der Plättchenaktivierung einerseits und in der Plättchen pro-koagulanten Aktivität andereseits untersucht. Die Konformationsänderung/Aktivierung von αIIbβ3-Integrinen und Sekretion von Granula sind charakteristisch für die Plättchenaktivierung. Calcium-(Ca^{2+})-abhängige Phosphatidylserin (PS)- Expression auf der Plättchenoberfläche hingegen ist kennzeichnend für die pro-koagulante Aktivität. Der Beitrag von PKC zu den beschriebenen Plättchenzuständen war bisher unklar. In diesem Projekt wurde zum ersten Mal gezeigt, dass PKC eine doppelte Funktion in den Plättchen besitzt: einerseits fördert PKC die Plättchen-Aktivierung und –Aggregation, andererseits unterdrückt PKC die pro-koagulant Aktivität.

In dem dritten Unterprojekt wurde die Rolle der kleinen GTPase Rac1 in der Plättchen-Aktivierung und -Aggregation *in vitro* und *in vivo* an konditionalen Rac1 Mäusen analysiert.

Es wird berichtet, dass Rac1 für die GPVI abhängige Aktivierung von αIIbβ3-Integrinen und dem Freisetzen von Ca^{2+} in der Zelle, notwendig ist, sowie für GPVI abhängige Plättchen-Aggregation und Thrombus Bildung. Hiermit wird die GTPase Rac1 zum ersten Mal in den Signalweg unterhalb von GPVI eingeordnet und ihr zudem dort eine essentielle Rolle zugeteilt.

A) Introduction

A 1. Platelets in Hemostasis and Thrombosis

Platelets are the smallest cells of the blood system, however they are essential to seal a wound and prevent extensive blood loss at sites of injury. On the other hand under pathological conditions, platelet aggregation can result in thrombus formation and occlusion of the vessel leading to heart attack and stroke, which represent the most frequent causes of death in the western world.

Platelets circulate in discoid shape in the blood. Upon endothelial injury they come in contact with components of the exposed subendothelial layer and get activated. Activated platelets change shape from discoid to spherical, start to extend filopods and finally spread and form lamellipodia on the matrix to increase the contact surface and stabilize the matrix-platelet contacts (see Figure 1A and B). During activation, platelets recruit other platelets from the flowing blood stream, they aggregate and stabilize the platelet clot. Many aspects of platelet activation and aggregation are unclear but are important to understand in order to improve the efficancy and safety of antithrombotic therapy.

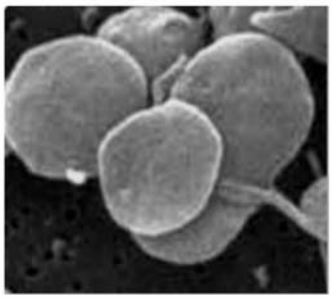


Fig. 1A:Unactivated platelets in discoid shape (http://www.ams.rdg.ac.uk/DMCB/platelets/plateletsmall2.jpg)

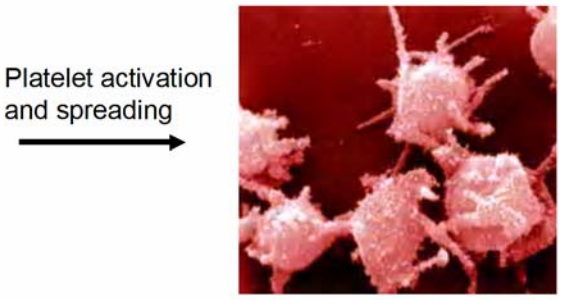


Fig. 1B: Activated platelets change shape, aggregate and start to extend filopodia (from www.cbr.ubc.ca)

A 2. Platelet formation and morphology

2.1. Platelet formation

Platelets represent with a diameter of 1-3 µm the smallest cells in the blood system. They arise from megakaryocytes, which develop from myeloid progenitor cells in the bone marrow. A widely accepted model of platelet formation is the classical proplatelet-formation theory, according to which platelets arise from pseudopodial elongations of the megakaryocyte (5). Megakaryocytes are polyploid cells, with a diameter of up to 150 µm and with multiples of the normal diploid chromosome content in a nucleus, attained by endomitotic cycles (6;7).

Platelets, in contrast, do not contain a nucleus, and thus display only limited ability for protein *de novo* synthesis (8;9). Platelets receive their final protein content from megakaryoctes which transfer the cell material into the proplatelets during their formation. Proplatelets consist of long lines of microtubules extended from the megakaryocyte cell body (10) with bulbous ends from which the mature platelets get released. Microtubules at the end of the extensions coil and lead back to the proplatelet shaft at the megakaryocyte body. They presumably represent the driving force for this extension, with an elongation rate of 1 µm/min (11). In addition, microtubules serve as tracks for the package material transferred to the proplatelet at the end of the extensions. In that way, proplatelets get loaded with organelles and cytoskeletal proteins like spectrin, adducing filamin and actin as well integrins and other glycoproteins (12). Another important component for the proplatelet extension process is the cytoskeletal protein actin, it is responsible for branching and formation of new extensions.
Details for the release of platelets are missing (11). Possibly, mature platelets get shed into the blood from the megakaryocyte body (13) and released in an immature form, in which platelets still might be attached to another by cytoplasmic projections. They are then supposed to attain their final mature shape in the peripheric blood. The characteristic discoid shape of platelets is presumably established before or during this shedding process due to the high shear forces to which platelets are submitted in the blood stream (11). In humans, about $3x10^8$ platelets circulate per ml blood, whereas in mice the platelet count is approximately $1x10^9$ platelets/ml blood. The lifespan of platelets in humans is about 10 days, in mice about 5 days before they are cleared by the spleen (14).

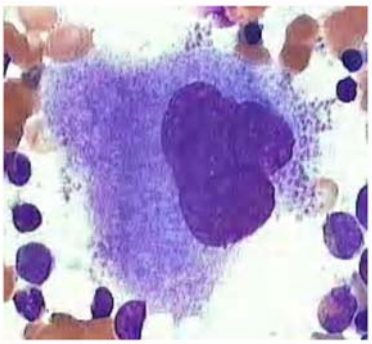


Fig. 2A: Megakaryocyte surrounded by platelets and red blood cells (from www.med-ed.virginia.edu). The nucleus is stained in purple (middle), seen as highly lobulated multilobed feature.

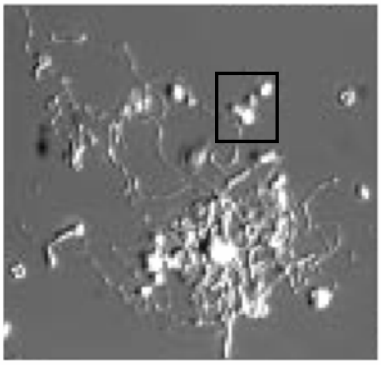


Fig. 2B: Production of proplatelets, that extend from the megakaryocyte cell body (from Larson MK and Watson SP, Blood 2006, (2)). Swelling ends (rectangle) are supposed to be nascent platelets at the end of the proplatelet (20 x objective used).

2.2. The platelet cytoskeleton

The main skeletal component is actin, with 2 million copies and making up a content of 0,55 mM in a platelet it is the most abundant of all proteins in these cells (15). Actin exisits in monomeric and polymeric form in the platelet cytoplasm; in the resting platelet about 40% of the actin molecules are assembled in linear actin polymers, which form the basic membrane. (16). The monomeric actin molecules are complexed with β4 thymosine in the cytoplasm. Upon platelet activation, the actin monomers are served onto exposed barbed ends of existing actin filaments (17-19). Spectrin, another membrane skeleton molecule, is expressed in a copy number of ~2.000/platelet. Spectrin is connected to the plasma membrane all along the cell (16;20). The actin binding molecule adducin, expressed at ~8.000 copies per platelet, is responsible for targeting the actin network to the spectrin-based membrane skeleton (16;21;22). The rigidity of the cytoplasmic network is further supported by the actin crosslinking protein filamin. Present in ~6.500 copies per resting platelet it interconnects at various points the actin network (23). It is a scaffolding protein binding to various signaling molecules and in that way bringing them close to the membrane (11). Filamin also binds to the cytosolic region of the GPIbα subunit of the transmembrane GPIb-V-IX complex. The filamin-GPIbα association seems to be essential for the unique discoid shape and the circulation of platelets in the blood (24-28).

2.3. Granules

Embedded in the cytoplasm of platelets are membrane remains of the endoplasmatic reticulum, also known as dense tubular system, organelles like mitochondria, endosomes and granules like lysosomes, α granules and dense granules. The granules are packed with proteins and platelet stimulating factors, which get released upon platelet activation during secretion. The most prominent granules are α and dense granules.

2.3.1 α Granules

α granules are 0.2-0.4 µm in diameter and with a number of ~80 per cell highly abundant in the platelet (29;30). α granules store megakaryocyte-produced molecules like β-thromboglobulin, platelet factor IV, thrombospondin and coagulation factor V as well as P-selectin and von Willebrand factor (vWf). In addition, α granules contain the adhesive molecules fibrinogen, fibronectin and vitronectin, which are taken up by endocytosis (31-34).
α granules also store a portion of major platelet surface receptors as GPVI, GPIb-IX-V complex and the integrin αIIbβ3. These receptors are expressed on the resting platelet, but get additionally redistributed onto the surface during platelet activation (35). P-selectin is expressed only on the platelet surface of the activated cell and therefore represents a specific marker for platelet activation (36;37).

2.3.2 Dense Granules

Dense granules are only about 0.15 µm in diameter. Human platelets carry 3-9 granules (38), whereas mouse platelets carry 5-6 per platelet (39). Dense granules store soluble platelet agonists like ADP, ATP, and serotonin as well as the divalent cations, calcium and magnesium and inorganic polyphosphates (40), which serve to enhance the activation state of platelets and accelerates the coagulation cascade, once released.

A 3. Platelet surface receptors

3.1. G-protein Coupled Receptors

G-protein coupled receptors (GPCRs) are receptors for soluble agonists such as ADP, thromboxane A2 (TxA2) or thrombin. They are involved in the second wave of platelet activation, which follows the initial steps of platelet adhesion, activation and granule secretion. There are only a hundred to thousand copies present per platelet. The characteristic feature of the GPCRs is a single chain of seven transmembrane helices spanned through the plasma membrane. The N-terminus is exposed to the extracellular region and the C-terminus to the intracellular region, where it couples to a heterotrimeric G-protein complex. The complex of the heterotrimeric G protein consists of a large α– and a small βγ subunit.

3.1.1 GPCR signaling

Upon ligand binding to the extracellular N-terminal region of the GPCR the α subunit of the G-protein gets activated by exchange of GDP to GTP. The active α-GTP dissociates from the βγ subunit and exerts its function on different downstream molecules. i.e. adenylat cyclase

(Gαi and Gαs), PLCβ (Gαq) or the GTPase activating molecule RhoGEF (Gα12/13), depending on the agonist and the thereby specifically activated G-protein. A list of GPCRs and their coupling is shown in table1 (41).

Among the best studied GPCRs are the thrombin receptors, named the protease-activated receptors, PAR1, 3 and 4 (PAR1&4 in human and PAR3&4 in mouse platelets). The activation mechanism of these receptors is unique: Thrombin functions as a protease and cleaves an extracellular N-terminal peptide of the receptor. The new free N-terminal region refolds and autoactivates the receptor.

The thrombin receptor PAR1 couples to the G-proteins $G_{12/13}$ and G_q and possibly also to Gi (42;43). PAR3 and PAR4 couple to Gq and $G_{12/13}$. The best studied and predominant receptor for thrombin in human platelets is PAR1, which is expressed in about ~2500 copies on the surface of human platelets. PAR1 in human has a high affinity for thrombin, whereas PAR4 exhibits a lower affinity (44;45), PAR3 is not expressed on human platelets. Conversely, mice platelets do not exhibit PAR1. In mice PAR3 is a cofactor for PAR4. Activation of platelets through thrombin is highly effective (already at nM-range) and results in shape change and spreading, integrin activation and aggregation.

Other GPCRs identified on platelets so far are the ADP receptors $P2Y_1$ and $P2Y_{12}$, coupling to Gq and Gi, respectively and the thromboxane A2 (TxA2) receptor, TPα/β, that couples to Gq as well as to $G_{12/13}$. Further, the receptor for epinephrine, α2A adrenergic receptor, that couples to the Gi-protein family member Gz and the receptor for prostaglandine2 (PGI2), termed IP, couples to the stimulatory Gs protein (see also table1).

Agonist	Receptor	G-protein	function
Thrombin	PAR1 (human)	Gq, Gi, G12/13	IP/DAG ↑, cAMP ↓ RhoGEF (to actin) ↑
	PAR3 (mouse)	No proprer signal function	
	PAR4 (mouse&human)	Gq, G12/13	IP/DAG ↑, RhoGEF ↑
ADP	P2Y1	Gq	IP/DAG ↑
	P2Y12	Gi	cAMP ↓
TxA2	TPα, TPβ	Gq, G12/13	IP/DAG ↑, RhoGEF ↑
Epinephrine	A2-adrenergic	Gz (Gi family member)	cAMP ↓
PGI2	IP	Gs	cAMP ↑

Table1: G-protein coupled receptors and their respective agonists and G-proteins in human and mouse platelets.
G_q signaling induces the production of IP3 and DAG, wereas G_i inhibits adenylyl cyclase. Activation of G_{12} through either PAR1 and PAR4 or TPα and TPβ signals to the GTPase exchange factor RhoGEF and induces actin rearrangement.

3.2. Integrins

Integrins are heterodimeric transmembrane receptors composed of an α and β subunit, each of them consisting of a long extracellular domain and a short intracellular tail. They exist in a closed inactive and an open active form on the surface. Integrins, namely the β tail, provide a link between ligands bound on their extracellular region and the intracellular complex of various adapter molecules connecting to the actin cytoskeleton. They are able to signal through the membrane in either direction: *inside-out* to adopt the active confirmation and from *outside-in* to transfer signals to the cytoskeleton and promote actin filament assembly (46;47). Platelets express six different types of integrins, belonging to either the β1, or β3 family.

3.2.1 The β1 family of intergins

The β1 familly of integrins consists of the fibronectin α5β1 receptor, the laminin α6β1 receptor and α2β1, also known as GPIaIIa, a major collagen receptor (along with GPVI). Integrin α2β1 gets activated through GPVI and then tightens the connection to the collagen matrix and supports GPVI signaling (48);(49;50). The recognition site for α2β1 on collagen appears to contain a glycine-glutamic acid-arginine sequence (51) and is less defined than that for GPVI.

3.2.2 The β3 family of integrins

On platelets, the β3 family of integrins covers the vitronectin receptor αvβ3, which is only expressed at about 100 copies/platelet and the fibrinogen receptor αIIbβ3. Integrin αIIbβ3, also known as GPIIbIIIa-complex, is expressed on platelets and megakaryocytes. With 50.000 to 80.000 copies/cell it is the most abundant integrin on the platelet surface. Integrin αIIbβ3 gets activated by *inside-out* signaling initiated by activatory receptors, such as GPVI and GPCRs (48;52-54). Upon platelet activation through these receptors αIIbβ3 undergoes conformational changes and shifts from a low (inactive) to a high affinity (active) state, ready to bind its ligand fibrinogen (55;56). Intergin αIIbβ3 is the major fibrinogen receptor, but also binds to vWf immobilized on collagen (57) and to collagen itself. Once engaged to its ligand several αIIbβ3 molecules cluster in the cell membrane and intracellularly at their β3 tail associate with various adapter molecules that transfer the signal to the cytoskeleton. This so called *outside-in* signaling leads to activation of actin binding proteins, actin reorganization and finally platelet spreading.

In addition to its important role in actin dynamics αIIbβ3 integrin also contributes to stable platelet-platelet contacts through bound fibrinogen on the platelet surface and thereby to platelet aggregation.

3.3. Glycoprotein VI

GPVI is a megakaryocyte/platelet specific protein of the immunoglobulin superfamily that is constitutively expressed on the surface. The 60 to 65 kD glycoprotein was first detected in the early eighties by Clemetson et al (58). Its role in platelet adhesion and as collagen receptor became evident when patients were identified who displayed bleeding disorders and whose platelets were unable to respond to collagen. Those lacked GPVI but expressed normal levels of $\alpha 2\beta 1$ (59;60;61). Nieswandt *et al.* demonstrated that mouse platelets lacking GPVI are resistant to activation by collagen and collagen-related peptide (CRP) under static and flow conditions (48;62). Now, GPVI is established as the major receptor for collagen on platelets and shown to play a role in platelet adhesion in vivo (48;62;63). The recognition site for GPVI appears to be the basic triple Glycin-Proline-Hydroxyproline (GPO) motif of collagen. GPVI consists of two Ig-C2-like extracellular domains linked by disulfide bonds, a transmembrane region and a short (~50 amino acids) intracellular tail. The transmembrane domain and the intracellular part of GPVI are non-covalently associated with the FcRγ-chain. The association with the FcRγ-chain is indispensable for the expression of GPVI (64). The GPVI cytoplasmic tail associates with the Ca^{2+} binding protein calmodulin (CaM) and contains two proline rich domains to which the Src kinases Fyn and Lyn bind via their SH3 domains (Src homology 3 domain), (see Figure 3A).

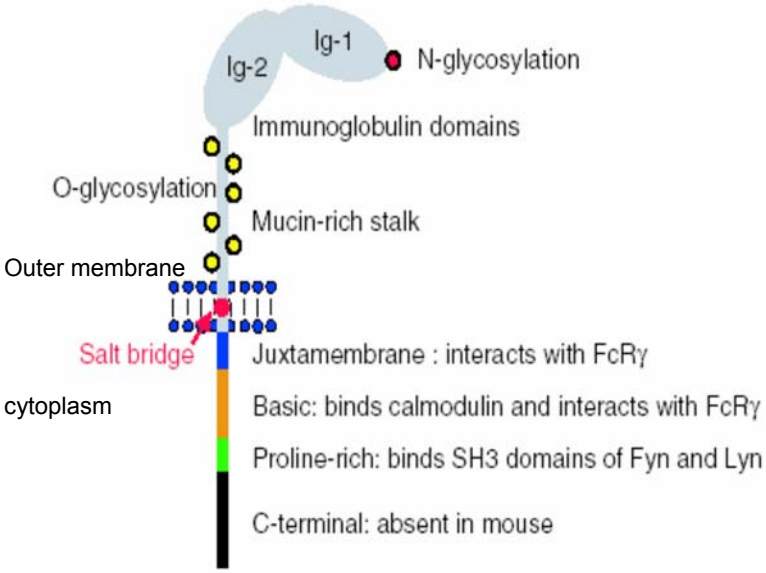


Fig 3A: Structure of the main collagen receptor GPVI on platelets. From Watson et al. 2005, (4). The extracellular part of GPVI contains two Ig like domains, with one N-gylcolysation site, followed by a stalk domain, which is strongly o-glycosylated. The transmembrane domain contains a salt bridge to which the FcRγ is associated. The cytoplasmic tail associates with calmodulin and the Src kinases Fyn and Lyn

3.3.1 GPVI signaling and function in platelets

GPVI stimulation initiates a cascade of several tyrosine phosphorylation steps, which results integrin activation, calcium mobilization and granule secretion. In addition to its role in platelet activation, recently the importance of GPVI in mediating platelet procoagulant activity was described. Mice deficient in GPVI displayed reduced thrombus formation and reduced procoagulant activity following vessel wall injury *in vivo* as compared to wild type mice (65).

Specific binding of GPVI to the (GPO)-motif of collagen clusters two GPVI molecules together. Like the B-cell receptor and T-cell receptor, GPVI has no intrinsic protein kinase activity; this activity is acquired by recruitment of a number of different non-receptor tyrosine kinases. GPVI clustring approaches fyn and lyn to their substrate, the FcRγ-chain, of the neighbored GPVI molecule. This enables the phosphorylation of two tyrosine residues in a conserved region present in the cytoplasmic tail of FcRγ-chain (66), termed the immunoreceptor tyrosine activation motif (ITAM). ITAM motifs are also found in association with cell surface Ig-receptors and T-cell receptors (67). The phosphorylation of the ITAM generates docking sites for Syk kinase, which then becomes autophosphorylated and initiates the phosphorylation of the adaptor molecule LAT (linker for activation of T-cells) (68). LAT initiates the activation of phosphoinositide-3kinase (PI3k) and phospholipase Cγ2 (PLCγ2) and their translocation to the plasma membrane in the vicinity to their substrates, the phosphoinositides (PIs) (69-71). In addition, PLCγ associates with a number of other tyrosine phosphorylated proteins that regulate the enzyme independently of LAT (see Figure 3B). At the plasma membrane, PI3k mediates the hydrolysis of phosphoinositides leading to the formation of PIP3 (PI-3,4,5-triphosphate), which is additionally needed to recruit PLCγ2 to the membrane (70;71). PLCγ2 mediates the hydrolysis of PI-4,5-phosphate (PIP2) – as it is also known for PLCβ – which results in the formation of the second messengers DAG and IP3 (see Figure 3B). DAG activates PKC and IP3 opens channels of intracellular calcium stores. PKC in synergy with released calcium triggers granule release and integrin αIIbβ3 activation.

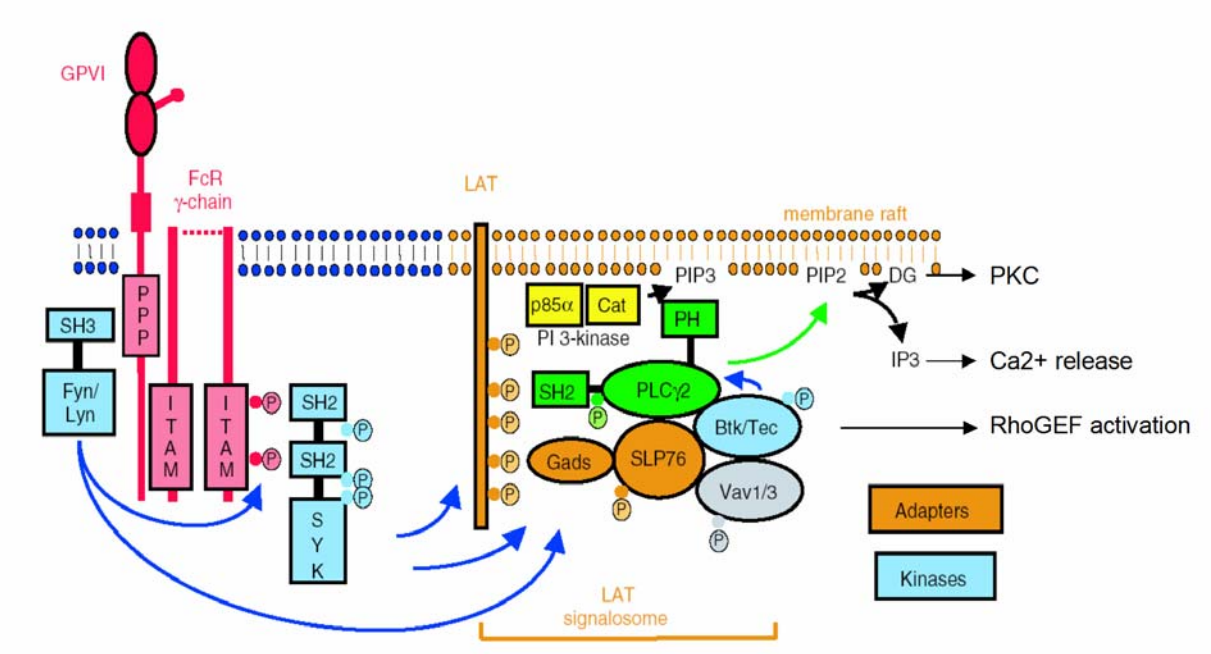


Fig. 3B: GPVI signaling in platelets. From Watson et al. 2005, 3, (4), slightly modified. For description see text.

Although GPVI has an important role in platelet adhesion and platelet activation, little is known about the regulation of this receptor. Nieswandt *et al*. demonstrated the down-regulation of GPVI *in vivo.* Five days after injection of a GPVI specific antibody, JAQ1, JAQ2 or JAQ3 in mice, GPVI is completely and irreversibly depleted from the platelet surface (62;72). However, following *in vitro* treatment of platelets with JAQ1, GPVI was not down-regulated from the surface. The nature of this mechanism is not clear, but it was shown that GPVI can be internalized *in vivo* upon antibody binding.

3.3.2 GPVI agonists

a) The physiologic GPVI agonist collagen

Endothelial damage exposes the subendothelial layer to cells of the flowing blood. The main constituents of the subendothelial layer are collagens. There exist nine different types of collagen in the vessel wall, of which the most prominent types are fibrillar collagen type I and III. Collagens contain a repeated glycine-proline-hydroxyproline (GPO) motif, which represents 10% of the molecule. In the monomer, the GPO- repeat sequences form a single left handed- helix, in association with two other chains it forms a superhelix. In collagen type I and type III about 1000 amino acid sequences form a helix, flanked by non helical sequences. The crosslinking of these collagen compositions leads to fibrillar collagen structures. Fibrillar collagen mostly contains more than one type of collagen and also other matrix proteins. For *in vitro* platelet activation, mostly a soluble mixture of fibrillar collagen

type I and fibrillar collagen type III is used, so called “Horm” collagen (50), therein the basic triple GPO is the major recognition site for the collagen receptor GPVI.

b) The synthetic GPVI agonist CRP

The discovery of the GPO motif by Morton *et al.* led to the preparation of synthetic collagens of GPO containing peptides. Only five GPO repeats were found to be sufficient to maintain the helical structure, which was shown to be important for the reactivity of collagen (73). The GPO containing peptide was termed *collagen related peptide* (CRP) and proved to be specific for GPVI binding independent of $\alpha 2\beta 1$ (74;75).

c) The snake venom convulxin

Many snake venom proteins are known to interact with platelets, thereby inducing either inhibitory or activatory effects. The snake venom convulxin, a 72 kDa protein isolated from rattle snake *Crotalus durisus terrificus* is a potent platelet agonist. Jandrot Perrus *et al.* could identify convulxin as a GPVI specific agonist (76).

3.4. Glycoprotein - Ib-V-IX complex

The GPIb-V-IX complex is constitutively expressed on platelets. Within this complex, GPV is flanked on each side by a GPIb and a GPIX molecule, giving a stoichiometry of 2:2:1, expressed in 25.000 copies per cell (77;78). The transmembrane proteins are non-covalently associated with each other, and only GPIb consists of two subunits, α and β, covalently linked through a disulfide bond (see Figure 4). With 135 kDa, GPIbα represents the largest subunit of the complex, followed by GPV (82 kDa), then GPIbβ (25 kDa) and GPIX (22 kDa). GPIb-V-IX belongs to the leucine-rich repeats (LRR) family of proteins and is a major receptor for immobilized vWf. It is also known to bind the coagulation factors XI, XII and thrombin and the membrane glycoproteins P-selectin and Mac1 (3). The extracellular LRR on GPIbα are essential for most of the ligand interactions with the complex (3). GPIb-V-IX binds to vWf immobilized on collagen through its A1 domain. The interaction is crucial for the first contact of platelets to the vessel wall. Flowing platelets slow down and adhere to the site of injury under conditions of high shear forces. This contact formation between GPIb-V-IX and vWf precedes platelet activation and stable adhesion.

GPIbα intracellular domains associate with actin binding proteins, importantly filamin, that anchors the actin cytoskeleton to the membrane (16). This is supposed to strengthen the vWf – platelet contacts under high shear and is important for platelet adhesion and signaling (79). In turn the palmitylated cytoplamic tail of GPIbβ and GPIX may contribute to the stable anchorage of the complex into the plasma membrane (80). Absence of both or either GPIb or GPIX subunits in humans is known as Bernard-Soulier-Syndrome (BSS) and results in giant

platelets, severe bleeding and thrombocytopenia, reflecting the importance of the complex for platelet structure and platelet adhesion (63; 81). GPIbβ and GPV are intracellularly associated with calmodulin, a calcium binding molecule. Upon ligand binding, calmodulin dissociates and relocates to the cytoskeleton where it mediates uptake of free Ca^{2+} (82).

The contribution of GPIb-V-IX complex to platelet signaling and activation, has been proposed to be dependent on the physical association with the ITAM containing receptor FcγRIIA which is expressed in vicinity (83). But also direct signaling of GPIbVIX to integrin αIIbβ3 could be observed involving the action of Src, PI-3kinase and intracellular calcium oscillation (84). In addition, GPIb and GPV cytoplasmic tails also bind to 14-3-3-ζ, a 30 kDa protein belonging to the 14-3-3 protein family, that regulate the function of several signaling molecules (3) and thus also may be involved in integrin activation upon vWf binding to GPIb-V-IX on platelets (85).

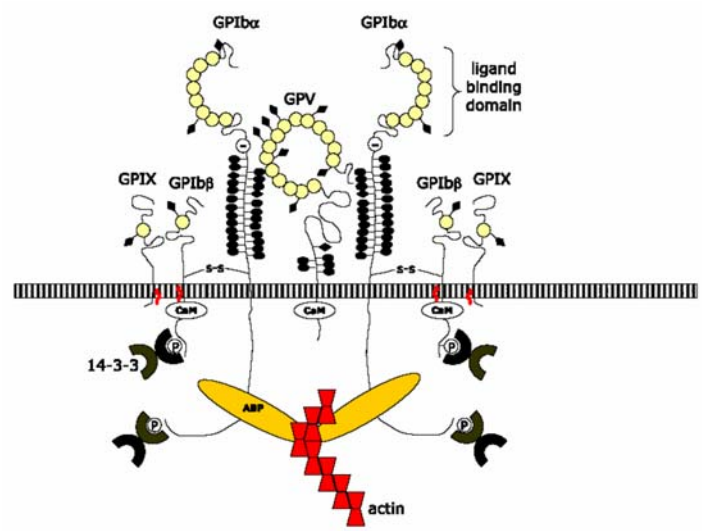


Fig. 4: Main features of the GPIb-V-IX complex on the platelet surface.
From Cannobio I et al., 2004, (3). The GPIb-V-IX complex consists of four different subunits, GPIbα and β, GPV and GPIX , which are non-covalently associated in the platelet membrane.The extracellular domains contain one or more leucine rich regions (Yellow circles). The stalk domain is slightly o-glycosylated (black circles), the ligand binding domain is N-glycosylated (black diamonds). Intracellular tails are associated with calmodulin (CaM), actin binding protein (ABP) or 14-3-3-ζ.

3.4.1 Structure of GPV

GPV has a molecular mass of 83 kDa with 544 residues and is non-covalently associated with the adjacent GPIbβ molecules of the GPIb-V-IX complex. It possesses a single transmembrane domain and a short intracellular tail of 16 amino acids. On the extracellular domain, GPV contains a thrombin cleavage site. Cleavage of GPV from the platelet surface by thrombin releases a 69 kDa soluble fragment (GPVf1) of yet unknown function (86;87).

3.4.2 Function of GPV

The function of GPV although being part of one of the most abundant protein complexes on platelets is a controversial issue. Mutations in GPIb or GPIX are responsible for the phenotype in Bernard Soulier syndrome (BSS) patients and thus are essential for normal platelet function (63, 87). GPV mutation or deletion in mice does not lead to the pronounced phenotype of BSS (88;89).

Studies on GPV deficient mouse platelets led later again to the assumption that GPV is involved in thrombin signaling and possibly exhibits a negative function on binding of thrombin to the GPIb-V-IX complex. It was shown that proteolytically inactive thrombin was able to activate platelets from GPV deficient mice. In addition enhanced responsiveness of GPV deficient platelets to thrombin was reported and the mice displayed shorter bleeding times and increased thrombosis (89;90). Cleavage of GPV is therefore supposed to unmask the thrombin binding site on GPIbα and enable ligation of thrombin to the receptor.

Other work reported an impaired thrombus formation in GPV deficient mice *in vivo* and observed a collagen binding defect (91), suggesting an additional role for GPV as a putative collagen receptor.

In patients with acute myocardial infarction increased levels of thrombin-cleaved GPV fragment, GPVf1, have been detected, as a result of enhanced platelet activation by thrombin. Early detection of GPVf1 levels in plasma of patients were proposed to present a tool to monitor the development of an acute myocardial infarction (92;93).

In addition to thrombin, elastase and calpain also are capable of directly cleaving GPV independent of platelet activation, releasing 74 kDa and 82 kDa fragments respectively (94).

A 4. Regulation of cell surface receptors

4.1. Ectodomain shedding of cell surface receptors

Several cell surface receptors underlie regulatory mechanisms including either internalization or shedding. Internalization mostly follows ligand binding and involves endocytosis of the ligand–receptor complex and intracellular degradation (95). It is supposed to down-regulate the signals that have been transduced and transmitted from the receptor to the inside of the cell and thus is aimed to prevent continuation of signaling (96). In contrast, cleavage of the receptor, also referred to as ectodomain shedding, is mediated by proteases and results in the release of mostly large extracellular fragments.

Cleavage occurs mainly at the stalk ectodomain region of the receptor in a domain close to the outer plasma membrane. Transmembrane protein cleavage may generate an extracellular fragment with ligand properties. The soluble forms bind to a membrane bound

receptor and induce cell signaling, that trigger physiological processes, which are important for normal cell development, immune responses and apoptose signaling (97-99). A prominent example for ectodomain shedding of a transmembrane protein is the cleavage of tumor necrosis factor a (TNFα) on macrophages. The released factor subsequently stimulates the expression of adhesion molecules on endothelial cells and activates neutrophils, B- and T-cells and thereby importantly contributes to the inflammatory response (97). In other cases generation of soluble functional domains may lead to uncontrolled signaling, tumor progression and metastasis. An example is the shedding of membrane anchored ligands for the epidermal growth factor receptor (EGFR). The ligands are functional in both, membrane bound and soluble forms. Inhibition of cleavage of EGFR ligands was found to be effective in treatment of tumor progression, as it reduced cell proliferation and migration (100;101). Conversely, shedding can also, similar to internalization, simply serve to down-regulate an active receptor, preventing hyperresponsiveness (102;103).
The proteins undergoing ectodomain shedding are diverse and a consensus sequence could not be identified. In platelets shedding of P-selectin was reported and the soluble fragment was supposed to negatively mediate platelet adhesion and thrombus growth, by competitive inhibition of platelet P-selectin binding sites on endothel cells (102). Furthermore, also CD40 ligand (CD40L), which - like P-selectin - is only expressed on activated platelets, is subsequently cleaved from the platelet surface to generate an active soluble form that contributes to thrombus stability (104). In addition cleavage of GPIbα upon platelet activation was described, releasing a 130 kDa fragment known as glycocalicin, with unknown function. Recently TNFα converting enzyme, ADAM17, was identified to be the responsible metalloproteinase for GPIb shedding *in vitro* and *in vivo* in platelets (105).

4.2. ADAM17 (TNFα converting enzyme)

The proteinases, responsible for shedding are mostly zink-dependent matrix metalloproteinases and proteinases of the *a disintergin and a metalloproteinase (ADAM)-family.* ADAMs are part of the snake venome metalloproteinases, the adamalysins. They contain a signal-domain, followed by an inhibitory pro-domain, a metalloproteinase-domain, containing the zink binding site, followed by a disintergin-, a cystein rich-, a transmembrane-domain and a cytoplasmic tail. The metalloproteinase ADAM17 was first described in the early nineties as being responsible for shedding of tumor necrosis factor-α (TNFα) *in vitro* and *in vivo* and was therefore known as *TNFα converting enzyme* (TACE) (106;107). Thereby ADAM17 represented the first metalloproteinase to which a specific substrate could be attributed. ADAM17 is a 85 kDa transmembrane molecule expressed on the cell surface in a wide range of cells. It executes a very low basal activity in unstimulated cells, which rises upon cellular stimulation (108). The detailed pathway that activates ADAM17 is not clear. In

many cases shedding by ADAM17 could be induced by phorbol esters, like PMA, which directly activate PKC and subsequently the mitogen-activated-protein (MAP) kinase pathway. However, another mechanism, independent of PKC, was shown to efficiently induce ADAM17 mediated L-selectin shedding from leukocytes: calmodulin (CaM) binds on the intracellular domain of L-selectin and prevents its shedding, which consequently can be induced by CaM inhibitors (109). Besides TNFα several other molecules have now described to be specifically cleaved by ADAM17, including GPIb on platelets, L-selectin on endothel cells and the EGFR ligand TGFα on T-cells (110;111). Homozygous deletion of the zink binding domain in ADAM17 results in perinatal lethally of the mice, reflecting the importance of that metalloproteinase for normal development (99;109) and indicating a far more complex role of ADAM17 in the organism than its initial name (TACE) might implicate.

A 4. Platelet signaling molecules

4.1. Protein kinase C

Protein kinase C (PKC) is a 77 kDa cytoplasmic enzyme, that phosphorylates several downstream target molecules at serine or threonine residues. In platelets at least six PKC isoforms have been detected, namely $\alpha, \beta, \delta, \eta, \theta$ and ζ. They are divided into three classes of PKC forms (112;113):

- The *classical*, calcium and 1,2- diacylglycerol (DAG)- regulated PKCs,
- The *novel*, DAG- regulated but calcium independent PKCs
- and the *atypical forms*, independent of both, calcium and DAG.

In platelets, all three classes of PKC isoforms have been reported (114;115). The PKC proteins contain two domains. One regulatory domain, that interacts with phosphatidylserine and - depending on the isoform- with DAG and calcium and a second, catalytic domain, that binds ATP and the protein substrate. Both domains provide targets for PKC inhibition and there exists a wide range of different PKC inhibitors (116), among which the bisindolylmalmeide derivatives RO318425 and GF109203X have been shown to be the most effective (116;117).

4.1.1 Protein kinase C signaling and function in platelets

Among the three classes of PKCs, an important role was attributed especially to the classical PKC isoforms α and β in platelets. They are supposed to lie downstream of PLCβ and γ activated by GPCRs and GPVI, respectively. PKC gets activated upon DAG binding, a

phorbol ester that arises from hydrolysis of phoshinositol-4,5-bisphosphate (PIP2) by PLC (118). IP3 in parallel to DAG, opens calcium channels of the intracellular calcium stores and releases calcium into the cytoplasm (see also Fig.5). The active form of PKC relocates to the plasma membrane (119) and in synergy with calcium promotes granule secretion and the activation of integrins (120).

A major substrate for PKC is pleckstrin (p47) a 47 kDa molecule, of so far unknown function. Upon platelet activation by thrombin or collagen it gets rapidly phosphorylated (121). Additionally, PKC was reported to lie downstream of integrin αIIbβ3 (122). Integrin αIIbβ3 signaling contributes to cytoskeletal reorganization and platelet spreading leading to stable platelet attachment. Consequently, in addition to inside-out signaling, PKC also takes part in outside-in signaling initiated by αIIbβ3 (122;123).

However, in addition to the activating functions of PKC, also inhibitory effects have been reported. Therein PKC was shown to negatively regulate calcium influx into platelets (124-126), thus the role of PKC in platelets is yet not clear.

4.2. The Rho family of small GTPases

Rac1 belongs to the Rho-subfamily of small GTPases, which includes 14 distinct members, with a molecular mass of 20 to 25 kDa (127). These can be broadly divided into four subgroups: Rho, Rnd, Cdc42 and Rac.

Rac, with a molecular mass of 21 kDa, comprises three different isoforms with tissue specific expression pattern: the Rac1 isoform is expressed ubiquitously, Rac2 in the hematopoietic cell system and Rac3 exclusively in the brain (128). In platelets, Rac1 appears to be the predominant form (129).

A common feature for GTPases in general is the GDP-GTP exchange. Binding of GTP turns the GTPase in its active state while hydrolysis of GTP to GDP inactivates the GTPase, mediated by conformational changes. Cycling between the active and inactive state is under the control of small factors. For instance, the low inherent hydrolytic activity of GTPases is increased by the means of GTPase activating proteins (GAPs). In turn the replacement of GDP by a GTP is performed by GTPase exchange factors (GEFs). GDP dissociation inhibitors (GDI) maintain the GDP bound, inactive state of the GTPase in the cytoplasm until a signal initiates the activation of a GEF and the release of GDI (Fig. 5) (1). Over the last decade knowledge about role and function of small GTPases has increased by the use of cell models with overexpressed or blocked activity of a specific GTPase (130;131). Rho-subfamilly GTPases are involved in various physiological processes, like cell proliferation, cell migration, endocytosis, and cell spreading (132;133), but under pathological conditions

they contribute also to cell transformation and metastasis (134). In platelets, the Rho-familly of GTPases have only been intensively studied during the last few years.

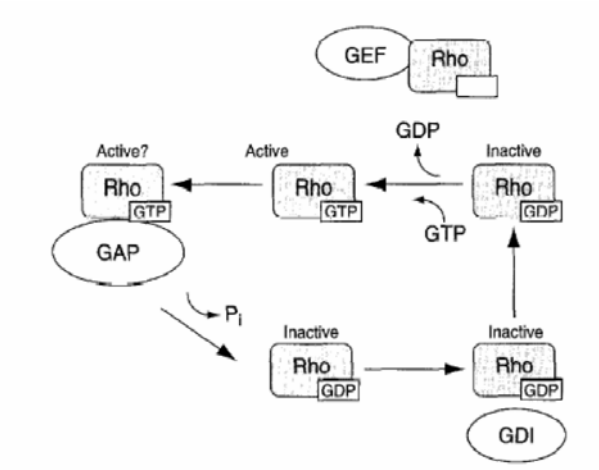


Fig. 5: Activation cycle of RhoGTPases, as an example for GTPases in general
(From Machesky LM, and Hall A, 1996 (1)).
The GTPase is active in the GTP bound form, which is attained through GDP-GTP exchange triggered by a GDP-GTP exchange factor (GEF). GTPase activating protein (GAP) activates the hydrolysis of GTP to GDP, driving the GTPase again in its inactive state. GDP dissociation inhibitor (GDI) maintains the GTPase in this inactive form.

4.2.1 The small GTPase Rac1, signaling and function in platelets

Three members of the Rho-subfamilly of small GTPases, RhoA, Cdc42 and Rac1 are strongly expressed in platelets. In several studies with fibroblasts using over-expression of specific GTPases, it could initially be shown that Rac1 is responsible for the specific formation of lamellipodia, while Cdc42 promotes filopodial extension and RhoA the specific formation of stress fibers upon stimulation with growth factors (135-137).

Rac1 deficiency leads to embryonic lethality already at day E 8.5 (138). Therefore, conditional Rac1 deficient mice were generated and lately described (139). Rac1-deficient hematopoietic stem-cell/progenitors showed reduced reconstitution of hematopoiesis in the bone marrow in an engraftment model. This was attributed to defects of those cells in migration and adhesion, which is dependent on Rac1 mediated actin reorganization (140). Gu et al. reported essential but overlapping function of Rac1 and Rac2 in B cell development and consequently an essential role to Rac isoforms in B-cell signaling was credited (139). Activation of Rac1 in platelets was demonstrated to be dependent on stimulation of GPCRs by thrombin, ADP and TxA2 by the means of specific inhibitors or dominant negative mutants and Rac-GTP pull down assays (141-143).

During the course of this work McCarty et al. (140) described a spreading defect on immobilized collagen of platelets derived from Rac1 deficient mice, which was reported to be due to defective GPCR signaling, following release of second wave mediators ADP and TxA.

In other cells, i.e. fibroblasts, Rac1 was shown to lie downstream of integrins and to be required for integrin mediated cell spreading (144-146). In platelets it was demonstrated that Rac1 migrates to the cell membrane upon platelet stimulation with the GPCR ligand thrombin (141), where it activates membrane bound lipid kinases that regulate phosphoinositide synthesis and actin filament uncapping and polymerization (147). Although the effects and mechanism following Rac1 activation are not fully understood, it is known that an effector

molecule for Rac1-GTP represents p21-activated kinase (PAK1), that binds to the PAK1-binding domains (PDB) of both GTPases, Cdc42 and Rac1 (148;149). PAK1 is supposed to couple GTPase signaling to actin dynamics (150). A potential GEF for Rac1 in platelets is Vav1, which is exclusively expressed in hematopoietic cells (151). Vav1 gets rapidly tyrosine phosphorylated upon integrin stimulation and has been shown to regulate Rac1 activity in T-cells (151;152). Recently it was published that PLCγ2, downstream of the B-cell receptor (BCR), is activated by Rac GTPases and translocated to the membrane independently of PLCγ tyrosine phosphorylation or PIP3 production (153). TCR and BCR signaling induce a phosphorylation cascade similar to the GPVI signaling pathway and several molecules downstream of GPVI were discovered in first place by studying TCR engagment and the downstream tyrosine phosphorylation cascade. Nevertheless, in platelets still little is known about the role of Rac1 in tyrosine kinase activation mediated by the main collagen receptor GPVI.

A 5. Role of Platelets in Coagulation

Injury of the endothelial vessel wall induces two parallel processes, intended to seal the wound and inhibit extensive blood loss:

- Initiation of the coagulation cascade, started by released/expressed tissue factor and followed by sequential action of coagulation factors

and

- Platelet adhesion to the site of injury followed by subsequent platelet activation, aggregation, and display of platelet procoagulant activity (PS exposure).

Upon vessel wall damage, components of the subendothelial layer are exposed to cells of the flowing blood. Subsequently, platelets adhere to the site of injury by interaction of GPIb-V-IX with vWf, immobilized on subendothelial collagen.

Through interaction of GPIb-V-IX with vWf, platelets tether to the subendothelium, slowly rolling over the matrix. The collagen receptor GPVI can now directly interact with its ligand, supported by integrin α2β1 (48). An intracellular tyrosine signaling cascade starts that leads to cytoplasmic calcium increase, integrin αIIbβ3 activation, granule secretion, and subsequent enhancement of the coagulation process (65;154;155). Active αIIbβ3 binds soluble fibrinogen and recruits further platelets from the flowing blood, forming platelet–platelet contacts via integrin bound fibrinogen, the basis for platelet aggregation and thrombus formation. Secreted granules release the soluble agonists ADP and TxA2 into the plasma, they bind to their respective GPCR on the platelet surface and locally reinforce platelet activation. Integrin αIIbβ3 outside-in signaling in turn amplifies calcium increase

(156;157) and signals to the cytoskeleton, that starts to reorganize and thereby stabilize platelet-matrix contacts (158). Calcium is essential for platelet activation; it supports integrin activation and in synergy with PKC induces granule secretion. Calcium also triggers the exposure of PS onto the surface of the platelet membrane, characteristic of platelet procoagulant activity (159). The negatively charged PS represent a adhesion surface for the prothrombinase complex formed by coagulation factor V(a) and its co-factor Xa from the coagulation cascade. The prothrombinase complex bound to the PS surface displays enhanced proteolytic activity and leads to increased thrombin formation by cleavage of prothrombin (160;161). Thrombin amplifies platelet activation signals by binding to PAR receptors and in addition cleaves soluble fibrinogen into fibrin, which results in the formation of a fibrin-network that stabilizes the platelet plug (see Fig. 6). The exposure of PS on the surface of procoagulant platelets is a key regulatory mechanism in blood coagulation as it acts as interface of coagulation and platelet activation, sustaining and amplificating the coagulation process.

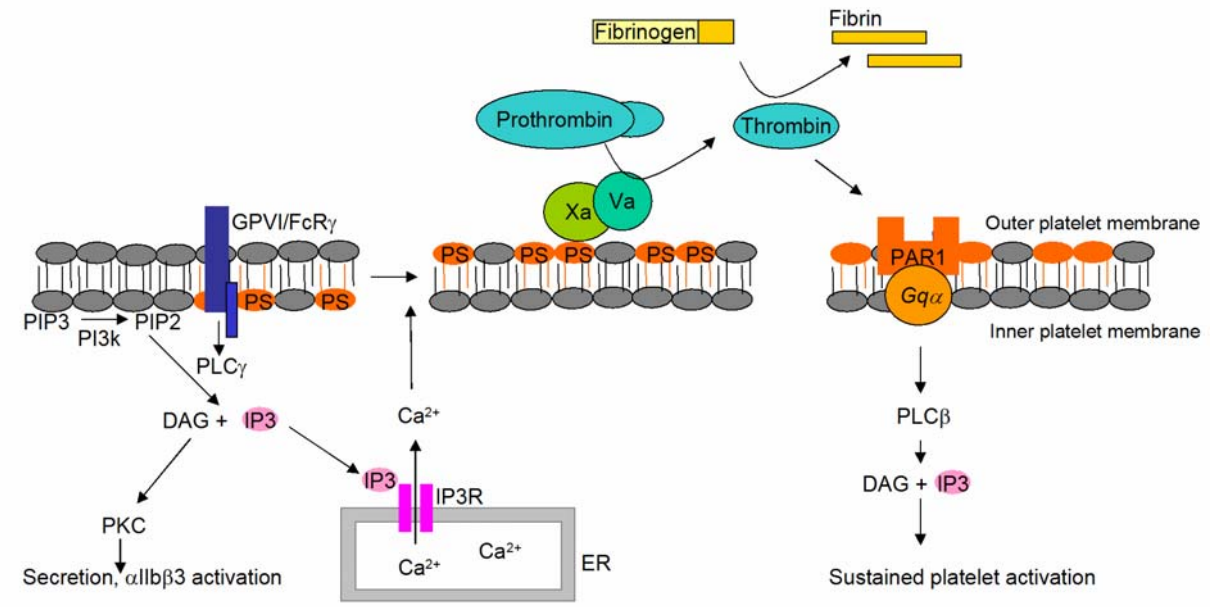


Fig. 6: Intracellular calcium release leads to PS exposure and enhanced thrombin generation. Schematic presentation of the processes occurring during platelet activation and their contribution to coagulation. For description see text above. Abbreviations: PIP3: Phosphatidyl-3,4,5-triphosophate; PIP2: Phosphatidyl-4,5-bisphosphate; PI3k: Phosphatidylinositol 3-Kinase; PS: Phophatidylserine, PLCγ/β: Phospholipase Cβ/γ, DAG: Diacylglycerol, PKC: Protein kinase C, IP3: Inositoltriphosphate, IP3R: IP3 receptor; ER: endoplasmatic reticulum, Va:Coagulation factor Va, Xa: Co-factor Xa, PAR1:Protease-activated receptor 1 (thrombin receptor).

A 6. Aim of the Study

Due to the significance of platelets for hemostasis and thrombosis in the human body, the aim of this study was to elucidate main features of platelet receptor regulation, platelet activation, aggregation and platelet procogulant activity. Three subprojects were conducted, dealing with the following questions:

1. What mechanism underlies the downregulation of the platelet surface receptors GPVI and GPV; are the glycoproteins shed from the membrane or rather internalized?
2. What is the role of PKC downstream of GPVI in platelet activation on one hand and its contribution to platelet procoagulant activity on the other?
3. What is the function of Rac1 in platelet activation?

The results presented here may contribute to a better understanding of platelet biology and could help in the development of medical treatments against thrombosis, heart attack and stroke.

B) Material and Methods

B 1. Materials

ADP	Sigma (Deisenhofen, Germany)
Alexa Fluor® 488 phalloidin	Invitrogen™ Molecular Probes™ (Orgeon, USA)
Annexin A5 labeled with Alexa fluor (AF)647	Invitrogen™ Molecular Probes™ (Orgeon, USA)
Annexin A5 labeled with fluorescein isothiocyanate (FITC)	NeXins Research BV. (Hoeven, the Netherlands)
Apyrase (grade III)	Sigma (Deisenhofen, Germany)
Bovine serum albumin (BSA)	Applichem. (Darmstadt Germany)
Brij 35 (polyoxyethylene (23) lauryl ether)	Sigma (Deisenhofen, Germany)
Carbonyl cyanide m-chlorophenylhydrazone (CCCP)	Sigma (Deisenhofen, Germany)
Complete Mini (Protease inhibitor mix)	Roche Diagnostics (Mannheim, Germany)
5-carboxyfluorescein diacetate succinimidyl ester (DCF)	Invitrogen™ Molecular Probes™ (Orgeon, USA)
Enhanced chemoluminiscence (ECL) detection substrate	MoBiTec (Göttingen, Germany)
ELISA detection kit TMB one	Europa Bioproducts Ltd., Cambridge, UK
EDTA	Applichem. (Darmstadt Germany)
EZ-Link sulfo-NHS-LC-biotin	Pierce (Rockford, IL,USA)
Fibrillar type I collagen (Horm)	Nycomed (Munich, Germany)
Fluorescein-isothiocyanate (FITC)	Molecular Probes (Oregon, USA)
Formaldehyde (37 %)	Merck (Darmstadt, Germany)
Fura-2 and Fluo-3 acetoxymethyl esters	Molecular Probes (Oregon, USA)
GF109203X	Molecular Probes (Oregon, USA)
GM6001	Calbiochem (Bad Soden, Germany)
GW280264X	GlaxoSmithKline (Stevenage, UK)
H-Phe-Pro-Arg chloromethyl ketone (PPACK)	Calbiochem (Bad Soden, Germany)
High molecular weight heparin	Sigma (Deisenhofen, Germany)

Hirudin	Aventis (Frankfurt, Germany)
Horseradish peroxidase (HRP)-labeling kit	Zymed (Berlin, Germany)
Human fibrinogen	Sigma (Deisenhofen, Germany)
Igpal	Roche Diagnostics (Mannheim)
Indomethacin	Instituts pharmacy (Würzburg, Germany)
Sodium-orthovanadat (Na3Vo4)	Sigma (Deisenhofen, Germany)
Oregon green (OG)488-conjugated fibrinogen	Molecular Probes (Oregon,USA)
PD-10 column	Pharmacia (Uppsala, Sweden)
Phorbol 12-myristate 13-acetate (PMA)	Sigma (Deisenhofen, Germany)
Polyinosinic-Polycytidylic Acid (polyIC)	Amersham/GeHealthcare (Munich, Germany)
Protein G sepharose	Pharmacia (Uppsala, Sweden)
Prostaglandine 2 (PGI2)	Sigma (Deisenhofen, Germany)
Recombinant human (rh) ADAM17	R&D Systems (Minneapolis, USA)
RO318425	Calbiochem (Bad Soden, Germany)
R-phycoerythrin (PE)	EUROPA (Cambridge, UK)
RPMI media	PAN (Aidenbach, Germany)
TNFα protease inhibitor-2 (TAPI-2)	Calbiochem (Bad Soden, Germany)
Thrombin	Roche Diagnostics (Mannheim, Germany)
3,3,5,5-tetramethylbenzidine (TMB)	EUROPA (Cambridge, UK)
Triton X-100	Sigma (Deisenhofen, Germany)
Tween 20	Roth (Karlsruhe, Germany)
U46619	Alexis Biochemicals (San Diego, USA)
W13	Calbiochem (Bad Soden, Germany)
W7	Calbiochem (Bad Soden, Germany)
Z-Gly-Gly-Arg aminomethyl coumarin	Bachem (Weil am Rhein, Germany)

Collagen related peptide (CRP) was kindly provided by S.P Watson (University of Birmingham, UK). Convulxin was obtained from the venom of the tropical rattlesnake *Crotalus durissus terrificus* and was kindly provided by M. Leduc and C. Bon (Institute Pasteur, Paris, France).

All other chemicals were obtained from Sigma (Deisenhofen, Germany) or Roth (Karlsruhe, Germany).

1.1. Antibodies

1.1.1 Commercial monoclonal antibodies (mAbs)

Anti-CD62 (anti human P-selectin antigen)	Sanquin (Amsterdam, The Netherlands)
4G10 (mouse anti-mouse phospho-tyrosin antigen)	Upstate cell Signalling Solutions (Lake Placid, USA)
Mouse anti-mouse Rac1 antigen	BD Biosciences Pharmingen (Franklin Lakes, USA)
PAC1-FITC (anti human αIIbβ3)	BD Biosciences Pharmingen (Franklin Lakes, USA)

1.1.2 Commercial polyclonal antibodies (pAbs) /secondary reagents

Rabbit anti-rat IgG-HRP	DAKO (Hamburg, Germany)
Rat anti-mouse IgG-HRP	DAKO (Hamburg, Germany).
Streptavidin (-HRP, -FITC)	DAKO (Hamburg, Germany)

1.1.3 Monoclonal rat anti-mouse IgG antibodies generated and modified in our laboratory

Antibody	**isotype**	**Antigen**	**described in**
JAQ1	IgG2a	GPVI	(62)
DOM1	IgG1	GPV	(162)
DOM2	IgG2a	GPV	(162)
JON/A	IgG2b	GPIIb/IIIa	(163)
ULF1	IgG2a	CD9	(162)
p0p4	IgG2b	GPIbα	(162)
p0p6	IgG2b	GPIX	(162)
21H4	IgG2b	α2 integrin	Unpublished
BRU1	IgG1	P-selectin	Unpublished

1.2. Equipment

Aggregometer	LaborGmbH, Ahrensburg, Germany
Cool Snap Camera	Cool Snap ES, Visitron Systems GmbH, Puchheim, Germany
Elisa Reader	Anthos Microsystems, Krefeld, Germany
Master Cycler Gradient	Eppendorf, Hamburg, Germany
Multifuge 3S-R	Thermo, Darmstadt, Germany
Flow Cytometer	FACScalibur, Becton-Dickinson
Fluoroscan Ascent well plate reader	Thermolab Systems, Helsinki Finland (in the Department of Biochemistry and Human Biology, Maastricht, NL)
Fluorescence and Phase Contrast Microscope	Axiovert 200, Carl Zeiss, Jena, Germany
Fluorescence lamp	100-W mercury short arc photo optic lamp (HBO) for epi illumination HBO100
Objective 100x/ 1,4 Oil DIC	Carl Zeiss, Jena, Germany
Objective 63x/ 0,75 Korr Ph2	Carl Zeiss, Jena, Germany
Objective 40x/ 0,60 Korr Ph2	Carl Zeiss, Jena, Germany
Photo Camera	Power Shot A620, Canon, Tokio, Japan
Photometer	BIOPhotometer, Eppendorf, Hamburg, Germany
Tablecentrifuges 5415C, 5415D, 5415R	Eppendorf, Hamburg, Germany
Thermomixer comfort	Eppendorf, Hamburg, Germany
Two photon laser scanning microscope	Bio-Rad 2100 Multiphoton System (in the Department of Biochemistry and Human Biology, Maastricht, NL)

1.3. Buffers

All buffers were prepared and diluted using aqua $_{bidest}$

- Acid-citrate-dextrose (ACD) buffer, pH 4.5

Trisodium citrate dehydrate	80 mM
Citric acid anhydrous	52 mM
Glucose anhydrous	183 mM

- Biotinylation buffer, pH 9.0

$NaHCO_3$	50 mM
NaCl	0.9 %

- Blotting buffer A

Tris, pH 10.4	0.3 M
Methanol	20 %

- Blotting buffer B

Tris, pH 10.4	25 mM
Methanol	20 %

- Blotting buffer C

ε-amino-n-caproic acid	4 mM
Methanol	20 %

- Coating buffer, pH 9.0

$NaHCO_3$	50 mM

- Coomassie Stain

Acetic acid	10 %
Methanol	40 %
Brilliant blue in H_2O	1 g

- Coupling buffer, pH 9.0

$NaHCO_3$	160 mM

Na_2CO3	80 mM

- Destaining solution

Acetic acid in H_2O	10 %

- DNA digestion buffer

Tris-HCl (pH 8.0)	50 mM
EDTA	100 mM
NaCl	100 mM
SDS	1 %
Proteinase K (10 mg/mL)	35 µL

- Hepes buffer (pH 7,45)

Hepes	10 mM
NaCl	136 mM
KCL	2,7 mM
MgCl2	2 mM

- Laemmli buffer

Tris	40 mM
Glycin	0.95 M
SDS	0.5 %

- 2x Lysis buffer (for tyrosin phosphorylation, pH 7,5)

NaCL	300mM
Tris/HCl	20mM
EGTA	2mM
EDTA	2mM
Igpal	2%

- Immunopreciptitation (IP) buffer

Tris/HCl (pH 8.0)	15 mM
NaCl	155 mM
EDTA	1 mM
NaN_3	0.005 %

- Phosphate-buffered saline (PBS), pH 7.14

NaCl	137 mM (0.9 %)
KCl	2.7 mM
KH_2PO_4	1.5 mM
$Na_2HPO_4x2H_2O$	8 mM

- PBS/EDTA

PBS	
EDTA	5 mM

- SDS sample buffer, 2X

β-mercaptoethanol (for red. conditions)	10 %
Tris buffer (1.25 M), pH 6.8	10 %
Glycerin	20 %
SDS	4 %
Bromophenolblue	0.02 %

- Storage buffer, pH 7.0

Tris	20 mM
NaCl	0.9 %
BSA	0.5 %
NaN_3	0.09 %

- Tyrode's buffer, pH 7.3

NaCl	137 mM (0.9 %)
KCl	2.7 mM
$NaHCO_3$	12 mM
NaH_2PO_4	0.43 mM
Glucose	0.1 %
Hepes	5 mM
BSA	0.35 %
$CaCl_2$	1 mM
$MgCl_2$	1 mM

- Washing buffer

PBS	
Tween 20	0.1 %

1.4. Animals

Specific-pathogen-free mice (NMRI, C57Bl/6) 6 to 10 weeks of age were obtained from Charles River, Sulzfeld, Germany. Rac1$^{fl/fl}$ mice were obtained from Cord Brakebusch (MPI, Martinsried, Germany) and bred with MxCre mice expressing Cre recombinase under the control of the interferon inducible Mx promotor (164) to obtain Rac1$^{fl/fl}$ MxCre$^{+/-}$ and Rac1$^{fl/fl}$ MxCre$^{-/-}$. Three to four week old Rac1$^{fl/fl}$ MxCre $^{+/-}$ (Ko mice) and Rac1$^{fl/fl}$ MxCre$^{-/-}$ (control mice) were injected two times over five days with 500 µl of 250 µg/ml Polyinosinic-Polycytidylic Acid (PIC). Ten days after the first injection, the mice were tested for successful knock out by western blotting.

B 2. Methods

2.1. DNA extraction and purification for genotyping

Mice ear pieces (3 mm^2) were lysed in 500 µl digestion buffer with 50 µg/ml proteinase K.
After over night shaking at 56°C (in Thermomixer, 700 rpm), 250 µl of saturated NaCl solution were added and the samples vortexed for 5 min.
Samples were centrifuged 14000 rpm (^ 20000 g) for 10min and the supernatent, without taking the very top, carefully transferred into new tubes. To precipitate DNA, 500 µl isopropanol were added and samples carfully shaken for 2 min (Thermomixer, 350 rpm, 25°C) and afterwards centrifuged (20000 g for 10 min). DNA pellets were washed 2x with 500 µl 70% ice cold ethanol (20000 g for 10 min), after last washing step, all ethanol was removed and samples were dried for 30 min at 37 °C, before solubilizing DNA in 50 µl H_2O bidest for 2 hrs at 37 °C.
DNA concentration was measured at a wavelength of A260 nm. The DNA concentration for PCR was adjusted to 200 µg/ml with H_2O bidest.

2.2. Genotyping Protocol

Buffers, nucleotides and Taq polymerase were from Fermentas (St Leon-Rot, Germany)
Primers were ordered from MWG (Martinsried, Germany)

Two genotyping protocols were carried out to determine the genotype of mice for MxCre (420 bp) and Rac1$^{fl/fl}$ (280 bp). As control DNA of wild type mice was used (Rac1, 200 bp).

PCR buffer (10x)	5 µL
$MgCl_2$ (10x)	5 µL
dNTPs	2 µL
Forward primer (10 pmol)	2 µL
Reverse primer (10 pmol)	2 µL
DNA (200µg/ml)	2 µl
Taq polymerase	0.5 µL
H_2O	ad 50 µL

2.2.1 MxCre (420 bp)

Forward primer 5´-AAC ATG CTT CAT CGT CGG-3´

Reverse primer 5´-TTC GGA TCA TCA GCT ACA CC-3´

PCR program

95 °C	3 min	
95 °C	30 sec	
63°C	30 sec (-1°C each cycle)	10 cycles touch down
72°C	30 sec	
95°C	30 sec	
53°C	30 sec	35 cycles
72°C	30 sec	
72°C	3 min	
4°C	pause	

2.2.2 Rac1fl/fl (280 bp)

Forward primer: 5´ -GTC TTG AGT TAC ATC TCT- 3´

Reverse primer: 5´-CTG ACG CCA ACA ACT ATGC- 3´

PCR program

95 °C	4 min	
95 °C	30 sec	
63°C	30 sec (-1°C each cycle)	10 cycles touch down
72°C	30 sec	

95°C	30 sec	35 cycles
53°C	30 sec	
72°C	30 sec	
72°C	4 min	
4°C	pause	

2.3. Washing and preparation of mouse platelets

Mice were bled under ether anesthesia from the retroorbital plexus. Blood was collected into a tube containg ACD buffer or 20 U/mL heparin in TBS, pH 7.3 (both 300 µL). Blood was centrifuged at 1800 rpm (^ 290.3 g) for 5 min. Supernatant was taken and centrifuged at 800 rpm for 6 min at RT to obtain platelet rich plasma (prp). To wash platelets, prp was centrifuged at 2800 rpm (^ 702.46 g) for 5 min in the presence of prostacyclin (PGI_2) (0.1 µg/mL) and the pellet was resuspended in Tyrode's buffer containing PGI_2 (0.1 µg/mL) and apyrase (0.02 U/mL) and left to incubate at 37 °C for 5 min. After a second centrifugation step, platelets were resuspended in the same buffer and incubated at 37°C for 5 min. Platelets were finally centrifuged as above, resuspended in Tyrode's buffer containing apyrase (0.02 U/mL) (500 µL) and left to incubate for at least 30 min at 37 °C before analysis.

2.4. Washing and preparation of human platelets

Blood was taken (1 vol) in ACD (5 vol), mixed gently and centrifuged at 1200 rpm (ˆ 260 g) for 15 min (without brake). Platelet rich plasma (prp was carefully taken off and transferred into a fresh tube. To 15-25 ml prp 1 ml of ACD and 0,1 U/ml apyrase were added, mixed and sample centrifuged at 2200 rpm (ˆ 870 g) for 15 min. Platelet poor plasma was poured off by turning the tube upside-down on a tissue in one movement. Pellet was resupended in 1 ml Hepes buffer, and depending on the original volume of prp, 10-20 ml additional Hepes were added. To 15 ml solution 1ml ACD and 0.1 U/ml apyrase were added and the sample again centrifuged at 870 g for 15 min. Subsequently, the pellet was resuspended in 1 ml Hepes, platelets were counted and adjusted to the appropriate concentration with Tyrode´s buffer.

2.5. Platelet count

For determination of platelet counts, blood (20 µL) was obtained from the retroorbital plexus of anesthetized mice using siliconized microcapillaries and immediately diluted 1:100 in Unopette kits (Becton Dickinson, Heidelberg, Germany). The diluted blood sample was allowed to settle for 20 minutes in an Improved Neubauer haemocytometer (Superior, Bad

Mergentheim, Germany), and platelets were counted under a phase contrast microscope at 400x magnification.

2.6. Platelet surface biotinylation

To biotinylate platelet surface molecules, washed platelets were suspended in biotinylation buffer at a concentration of 2 x10^9 platelets/mL. EZ-link sulfo-NHS-LC-biotin (3 mg/mL in biotinylation buffer) was added at a final concentration of 25 µg/mL and left to incubate for 10 min with rotation. Reaction was stopped by addition of 100 µL of 1M NH_4Cl solution and platelets were washed twice in PBS/EDTA. To check the efficacy of the biotinylation, a sample (1:20 dil. in PBS; 50 µL) was incubated with FITC-labeled streptavidin (1.5 µg/mL; 10 min, RT), reaction was stopped by the addition of 500 µL PBS, and samples were analyzed immediately by flow cytometry.

2.7. Immunoprecipitation

For immunoprecipitation, biotinylated platelets (1x10^9) were solubilized in 1 mL of IP buffer containing Igpal (1 %) by incubation at RT for 10 min, followed by centrifugation at 20000 g for 10 min. Supernatants were then incubated with 10 µg of the required antibody (supernatant from 1x10^9 platelets is sufficient for 3 different antibodies) for 30 min at 4 °C with rotation, followed by addition of 25 µL G-sepharose (washed 3x in IP buffer). Samples were left to incubate overnight at 4 °C with rotation. Samples were then washed once with IP buffer containing 1 % Igpal and twice with IP buffer (20000 g, 1 min), solubilized in 50 µl 2x SDS sample buffer and boiled for 5 min at 95°C. Before loading onto the gel, samples were centrifuged (20000 g, 1 min,) and transferred into new eppis.

2.8. Immunoblotting

For Western blot analysis, platelets were washed three times in PBS/EDTA and finally solubilized in 100 µL IP buffer and lysed with 1% Igpal. Samples were separated by 12 % SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. To prevent non-specific antibody binding, membrane was incubated in 10 % fat-free milk or 5 % BSA (dissolved in PBS) for 1 h at RT. After that, membrane was incubated with the required antibody (5 µg/mL) for 1 h at RT. For washing, the membrane was incubated three times with washing buffer for 10 min at RT under agitation. After washing, HRP-labeled secondary reagent was added and left to incubate for 1 h at RT under agitation. After three washing steps, proteins were visualized by ECL.

2.9. Platelet activation measurement with flow cytometry

Washed platelets (1 x 10^6), or whole blood (50 µl) were activated with the indicated agonists or reagents (10 min, RT), stained for 15 min with saturating amounts of fluorophore-conjugated antibodies, reaction was stopped by addition of 500 µL PBS, and sample was immediately analyzed on a FACScalibur. For a two-color staining, the following settings were used:

Detectors/Amps:

Parameter	Detector	Voltage
P1	FSC	E01
P2	SSC	380
P3	Fl1	650
P4	Fl2	580
P5	Fl3	150

Threshold:

Value	Parameter
253	FSC-H
52	SSC-H
52	FL1-H
52	FL2-H
52	FL3-H

Compensation:

Fl1	2.4 % of FL2
Fl2	7.0 % of FL1
Fl2	0 % of FL3
Fl3	0 % of FL2

2.10. Aggregometry

To determine platelet aggregation, light transmission was measured using washed platelets (for thrombin) or heparinized prp (for all other agonists) adjusted to a platelet concentration of 3 x 10^8 platelets/mL with Tyrode's buffer. Agonists or reagents were added as 100-fold concentrates and light transmission was recorded over 5 min on an Apact 4-channel optical aggregation system. Prior to activation with thrombin 500 µg/ml exogenous fibrinogen was added to the suspension of washed platelets. Before starting the measurements, Tyrode's buffer (for washed platelets) or plasma (for prp) was set as 100 % aggregation and washed platelet suspension (for washed platelets) or prp (for prp) was set as 0 % aggregation.

2.11. Mitochondrial damage

Washed platelets resuspended at a concentration of ~1.5 x 10^9 platelets/mL in Tyrode's buffer containing PGI_2 (0.1 µg/mL) were treated for 1 h at 37 °C with 100 µM CCCP (dissolved in H_2O) in the presence or absence of the broad spectrum metalloproteinase inhibitor GM6001 (100 µM; dissolved in DMSO). At the end of the incubation period, platelets were washed once (centrifuged at 702.46 g for 5 min) and resuspended in Tyrode's buffer.

2.12. Platelet treatment with recombinant ADAM17

Platelets were washed twice in Tyrode´s buffer in the presence of prostacyclin (0.1 µg/mL) and apyrase (0.02 U/mL) and finally resuspended in the absence of prostacyclin and apyrase to a concentration of 0,5 x10^9 platelets/mL. Recombinant ADAM17 (dissolved at a concentration of 100 µg/ml in 25 mM Tris, pH 9.0 containing 2.5 µM $ZnCl_2$ and 0.005 % Brij according to the manufacturer's instructions) was added to the platelet suspension at the indicated concentrations and incubated for 1h at 37°C. Subsequently, the cells were analyzed by flow cytometry.

2.13. ELISA

To detect cleaved GPV molecule with the ELISA system, platelets were treated with the indicated activators and the supernatants (from 300 µl of 0.5 x 10^9 platelets) were cleared by 10 min centrifugation at 15.000 g. For αIIbβ3 blocking: JON/A (50 µg/mL) was added 3 min prior to addition of the activators. Supernatants were transferred onto αGPV (DOM1, 20 µg/ml) coated ELISA plates, incubated for 1h at 37 °C and washed three times with washing buffer (1 x PBS, 0.1 % Tween). Secondary antibody (DOM2-HRP) was added at a concentration of 2.5 µg/ml followed by 1 h incubation at 37 °C and detected with TMB. The reaction was stopped with 0.5 M sulforic acid and analyzed by WinRead.

2.14. Adhesion under flow conditions

Mouse blood (1 vol) was collected into 20 U/mL heparin and diluted 2:1 in Tyrode´s buffer pH 7.4. Coverslips (24 x 60 mm) were coated with fibrillar (Horm) collagen (0.2 mg/mL) , or human plaque material, and blocked for 1 h with 1% bovine serum albumin. Perfusion studies were performed as follows. Transparent flow chambers with a slit depth of 50 µm, equipped with the coated coverslips, were connected to a syringe filled with the anticoagulated blood. Perfusion was performed using a pulse-free pump under high shear stress equivalent to a wall shear rate of 1000 s^{-1} (4 min), or at a shear rate of 150 s^{-1} (10 min). Thereafter, chambers were rinsed by a 10 min perfusion with Tyrode´s buffer at the same shear stress and phase-contrast images were recorded from at least five different microscope fields (40x objectives). Analysis was performed using MetaVue ® software.

For PKC inhibition studies, PPACK-anticoagulated human and mouse blood was used for perfusion experiments of collagen coated surfaces. Prior to perfusion, blood was incubated for 15 min with indicated inhibitors (RO318425 or GF109203X) and/or fluorescent probes (i.e. Annexin A5 648). After perfusion, chamber was rinsed with Hepes buffer pH 7.45 containing 2 mM $CaCl_2$ and 1 U/mL heparin and fluorescently labeled annexin A5 (0.5 µg/mL), brightfield phase-contrast images and non-confocal fluorescence images of adherent platelets were recorded using a two camera system (165).

Platelet surface coverage was analyzed with ImagePro software (Media Cybernetics) for phase-contrast images and Quanticell software (Visitech) for fluorescence images. At least 10 different microscopic fields were averaged per experiment (no image processing). To provide a measure of the proportion of procoagulant platelets independent of platelet deposition, the ratio of annexin A5-binding surface coverage to phase-contrast surface coverage was calculated and was termed procoagulant index (Pi) (165). Although the procoagulant area was slightly overestimated through fluorescent glare in the optics, Pi provided a means of distinguishing the treatments of procoagulant expression from those of platelet deposition and aggregation.

For single-cell Ca^{2+} measurements under flow, blood was supplemented with 5 % autologous Fluo-3-loaded platelets, and processed as described (166).

2.15. Measurement of PKC activity

PKC activity of platelets in buffer, plasma or blood was determined by measuring Ser phosphorylation of the modified PKC/PKA pseudo-substrate, RFARKG-S-LRQKNV (36), using a biotinylated mAb recognizing this phosphorylated form (Calbiochem). Washed platelets, prp or whole blood, were treated with RO31 or GF10 (20 µM), and then stimulated as appropriate, e.g. with PMA (100 nM). Samples of 1×10^8 cells were centrifuged in a small volume of ice-cold PBS, immediately sonicated on ice, and then further processed as indicated by the manufacturer. When required, prp was isolated from blood prior to assaying.

2.16. Measurement of $[Ca^{2+}]_i$ release in suspension

Changes in cytosolic $[Ca^{2+}]_i$ were measured in washed suspensions of Fura-2-loaded platelets (2×10^8/ml) by ratio fluorometry, as described (167). Final suspensions did not contain aspirin; activations were carried out in the presence of 1 mM $CaCl_2$. Control calibrations were performed when colored substances were present.

2.17. Measurement of Ca^{2+} release by flow cytometry

Prp was prepared from wild type and Rac1-deficient mice and centrifuged once at 1400 g for 5 min in the presence of 5 mM EGTA. Pellet, received from one mouse, was resuspended in

500 µl Ca^{2+} free tyrode´s and platelets were loaded for 10 min with 5 µM fluo4-AM. After incubation, 10 µl of the platelet solution were diluted in 500 µl Ca^{2+} free tyrodes, previously added into FACS tubes, activated with the given concentrations of agonists and immediately analyzed on FACScalibur. The increase in mean fluorescence FL1 was measured every 20 seconds over 2 min (CRP and thrombin) or over 5 min (rhodocytin).

2.18. Spreading Experiments

Cover slips were coated in a humid chamber over night at 37 °C with 1 mg/ml fibrinogen, 50 µg/ml collagen or 100 µg/ml CRP and blocked at the following day for 1h with 1% BSA. Washed platelets of Rac1-deficient and wild type mice were resuspended to a concentration of 0.5 x 10^6 platelets/ml and then further diluted 1:10 in Tyrodes buffer. To each sample 10 µM indomethacin and 2 U/ml apyrase was added and platelets were seeded on the previously coated and blocked cover slips. Prior to spreading on fibrinogen, platelets were activated with 1 U/ml thrombin. Platelets were allowed to spread for 25 min on fibrinogen and CRP and for 40 min on collagen. The reaction was stopped by adding 3,7 % formaldehyde. The coverslips were washed three times with PBS and then mounted onto the microscope and visualized using DIC and a Cool Snap Camera (Visitron Systems, Puchheim, Germany). At least 10 pictures of each sample were taken and analyzed with MetaVue® software (Visitron Systems, Puchheim, Germany).

2.19. Actin staining of spread platelets with alexa 488-phalloidin

Platelets were fixed on cover slips with 3,7 % formaldehyde for 10 min at room temperature and afterwards washed three times with PBS. The cells were permeabilized with 0,1% Triton X-100 in PBS for 30 min and again washed three times with PBS. To prevent nonspecific backround staining, samples were incubated with 1% BSA in PBS for 30 min prior to staining. To avoid evaporation samples were kept all the time in a humide chamber. For staining, 200 µl of sterile PBS containing 2 µl Alexa Fluor® phalloidin were added to the samples and incubated for 30 min. Prior to analysis with a fluorescence microscope samples were washed several times with PBS. Photos were taken using a Digital Camera from Canon (Tokio, Japan)

2.20. Two-Photon Laser Scanning Microscopy

For two-photon laser scanning microscopy (TPLSM), coverslips with thrombi were observed with a Bio-Rad 2100 multiphoton system (168). Excitation was achieved by a Spectra-Physics Tsunami Ti:Sapphire laser, tuned and mode-locked at 800 nm, producing pulses of 100 fs wide (repetition rate 82 MHz). Excitation at 647 nm was performed by a parallel-placed red diode laser. Fluorescence was detected using appropriate wavelength filters (29).

Thrombi in flow chambers, double labeled with OG488 fibrinogen and AF647-annexin A5, were scanned at the end of perfusions. Optical sections were recorded in Kalman filtering mode; no further image processing was performed.

2.21. Thrombin Generation

prp and platelet-free plasma (pfp) collected on citrate were used to measure thrombin generation using the thrombogram method (169). Briefly, normalized prp (1.5×10^8 platelets/mL) or pfp was preincubated with inhibitor for 15 min and then with collagen for 10 min. Samples of prp or pfp (4 vol.) were added to wells of a 96-well plate (Immulon 2HB; Dynex Technologies), containing 1 vol. of 20 mM Hepes, 140 mM NaCl, 5 mg/mL BSA and tissue factor (1 pM, final concentartion, f.c.). Coagulation was started by addition of 1 vol. thrombin substrate, Z-GGR-AMC. Pre-warmed plates were inserted into a Fluoroskan Ascent well-plate reader (Thermolab Systems) and processed at 37 °C. Fluorescence accumulation from cleaved AMC was measured, and first derivative curves of accumulation of fluorescence were generated; calibrations were performed with human thrombin (170).

2.23. Intravital microscopy of thrombus formation in the carotid artery

Mice were anesthetized and polyethylene catheters (Portex, Hythe, England) were implanted into the right jugular vein. Fluorescently labelled platelets (200 x 10^6/250 µL) of the same genetic background were infused intravenously. The right common carotid artery was dissected free and ligated vigorously near the carotid bifurcation for 30 seconds using a surgical filament to induce vascular injury. Prior to and following vascular injury, the fluorescent platelets were visualized in situ by *in vivo* video microscopy of the right common carotid artery using a Zeiss Axiotech microscope (x 20 water immersion objective; W x 20/0.5; Zeiss, Göttingen, Germany) with a 100-W mercury short arc photo optic lamp (HBO) for epi-illumination. Platelet adhesion was recorded for 5 minutes after the induction of injury and the videotaped images were evaluated using a computer-assisted image analysis program (Visitron, Munich, Germany). The number of adherent platelets was assessed by counting the cells that did not move or detach from the vascular wall for at least 10 seconds. In each mouse, 3 nonoverlapping fields (size, 100 µm x 100 µm) were analyzed for 30 seconds (2.5 - 3.0 minutes after injury) in a slow-motion modus. Clusters of 2 or more platelets were defined as microaggregates. The total number of adherent platelets or microaggregates at t = 3 minutes was calculated by the following formula that reflects concave shape of the vessel wall: vessel diameter (µm) x π (circle constant) x 2 x $\sin^{-1}$ (amplitude of measured area, in µm) x length of measured area (µm) and is presented per mm^2. All experiments performed on animals were approved by the German legislation on protection of animals.

2.24. Aorta occulsion model

The abdominal cavity of anesthetized mice was opened with a longitudinal incision, and the abdominal aorta was exposed. An ultrasonic flow probe was placed around the aorta, and the blood flow was measured for at least one minute. Endothelial denudation was induced by a single firm compression of the vessel with a forceps upstream of the flow probe. Blood flow was then monitored for 45 minutes or until complete occlusion occurred, the blood flow was equal to zero. After vessel occlusion, the flow probe was observed for another 40 minutes. If the occlusive thrombus was recanalized and blood flow was re-established, this was considered as embolisation.

C) Results

C 1. GPVI downregulation in murine platelets through metalloproteinase-dependent shedding

Recently it was reported by Bergmeier et al. that induction of mitochondrial damage induces various changes in platelets which are similar to those observed during in vitro storage. These changes include the rearrangement of the cytoskeleton, low levels of Phosphatidylserine (PS) exposure and P-selectin expression and, interestingly, profound shedding of GPIbα releasing a 130 kDa fragment, Glycocalicin (GC) into the supernatant (171). In collaboration with W. Bergmeier et al. from Harvard Medical School Boston, and T. Rabie from our group, the study was followed up to further characterize the effect of mitochondrial damage on platelet function and receptor regulation (172). The results obtained from this collaboration are summarized and discussed here:

To induce mitochondrial damage platelets were treated for 1h with carbonyl cyande m-chlorophenyl-hydrazone (CCCP), which compromises mitochondrial function by uncoupling the oxidative phosphorylation. After CCCP-treatment, GPVI was undetectable on platelets suggesting that the receptor had been cleared from the surface of these cells. Similarly, GPIbα levels were reduced by more than 95 %, as noted previously (171) whereas surface levels of other receptors, including β1- and β3-integrins, CD9, and GPIX were unchanged or slightly increased, probably due to translocation of these glycoproteins from internal pools to the surface membrane (Fig. 7A).

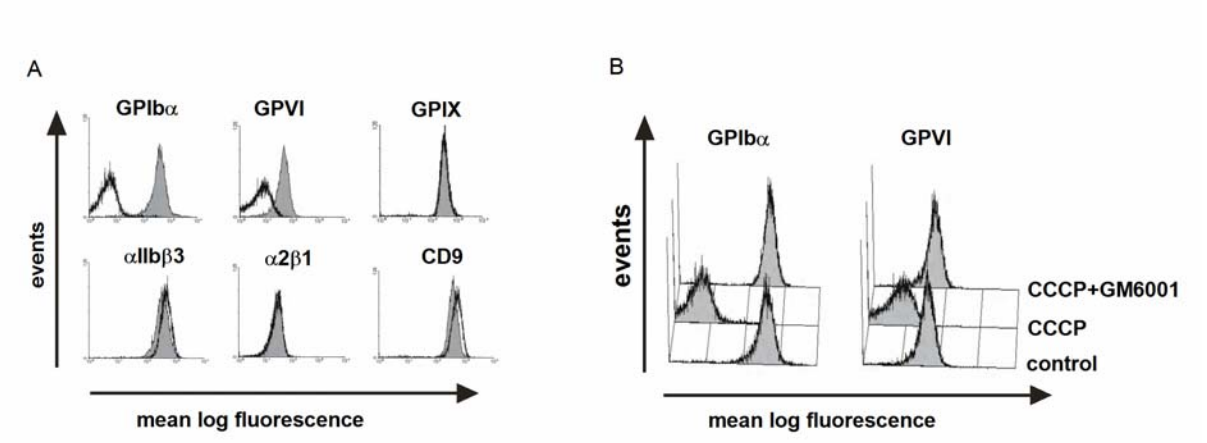


Fig. 7: GPVI is cleared from the platelet surface by metalloproteinase-dependent mechanisms. Flow cytometric analysis of glycoprotein expression. **(A)** Washed platelets were incubated in the absence (shaded area) or presence (black line) of CCCP for 1 h, stained with the indicated fluorophore-labeled antibodies for 15 min, and analyzed directly. **(B)** Washed platelets were incubated with CCCP for 1 h in the presence or absence of the broad range metalloproteinase inhibitor GM6001, stained with the indicated fluorophore-labeled antibodies for 15 min, and analyzed directly. (A, B) The data shown are representative of 6 individual experiments.

To test a possible involvement of platelet metalloproteinases in GPVI down-regulation, as it is was reported for GPIbα down-regulation (76), washed platelets were incubated with CCCP in the presence or absence of a broad range metalloproteinase inhibitor, GM6001. As shown in Fig. 7B, GM6001 blocked the down-regulation of both GPIbα and GPVI. Thus, GPVI can be efficiently cleared from the platelet surface by a metalloproteinase-dependent mechanism. In contrast to GPVI, the cellular regulation of GPIbα has been intensively studied and it is known that the receptor can be down-regulated also in response to agonist-induced platelet activation, in addition to CCCP treatment, by internalization of the GPIb-IX complex and/or proteolysis of GPIbα (173-175).

In present study it was tested if this is also true for GPVI. Interestingly, GPVI levels were unaffected by platelet activation with various agonists. Immunoprecipitation experiments confirmed the presence of cleaved GPIbα, but not GPVI, in the supernatant of thrombin- or CRP-activated platelets (Fig. 8). Only upon treatment with CCCP, GPVI was cleaved from the platelet surface, as detected by immunoblots with a 63 kDa fragment .

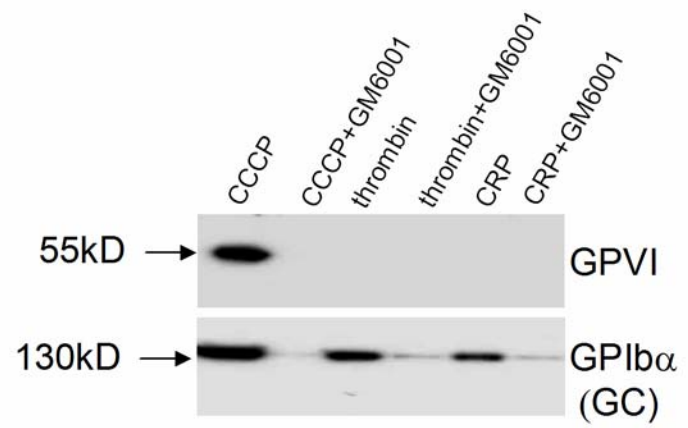


Fig. 8: Agonist-induced platelet activation leads to metalloproteinase-dependent shedding of GPIbα, but not GPVI. Washed and biotinylated platelets were left untreated or stimulated with CRP (5 µg/ml) or thrombin (0.3 U/ml) in the presence or absence of GM6001. Subsequently, GPVI or GPIbα were precipitated from the supernatant of the cells. Precipitates were separated by SDS-PAGE under reducing conditions, blotted onto a PVDF membrane and visualized using streptavidin-HRP and ECL. The data shown are representative of 3 identical experiments.

Together, these findings show that stimulation of the GPVI/FcRγ pathway or G-protein signaling is not sufficient to trigger GPVI-down-regulation and that this is distinct from GPIb down-regulation which occurs after platelet stimulation with CRP or thtrombin.

In summary, it is shown here that GPVI can be efficiently cleared from the platelet surface by proteolytic cleavage under conditions of metalloproteinase activation as seen in a model of mitochondrial injury.

C 2. Evidence for a Role of ADAM17 (TACE) in the Regulation of Platelet Glycoprotein V

As discussed above, surface receptors underlie defined regulatory mechanisms, that are responsible for receptor clearance from the cell surface. In most cases, shedding of ectodomains is mediated by membrane-anchored metalloproteinases (reviewed in 176-178). Calmodulin has been shown to play a role in shedding of L-selectin from leukocytes (179) a process that is mediated by the metalloproteinase ADAM17.

In platelets, it was reported that GPIbα is cleaved from the platelet surface upon mitochondrial injury which also induces mechanisms comparable to apoptotic events in nucleated cell (76). Similar, GPVI-downregulation as a consequence of mitochondrial damage is described here (see above, section C 1.). Both mechanism occur in a metalloproteinase dependent manner. However, different signaling pathways seem to be induced and/or different proteases seem to be involved in these processes (77). GPV is a member of the GPIb-V-IX complex and is known to be cleaved directly by the protease thrombin upon platelet activation with thrombin. Other mechanism for GPV downregulation have not yet been described and investigated. However, regarding the tightly controlled

metalloproteinase dependent shedding mechanisms that exist for GPIbα and GPVI on platelets and the potential role of GPV as negative modulator for platelet activation. It was likely that other regulatory mechanism- similar to these observed for GPIbα and GPVI- than thrombin cleavage- might control the number of GPV molecules on the platelet surface. Therefore in the following project the regulation of GPV was studied, the signals inducing GPV shedding were characterized, and the protease responsible for the process was identified.

2.1. Platelet activation induces metalloproteinase dependent shedding of GPV

To explore the initial stimuli underlying GPV regulation, human platelets were activated with phorbolester-myristate-acetate (PMA), ADP or collagen-related peptide (CRP) and surface levels of GPV were determined by flow cytometry. CRP activates platelets through the GPVI/FcRγ-chain complex (180), the major platelet collagen receptor (50) ADP signals through GPCRs and PMA, a DAG analogue that directly activates PKC. As a positive control, platelets were incubated with thrombin which directly cleaves the receptor, releasing a 69 kDa fragment, GPVf1. Monitoring GPV suface levels in flow cytometry, it was shown that GPV was downregulated (that is absent from the platelet surface) in response to PMA to a similar extent as in the thrombin control, whereas a weaker effect was observed in response to CRP. In contrast, ADP, which is only a weak platelet agonist, did not induce significant downregulation of GPV (Fig. 9A).

To investigate whether the decrease in GPV surface expression is accompanied by the cleavage or internalization of the entire GPIb-IX-V complex from the cell surface, the surface levels of GPIbβ were determined as this subunit is not cleaved upon platelet activation. GPIbβ surface levels were only slightly decreased or unchanged in PMA- or CRP-stimulated platelets, respectively, indicating that downregulation of GPV was rather the effect of shedding than internalization (Fig. 9B). PMA- or CRP-induced shedding of GPV occurred independently of thrombin activity as it was unaltered in the presence of the thrombin inhibitor, hirudin (not shown). In contrast, the broad spectrum metalloproteinase inhibitor GM6001 markedly blocked GPV shedding in response to PMA and CRP, but not thrombin (Fig. 9C).

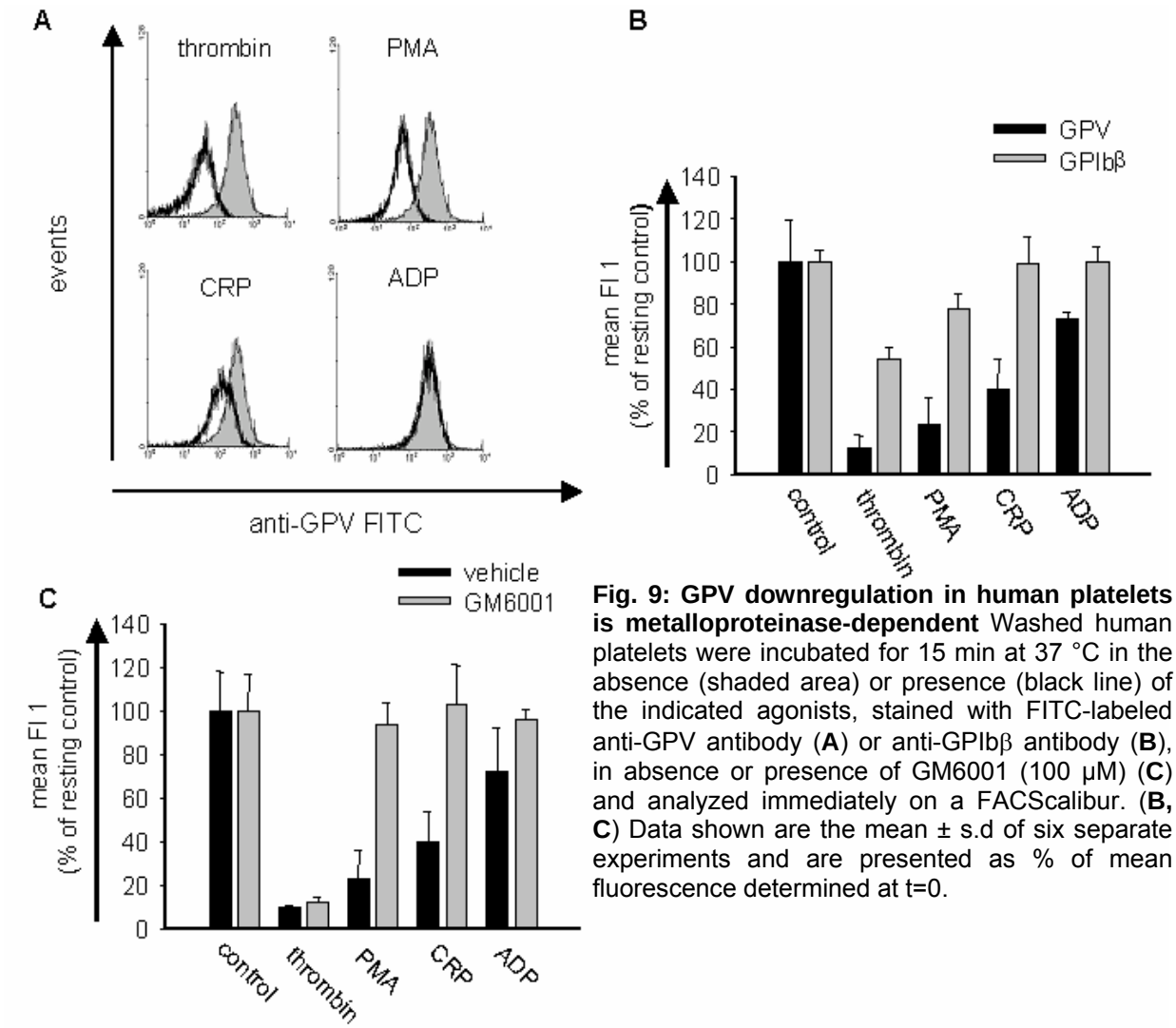


Fig. 9: GPV downregulation in human platelets is metalloproteinase-dependent Washed human platelets were incubated for 15 min at 37 °C in the absence (shaded area) or presence (black line) of the indicated agonists, stained with FITC-labeled anti-GPV antibody (**A**) or anti-GPIbβ antibody (**B**), in absence or presence of GM6001 (100 µM) (**C**) and analyzed immediately on a FACScalibur. (**B, C**) Data shown are the mean ± s.d of six separate experiments and are presented as % of mean fluorescence determined at t=0.

2.2. Metalloproteinase dependent GPV cleavage is distinct from thrombin mediated GPV proteolysis

Similar results as described above were obtained with mouse platelets; here, GPIX levels were monitored to assess whether the whole GPIb-V-IX complex is internalized. GPIX like GPIbβ is known in the GPIb-V-IX complex as not to be cleaved upon platelet activation. Thus GPIX levels were measured to distinguish between downregulation of the whole receptor complex or of single GPV molecules. GPIX levels remained virtually unchanged under all conditions, confirming that GPV shedding is not accompanied by downregulation of the entire GPIb-V-IX complex from the cell surface (Fig. 10A). Like in human platelets, GM6001 almost completely inhibited GPV shedding in response to PMA or CRP, but not thrombin (Fig. 10B). Immunoprecipitation experiments demonstrated that the metalloproteinase-generated fragment of GPV has a MW of ~82 kDa and thereby differs significantly from GPVf1 (~69 kDa) released by thrombin (Fig. 10C). As a control, immunoprecipitations were also performed with an anti-GPIIIa antibody which did not yield a band under any condition (Fig. 10.C lower panel).

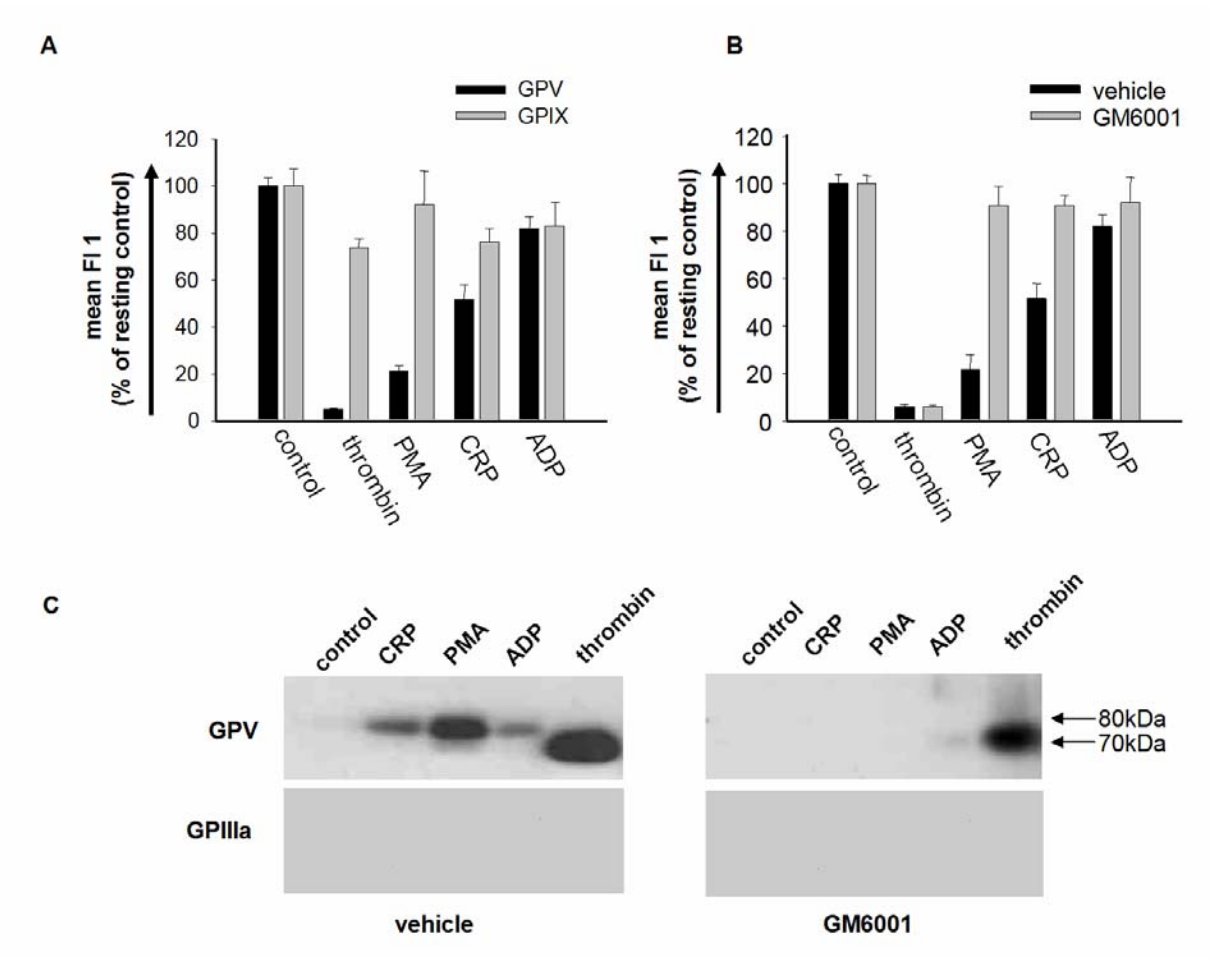


Fig. 10: Metalloproteinase- and thrombin–mediated GPV cleavage lead to the generation of different soluble GPV variants Washed mouse platelets were incubated with the indicated agonists for 15 min at 37 °C, stained with FITC-labeled anti-GPV or anti-GPIX antibody **(A)** in the presence or absence of GM6001 **(B)**, and analyzed immediately on a FACScalibur. The data shown are the mean ± s.d of six separate experiments and are presented as % of mean fluorescence determined at t=0. **(C)** Surface-biotinylated mouse platelets were incubated with the indicated agonists for 15 min at 37 °C in the absence or presence of GM6001 (100 µM). Subsequently, GPV and GPIIIa were immunoprecipitated from the supernatants of the cells. Immunoprecipitates were separated by SDS-PAGE under reducing conditions, blotted onto a PVDF membrane and visualized using streptavidin-HRP and ECL. The data shown are representative of 3 identical experiments.

2.3. The metalloproteinase ADAM17 is present in mouse and human platelets and responsible for GPV cleavage

The strong effect of PMA on GPV shedding leads to test a possible role of ADAM17 in this process, as this sheddase is known to be potently induced by PKC activators. Therefore, first platelets were tested for the expression of ADAM17 by western blot analysis. As shown in Fig. 11A, ADAM17 (~130 kDa) was specifically detectable in whole cell lysates of both human and mouse platelets. Next, the effect of the two potent ADAM17 inhibitors, GW 280264X (181;182) and TAPI-2 (183;184) on GPV downregulation was examined. Both compounds inhibited PMA- and CRP- induced GPV shedding in human and mouse platelets (Fig. 11B left and right), suggesting that ADAM17 mediates ectodomain shedding of GPV. To

test this directly, platelets were incubated with different concentrations of recombinant human ADAM17 ectodomain (rhADAM17) and GPV levels were determined by flow cytometry. Indeed, rhADAM17 dose-dependently downregulated GPV (Fig. 11C), but not GPIX or GPIIb/IIIa (not shown) from the platelet surface and this effect was abrogated in the presence of GM6001. Taken together, these results demonstrated that ADAM17 is expressed in platelets and strongly indicates that it may be the major sheddase that cleaves GPV.

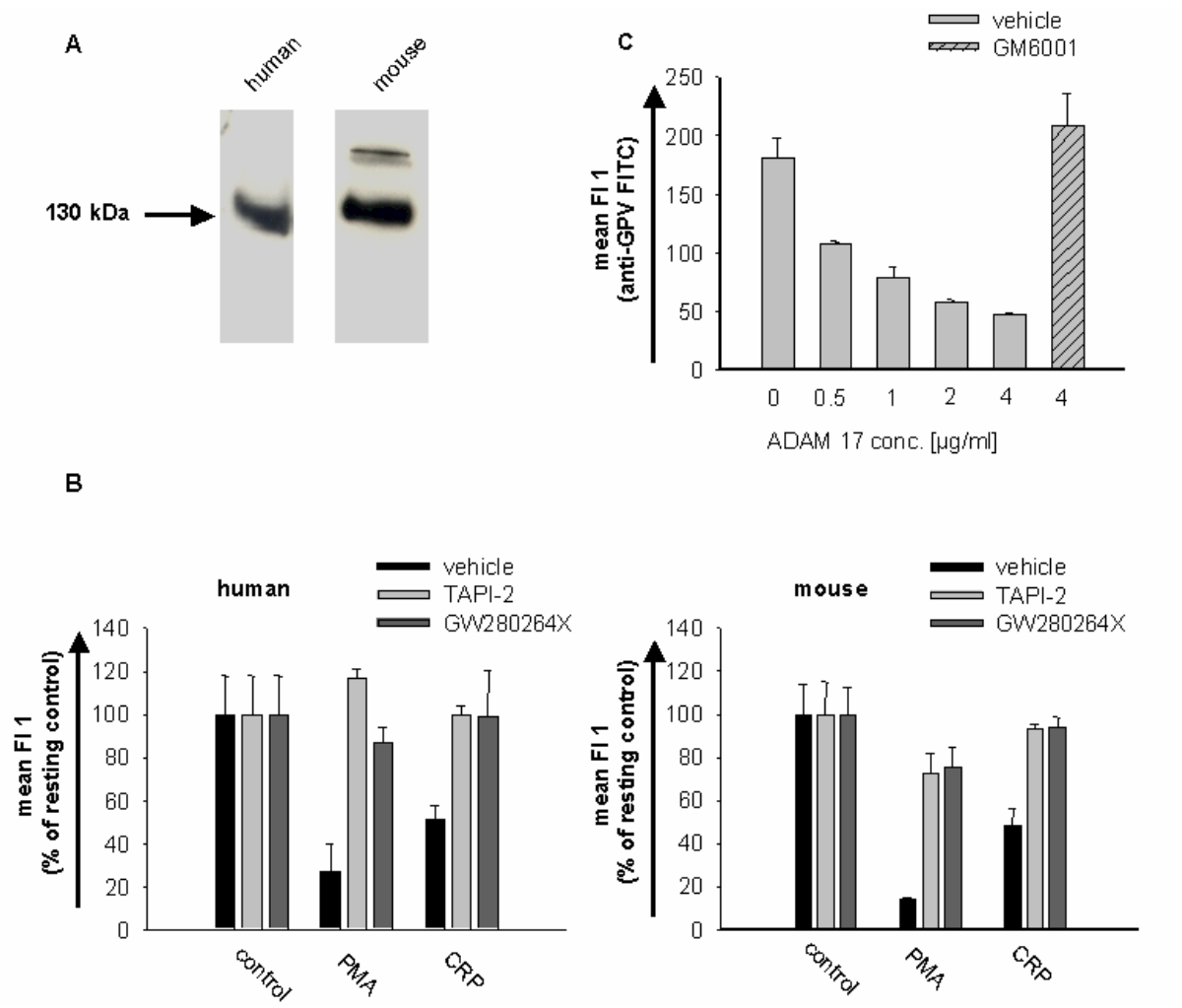


Fig. 11: Inhibition of ADAM17 blocks GPV shedding from the platelet surface (A) Whole platelet proteins were separated by denaturating SDS-PAGE and immunoblotted with anti-ADAM17 antibodies. **(B)** Human (left) and mouse (right) platelets were activated in the presence or absence of TAPI-2 (100 µM) or GW280264X (10 µM), stained with FITC-labeled anti-GPV antibody and analyzed immediately on a FACScalibur. Data shown are the mean ± s.d of six separate experiments and are presented as % of mean fluorescence determined at t=0. (**C**) Washed mouse platelets were incubated with the indicated concentrations of recombinant human ADAM17 for 1 h at 37 °C, stained with FITC-labeled anti-GPV antibody and analyzed immediately on a FACScalibur. Where indicated, the experiment was performed in the presence of GM6001 (100 µM). Data shown are the mean ± s.d of three separate experiments.

2.4. Calmodulin is involved in ADAM17 mediated GPV shedding

Besides GPV, a number of other receptors, including L-selectin on leukocytes (185) and GPVI on platelets (186) are known to be associated intracellularily with calmodulin and to

undergo rapid ectodomain shedding upon treatment with calmodulin inhibitors such as W13 (187;188). In order to examine the implication of calmodulin in ADAM17-dependent GPV shedding, platelets were incubated with vehicle or the calmodulin inhibitor W13 and GPV surface levels were determined at different time points. As shown in Figure 12A, GPV was rapidly downregulated in the presence, but not in the absence of W13 in both mouse and human platelets. Similar results were obtained with a second calmodulin inhibitor, W7 (not shown). As with CRP- or PMA-stimulated platelets, W13-induced GPV shedding was inhibited in the presence of GM6001, GW 280264X, or TAPI-2 (Fig. 12A and not shown). Moreover, immunoprecipitation experiments showed the accumulation of the ~82 kDa variant of soluble GPV in the supernatant of W13-treated platelets (Fig. 12B). Together, these results indicate that calmodulin is a negative regulator of ADAM17-mediated GPV cleavage.

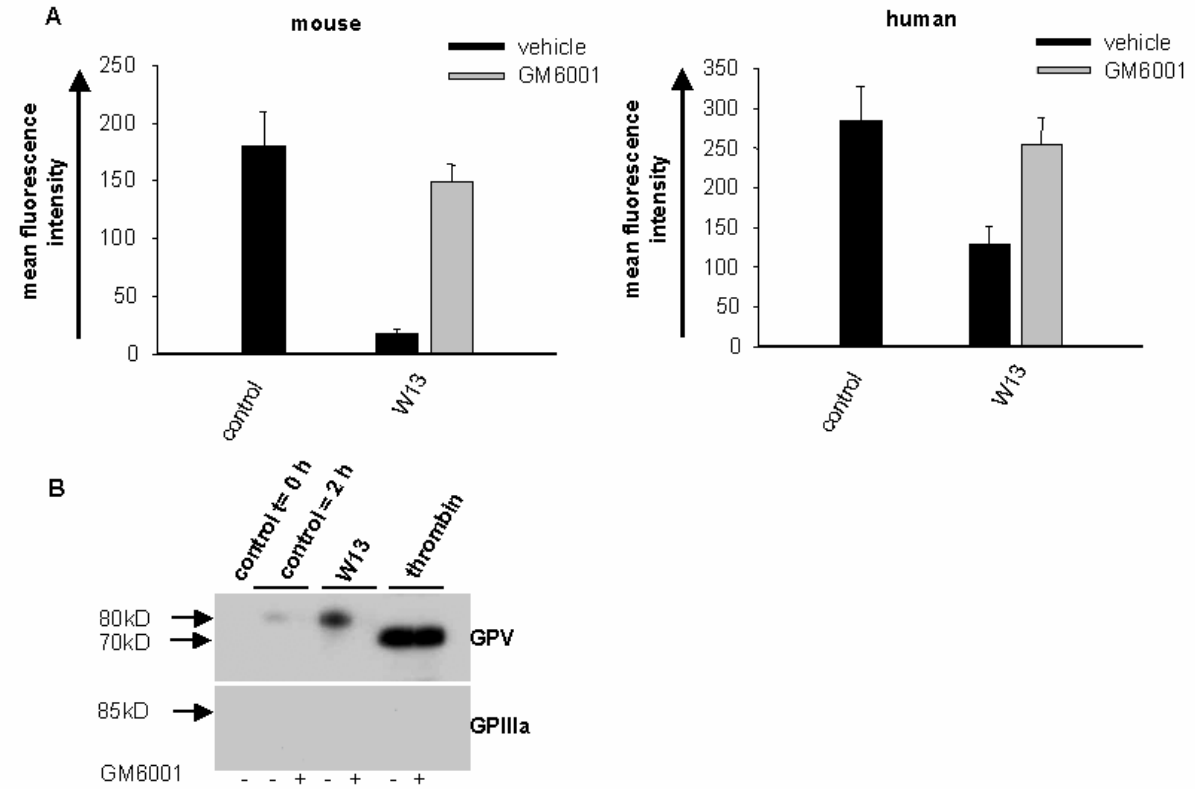


Fig. 12: Inhibition of calmodulin induces metalloproteinase-dependent shedding of GPV in mouse and human platelets. (A) Washed human and mouse platelets were incubated, in the presence or absence of GM6001, with W13 (200 µM) for 2 h at 37°C, stained with FITC-labeled anti-GPV antibody and analyzed immediately on a FACScalibur. The data shown are the mean ± s.d of six separate experiments. **(B)** Surface-biotinylated mouse platelets were incubated with W13 (200 µM) for 2 h at 37 °C in presence or absence of GM6001 (100 µM). Subsequently, GPV and GPIIIa were immunoprecipitated from the supernatants of the cells. Immunoprecipitates were separated by SDS PAGE under reducing conditions, blotted onto a PVDF membrane and visualized using streptavidin-HRP and ECL. The data shown are representative of 3 identical experiments.

2.5. GPV cleavage is independent of integrin αIIbβ3 signaling

In addition to GPV, a number of other platelet membrane glycoproteins can undergo ectodomain shedding on the surface of activated platelets, including P-selectin, CD40-L.

Shedding of CD40-L has been reported to be largely dependent on activated GPIIb/IIIa, suggesting that outside-in signaling through the integrin regulates the proteolytic process (189;190). In contrast, GPVI shedding occurs completely independent of GPIIb/IIIa signaling (186). To test the involvement of GPIIb/IIIa outside-in signaling in ADAM17-mediated GPV shedding, mouse platelets were stimulated with PMA, CRP, or thrombin under stirring conditions in the presence or absence of a blocking antibody against GPIIb/IIIa (JON/A, 50 µg/ml, ref. (191)). Cleaved GPV was quantified in the supernatant with a newly established ELISA system. While JON/A blocked platelet aggregation (Fig. 13A), it had no significant effect on GPV shedding in response to PMA, thrombin (Fig. 13B), or CRP (not shown), demonstrating that this process occurs independently of outside-in signaling through GPIIb/IIIa.

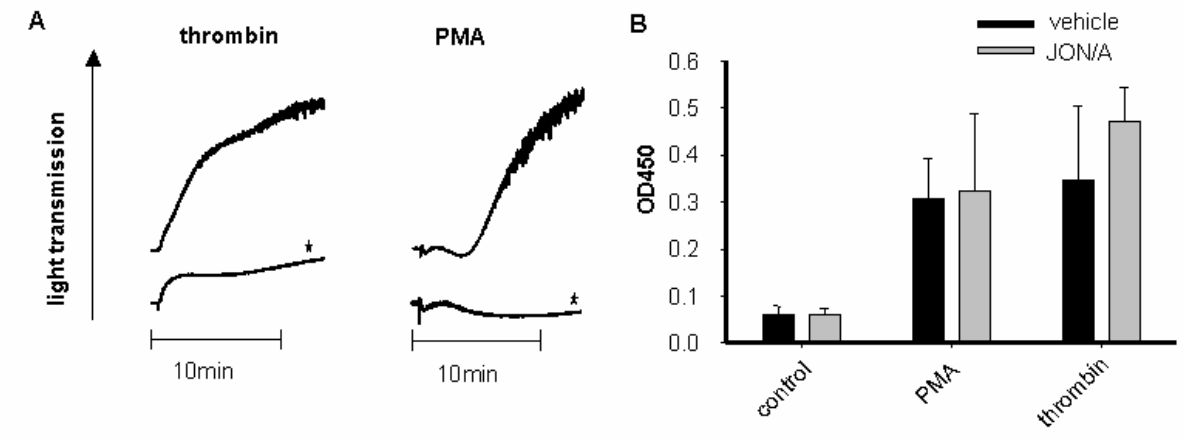


Fig. 13: GPV shedding occurs independently of GPIIbIIIa outside-in signaling Washed mouse platelets (thrombin) or heparinized prp (PMA) were incubated with the indicated activators in the absence or presence (*) of JON/A (50 µg/ml) and **(A)** light transmission was recorded on a Fibrintimer 4 channel aggregometer. **(B)** GPV levels in the supernatants were determined by ELISA as described in Experimental Procedures. Results shown are the mean ± s.d of six individual experiments.

C 3. Dual Role for Protein Kinase C in thrombus formation: stimulation of proaggregatory and suppression of procoagulant activity of platelets

Protein kinase C is known to become activated by DAG, downstream of PLCγ and PLCβ, which in turn get activated through GPVI/FcRγ and GPCR mediated signaling pathways respectively.

The contribution of PKC to platelet activation, aggregation and procoagulant response via these pathways is controversial as activatory effects in respect to α granule release and αIIbβ3 integrin activation as well as inhibitory effects of PKC concerning Ca^{2+} mobilization were observed (125;126). In a recently published study by Imke Munnix, Maastricht University, NL (data not shown), a key role for GPVI in platelet procoagulant activity along with its platelet proaggregatory effect was reported (65). In respect to this and to the previous

works, a study was carried out in collaboration with Johan Heemskerk, Maastricht University, NL, to investigate the role of PKC in both the platelet procoagulant response and platelet proaggregatory activity.

3.1. RO318425 and GF109203X efficiently inhibit PKC in washed platelets and whole blood

To investigate the regulation of thrombus formation by PKC, first tested several compounds with known selective PKC-inhibiting effect, particularly of α and β isoforms, on protein phosphorylation and platelet responses were tested. These compounds were added to platelets in plasma or in whole blood to establish their efficacy at physiological conditions. Dose-response experiments with several indolylmaleimide derivatives indicated that only RO318425 (referred to as RO31) and GF109203X (referred to as GF10) were capable to completely abolish PMA-induced aggregation in human PRP at concentrations ≤20 µM (Fig. 14). Inhibition of PKC was directly tested in platelets by measuring the Ser phosphorylation of the Ala→Ser modified PKC pseudo-substrate, RFARKG-S-LRQKNV added to the platelet lysate (192). When RO31 and GF10 were added to PRP at a concentration of 20 µM, the Ser phosphorylation was inhibited with 97±2 % and 93±3 % efficiency, respectively (not shown) (mean ± SEM, n=3). Addition of the compounds to whole blood gave a similar degree of inhibition, while in washed platelets 10 µM RO31 and GF10 blocked the phosphorylation with >99% and 93±1% (not shown). Both agents react with the ATP binding site of PKC, inhibiting the classical isoforms $\alpha/\beta_I/\beta_{II}/\gamma$ with an intracellular IC_{50} of about 10 nM (193). The relatively high concentration needed to block pseudo-substrate phosphorylation and PMA-induced aggregation in the presence of plasma or blood is due to the abundant presence of lipophilic substances and proteins in plasma.

3.2. Partial blockage of platelet aggregation by PKC inhibition

Platelet aggregation was studied in response to key agonists involved in thrombus formation: collagen activating GPVI, and ADP acting via the G protein-coupled receptors $P2Y_1$ and $P2Y_{12}$. Both RO31 and GF10 suppressed (with collagen) or abrogated (with ADP) aggregation of washed human platelets (Fig. 14A), but they only delayed the aggregation in response to collagen in PRP, while aggregation in response to ADP was unaffected (Fig. 14B). Both compounds suppressed aggregation of washed platelets with about 70%, when stimulated by the GPVI agonists convulxin and collagen-related peptide or by thrombin receptor-activating peptide. Aggregation of washed platelets was also prevented with ADP scavenging apyrase. Accordingly, the contribution of PKC isoforms to aggregation is partly indirect, that is via the secretion of ADP and fibrinogen, possibly through GPVI mediated signaling.

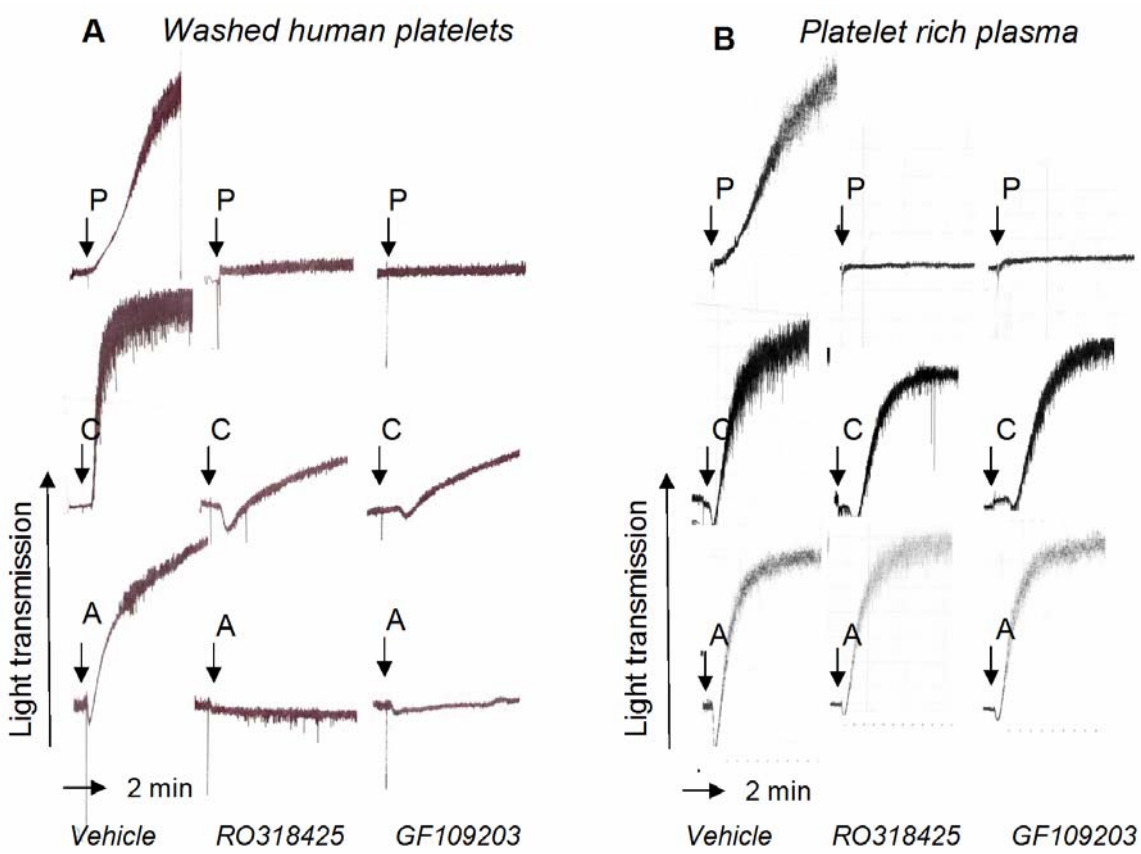


Fig. 14: PKC differentially contributes to aggregation of washed platelets and platelets in plasma. Human washed platelets **(A)** or prp **(B)** were activated with 100 nM PMA, P, 5 µg/ml collagen, C, or 10 µM ADP, A. Platelets were pretreated for 10 min with vehicle (DMSO), 20 µM RO31 or GF10. Traces of changes in optical transmission are representative for 3 or more experiments.

3.3. Platelet PKC contributes to αIIbβ3 activation and is required for secretion

Effects of PKC inhibition were measured on integrin αIIbβ3 activation and α-granule secretion by flow cytometry. Inhibitor-treated human platelets were stimulated with PMA, convulxin[1] or ADP. Activated αIIbβ3 was quantified with fluorescently labeled PAC1 mAb (194). In the presence of plasma, both RO31 and GF10 fully antagonized PMA-induced PAC1 binding (Fig. 15A, upper panel), confirming that PKC-induced integrin activation was completely inhibited. In contrast, they only partly reverted PAC1 binding in response to convulxin, and did not affect PAC1 binding with ADP (Fig. 15A, lower panels). The same results were obtained when washed platelets were stimulated with PMA, convulxin or ADP: RO31 reduced the PAC1 binding with 95±3, 63±6 or 5±4 % efficiency, respectively (mean ± SEM, n=3). These results extend earlier evidence for two pathways of integrin activation (120;195), only one of which seems to be PKC dependent.

Measure of P-selectin expression at the platelet surface with FITC labeled antibody was used to monitor α granule secretion (exocytosis). Both RO31 and GF10 completely abolished the high P-selectin expression induced by PMA or convulxin, and also the lesser P-selectin

[1] Collagen fibers were not used to prevent interference in the flow cytometric measurements.

expression with ADP, in either PRP (Fig. 15B) or washed platelets (not shown). That PKC-dependent secretion of fibrinogen is needed for aggregation of washed platelets was confirmed by the finding that addition of fibrinogen to washed, RO31-treated platelets fully restored the collagen- and ADP-induced aggregation responses (not shown).

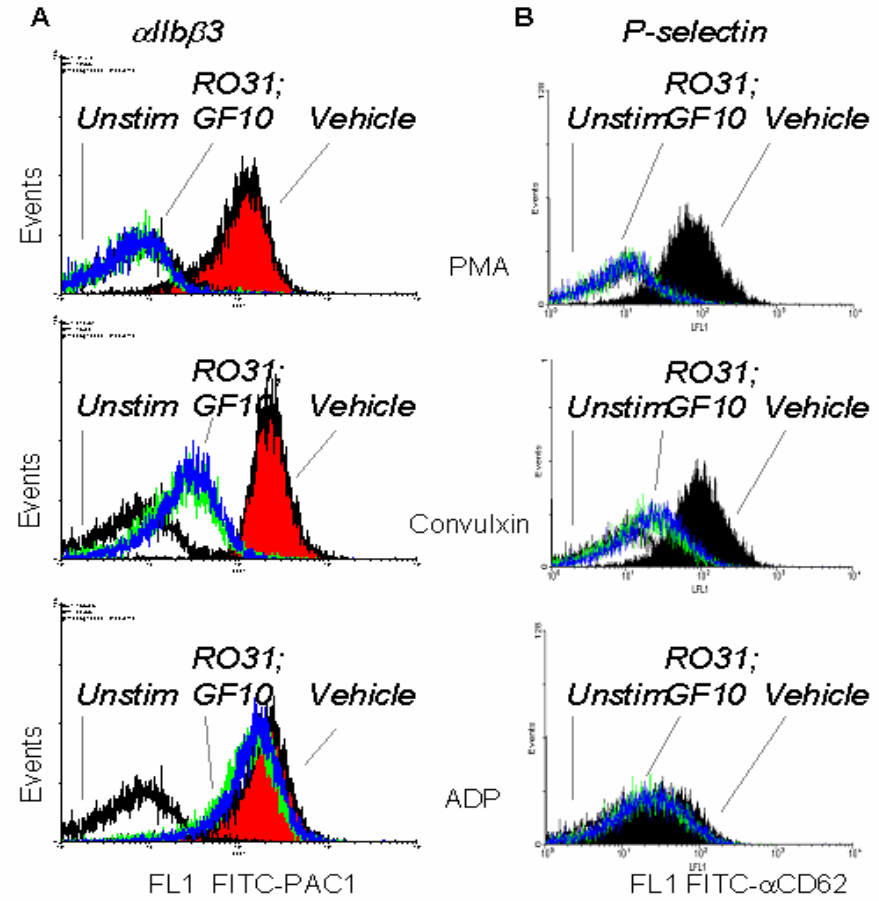


Fig. 15: PKC contributes to integrin activation and is required for secretion. Human prp was pretreated for 10 min with vehicle, or 20 µM RO31 or GF10. Platelets were then left unstimulated (unstim) or stimulated by 100 nM PMA, 50 ng/ml convulxin or 10 µM ADP. Measurement by flow cytometry of **(A)** integrin αIIbβ3 activation using FITC-labeled PAC-1 mAb, which recognizes the activated integrin, and **(B)** secretion using FITC labeled anti-CD62 (anti P-selectin) mAb, determining surface expression of P-selectin. Representative histograms are given of 5000 events (n=3-4).

3.4. Platelet PKC down-regulates Ca^{2+} and procoagulant responses

Calcium signal generation via GPVI and Gqα-coupled receptors is essential for platelet responses such as secretion, thromboxane formation and procoagulant activity (26). The effects of PKC antagonism on Ca^{2+} responses was monitored in washed, Fura-2-loaded platelets. Interestingly, platelet treatment with RO31 or GF10 substantially increased the Ca^{2+} signals by all tested agonists: ADP, convulxin or thrombin (Fig. 16A). Conversely, platelet treatment with PKC-activating PMA nearly completely (with ADP or thrombin) or substantially (with convulxin) blocked the Ca^{2+} responses. Time integrals of agonist-induced $[Ca^{2+}]_i$ increases were calculated to determine the Ca^{2+}-mobilizing potency of these agonists (167). Treatment with RO31 resulted in a 2-6 fold increase of Ca^{2+}-mobilization, while GF10

resulted in 2-4 fold increase (Fig. 16B).

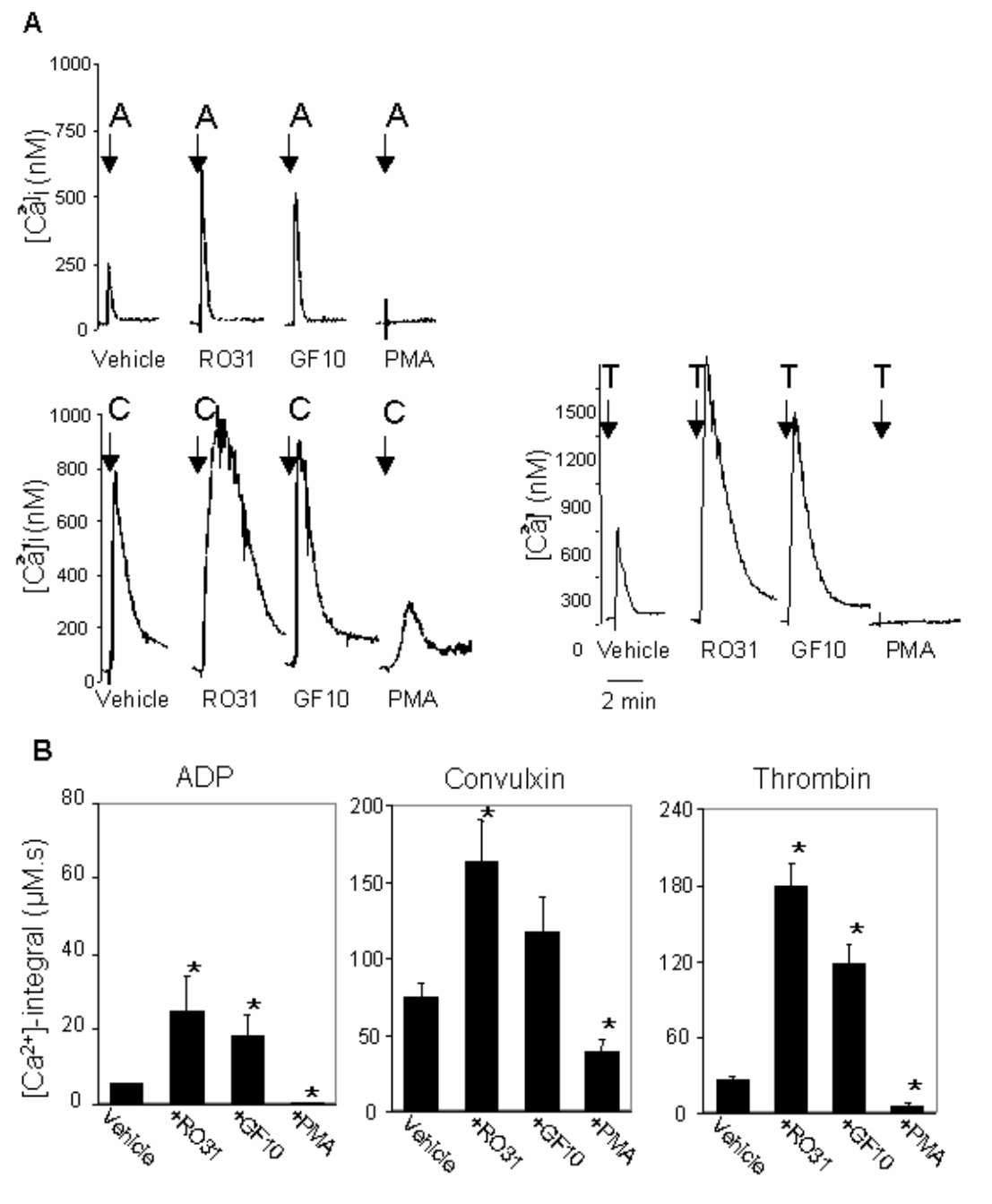


Fig. 16: PKC suppresses agonist-induced Ca^{2+} signaling. (A, B) Fura-2-loaded platelets were treated with DMSO vehicle, 20 µM RO31 or GF10, or 100 nM PMA for 10 min. Platelets were subsequently stimulated with 10 µM ADP, 20 ng/ml Convulxin or 2 nM Thrombin. Representative traces of changes in cytosolic Ca^{2+} concentration **(A)** and averaged time-$[Ca^{2+}]_i$ integrals of Ca^{2+} responses **(B)**. Mean values ± SEM (n=3-5), * $p < 0.05$.

The function of PKC in platelet procoagulant activity was established by measuring thrombin generation in PRP that was triggered with tissue factor and $CaCl_2$. In plasma thrombin is formed at the surface of activated platelets with high $[Ca^{2+}]_i$, exposing procoagulant PS (34). Both GF10 and RO31 greatly enhanced the thrombin generation process in PRP, and even enhanced the collagen-evoked (GPVI-mediated) increase in thrombin generation (Fig. 17A).

Control experiments indicated that neither RO31 nor GF10 influenced thrombin generation, if platelet activation was inhibited with cAMP-elevating iloprost (see Fig. 17B). The compounds did not interfere with the coagulation process itself when platelets were absent as was indicated by control experiments, when the measurement was performed in platelet pour plasma (not shown). Together, these results point to a clear suppressive effect of PKC on platelet Ca^{2+} signaling and ensuing procoagulant response.

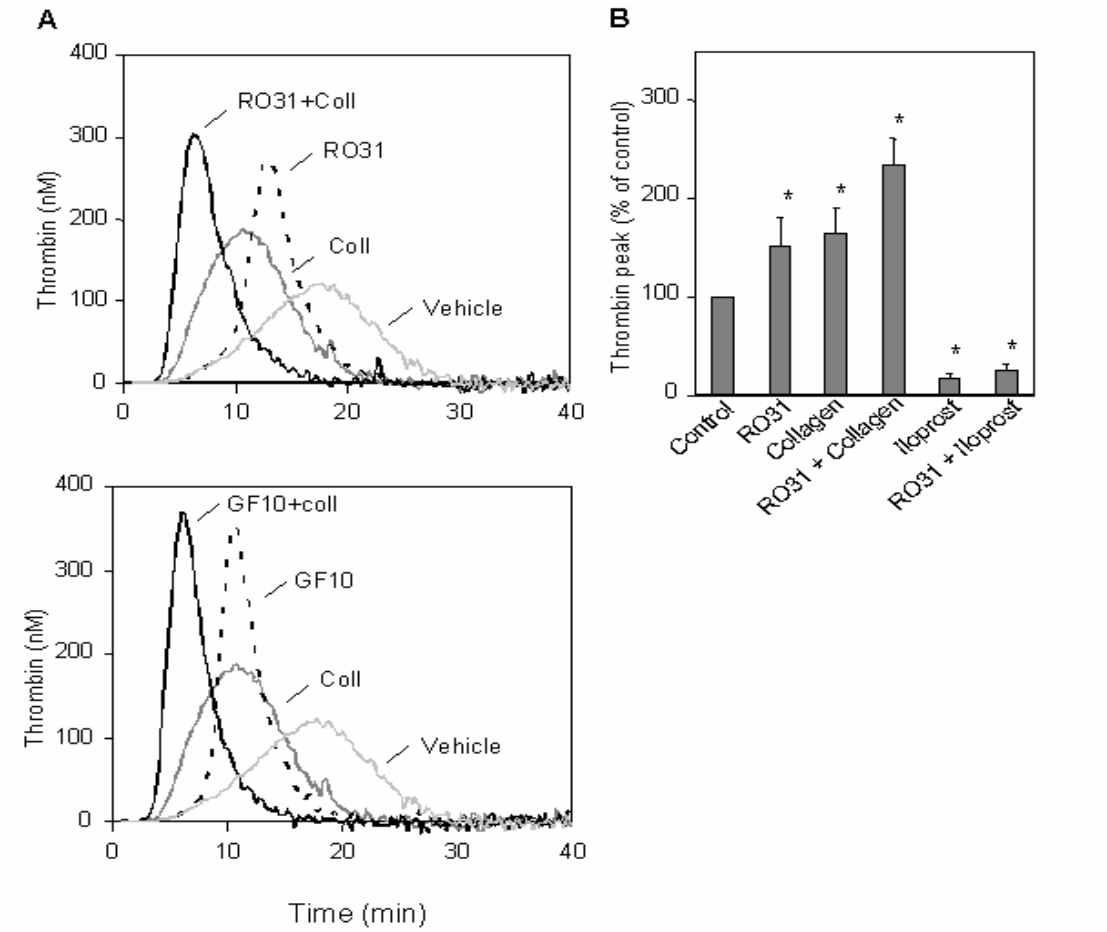


Fig. 17: PKC suppresses tissue factor-induced thrombin generation in PRP. Human PRP was incubated with vehicle or 20 µM RO31 or GF10, and then activated with 10 µg/ml collagen, as indicated. Coagulation in prp was initiated with 1 pM tissue factor and 16.6 mM $CaCl_2$. **(A)** Curves of thrombin generation in time, representative for at least three experiments, **(B)** Quantitative effect on thrombin peak levels, presenting rate of thrombin generation. Platelets in PRP were also pretreated with 1 µM iloprost in the presence or absence of RO31, as indicated (mean ± SEM, n=6-8).

3.5. Dual effects of PKC inhibition on shear-induced thrombus formation

The proaggregatory and procoagulant functions of platelets were simultaneously monitored in flow experiments, where whole blood was perfused over a collagen coated surface. At a moderately high shear rate of 1000 s^{-1}, representing arterial shear rate, flow over collagen rapidly results in GPVI-dependent formation of platelet aggregates and exposure of procoagulant PS (48). Under this condition, pretreatment of blood with RO31 or GF10 substantially reduced platelet adhesion and nearly abolished aggregate formation (Fig. 18A). Surface area coverage of the platelets was more than halved, while coverage with PS-

expressing platelets was not reduced (Fig. 18B). To determine the relative procoagulant expression of the deposited platelets, the ratio of PS-exposing surface coverage to total surface coverage with platelets was calculated (165). The so called procoagulant index (Pi) increased significantly with RO31 and GF10 (Fig. 18B, lower panel), thus pointing to increased procoagulant activity.

To directly compare the impact of PKC inhibition on αIIbβ3 activation and PS exposure under flow conditions, perfusions were performed in the presence of OG488-labeled fibrinogen and AF647-labeled annexin A5. Two-colored images were recorded using the high sensitivity of two-photon fluorescence microscopy. Treatment with RO31 considerably reduced the fibrinogen binding to collagen-adhered platelets, while again annexin A5 binding was less affected (Fig. 18C). By addition of autologous, Fluo-3-loaded platelets to the blood (165), changes in $[Ca^{2+}]_i$ of platelets upon adhesion under flow were evaluated. Control platelets had peak-shaped rises in $[Ca^{2+}]_i$ followed by a prolonged continuous rise; treatment with RO31 resulted in Ca^{2+} responses of similar shape, but increased amplitude (Fig. 19). After 30s of adhesion, vehicle- and RO31-treated platelets showed $[Ca^{2+}]_i$ increases of 232±25 nM and 602±95 nM, respectively ($p=0.01$, $n=31-38$). Since integrin activation, aggregate formation via ADP secretion, Ca^{2+} signaling and PS exposure are all GPVI-mediated events in this flow model (49;196), these data together point to a dual effect of PKC inhibition: on the one hand, reduction in secretion and integrin activation, leading to suppressed aggregation, and, on the other hand, enhancement of Ca^{2+} release and PS exposure.

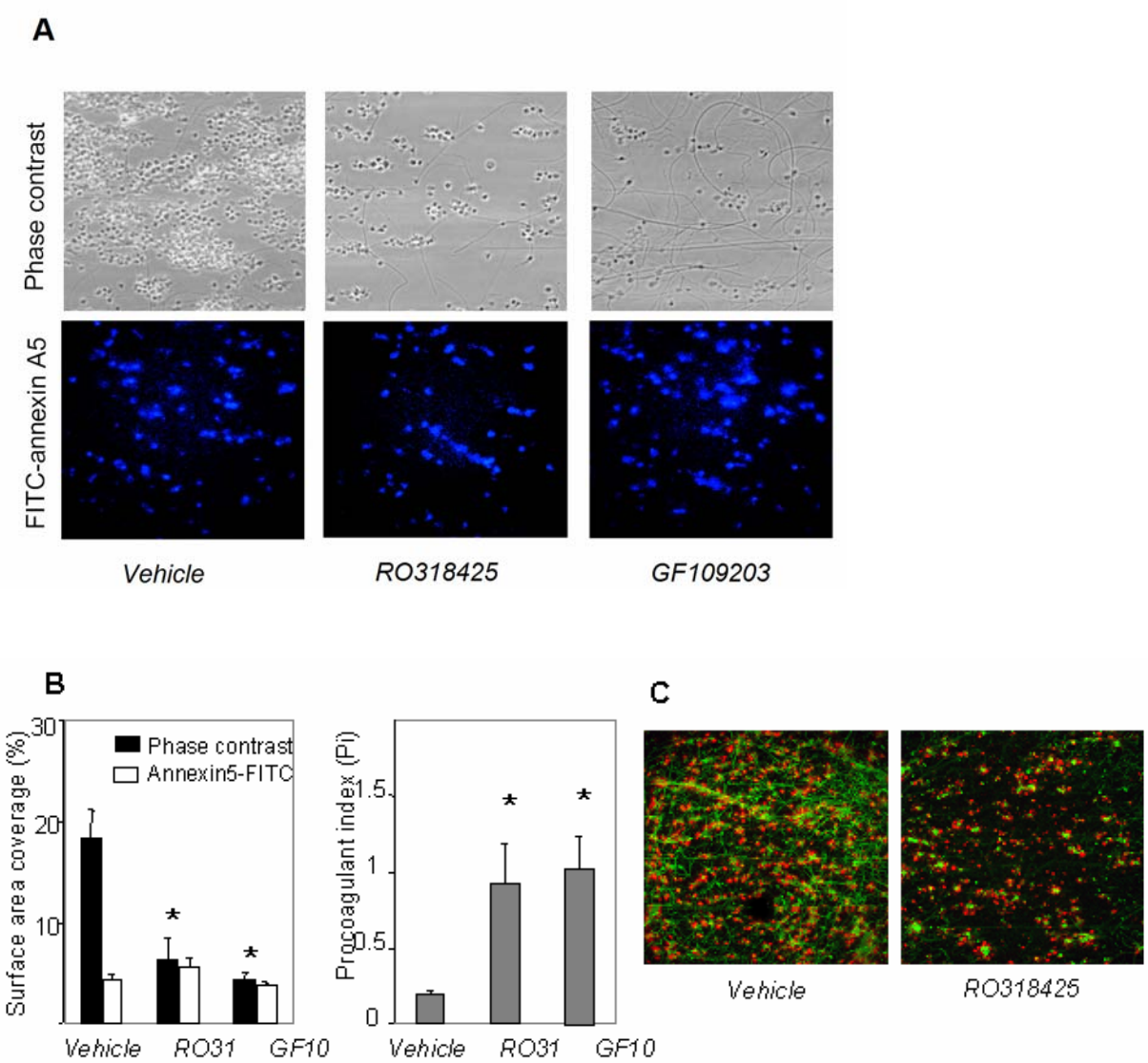


Fig. 18: PKC is required for thrombus formation and suppresses PS exposure under flow. Human, PPACK-anticoagulated blood was perfused over collagen at a shear rate of 1000 s^{-1} during 4 min, followed by staining with fluorescently labeled annexin A5 (0.5 µg/ml). Blood was pretreated with vehicle (veh), 20 µM RO31 or GF10 for 10 min. **(A)** Representative phase-contrast images (120×120 µm) and FITC-annexin A5 fluorescence images (150×150 µm). (**B)** Quantitative analysis of surface coverage with procoagulant, annexin A5-binding platelets and of total surface area coverage with platelets. The procoagulant index (Pi) was calculated per experiment as ratio of either parameter. **(C)** TPLSM images (309×309 µm) after perfusion with OG488-fibrinogen (green) and AF647-annexin A5 (red). Data are representative of 5 or more experiments with blood from different donors; data are mean ± SEM, * $p<0.05$.

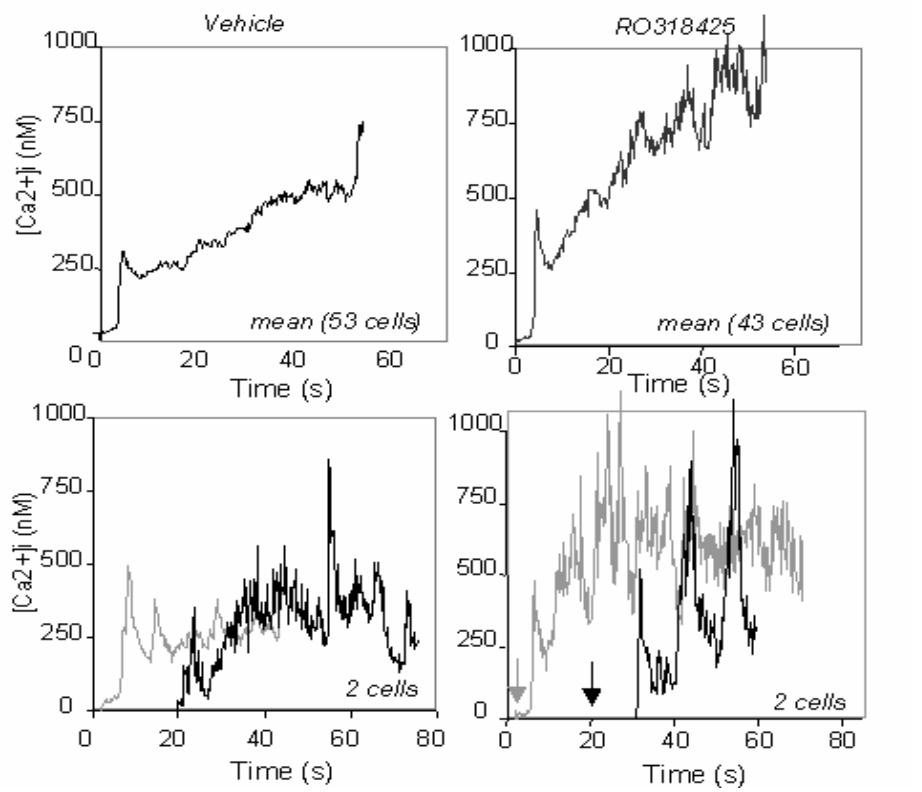


Fig. 19: PKC restricts collagen-induced Ca^{2+} responses under flow. PPACK-anticoagulated human blood was reconstituted with 5 % of autologous Fluo-3-loaded platelets, and perfused over collagen as described for Fig. 18. Blood was pretreated with DMSO vehicle or with 20 µM RO31. *Upper panels:* averaged Ca^{2+} responses from multiple adhered platelets during perfusion. *Lower panels:* representative Ca^{2+} responses from two individual platelets (grey and black line) adhering at indicated times (arrows).

3.6. Partial role of PKC in murine platelet aggregation and integrin activation

Effects of PKC inhibition were evaluated in wild-type mouse blood. With mouse PRP, it was confirmed that RO31 treatment inhibited PMA-induced pseudo-substrate phosphorylation by 89%. Both RO31 and GF10 inhibited the aggregation of washed mouse platelets induced by PMA, collagen peptide or ADP (Fig. 20A). In the presence of plasma similar to the human situation, only PMA-induced aggregation was abolished, while aggregation with other agonists was only delayed (Fig. 20B). Flow cytometric analysis was used to measure integrin activation in the presence and absence of PKC inhibitors. Activation of integrin αIIbβ3 was performed using fluorescently labeled JON/A mAb, which displays an increased binding to activated mouse αIIbβ3. Like RO31, GF10 antagonized PMA- and collagen related peptide-induced αIIbβ3 activation, but had only small effects on ADP- and thrombin-induced αIIbβ3 activation (Fig. 20C).

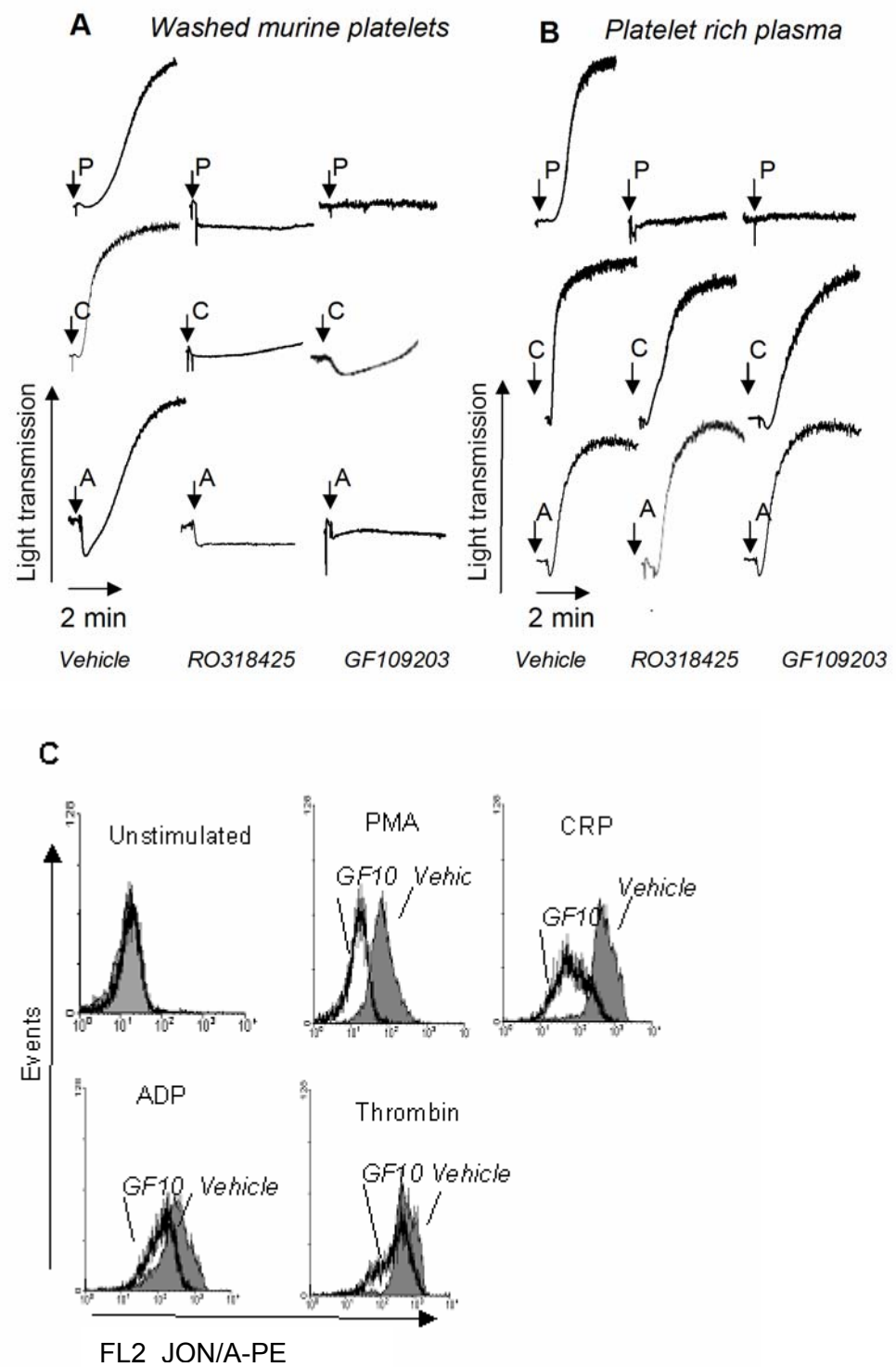


Fig. 20: Effect of PKC inhibition on aggregation and integrin activation of mouse platelets. Murine washed platelets (**A and C**) or prp **(B)** were stimulated with 100 nM PMA, 5 µg/ml CRP, 10 µM ADP or 2 nM thrombin. Platelets were pretreated for 10 min with vehicle (DMSO), 20 µM RO31 or GF10. **(A,B)** Representative aggregation traces are given (n=3-5).
(C) Flow cytometric analysis after staining with PE-labeled mAb against activated murine αIIbβ3 (JON/A). Representative histograms are given of FL1 fluorescence (n=3).

3.7. Effect of PKC inhibition on murine thrombus formation in the absence of Gq signaling

As mentioned earlier in this study, a pathway that is mediated by the second wave mediators ADP and thromboxane A2 and leads to integrin activation independent of PKC was reported (see Fig. 14 and Fig. 30) (197). To exclude the effect of this PKC independent pathway on the increased platelet procoagulant response in PKC inhibited platelets observed here, flow experiments over collagen were performed with blood from wild-type control mice and Gqα deficient mice. Gqα -protein couples to the GPCRs $P2Y_1$ and TP, whose ligands are the platelet secreted (autocrine) second wave mediators ADP and thromboxane A2 respectively. Consequently, Gqα deficient mice have abolished responses towards these second wave mediators (49;198). As a result all effects observed in this experiment are directly dependent on PKC signaling downstream of GPVI and in addition platelet aggregation should be diminished.

Flow of Gqα-deficient blood did not influence platelet adhesion to collagen, but resulted in greatly reduced thrombus formation with only small, two-layered aggregates remaining (Fig. 21A). The adhered Gqα deficient platelets still displayed a procoagulant response by exposing PS. Nevertheless, PKC inhibition with GF10 further reduced the aggregation of both wild-type and Gqα deficient platelets and, in comparison, increased the PS exposure, with as consequence a significantly increased procoagulant index Pi (Fig. 21B). Thus, even in the absence of Gqα signaling, where aggregation was suppressed, PKC still positively regulated aggregation and negatively regulated the procoagulant response.

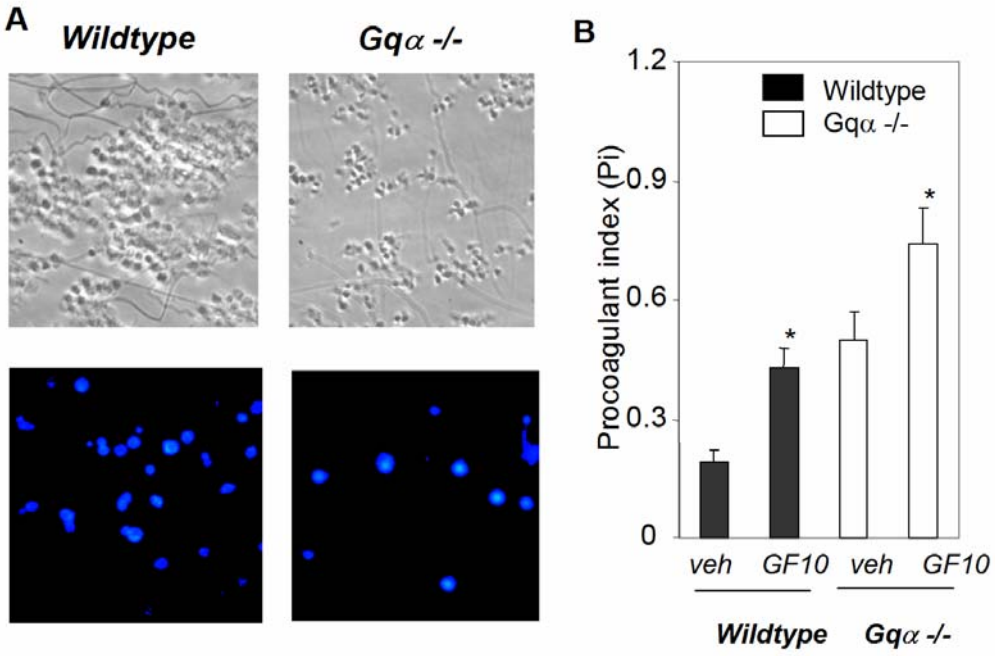


Fig. 21: Murine PKC suppresses procoagulant activity in the presence and absence of Gqα Wild-type control and Gqα deficient mouse blood was treated with vehicle or GF10 perfused over collagen, and post-stained with FITC-annexin A5, as described for Fig. 18. **(A)** Representative phase-contrast and fluorescence images of vehicle controls (120×120 µm). Numbers indicate mean surface area coverage (%). **(B)** Quantitative analysis of the procoagulant index (Pi), as a measure of the relative procoagulant activity of adhered platelets. Data are mean ± SEM (n=5-8), * $p<0.05$ compared to vehicle control.

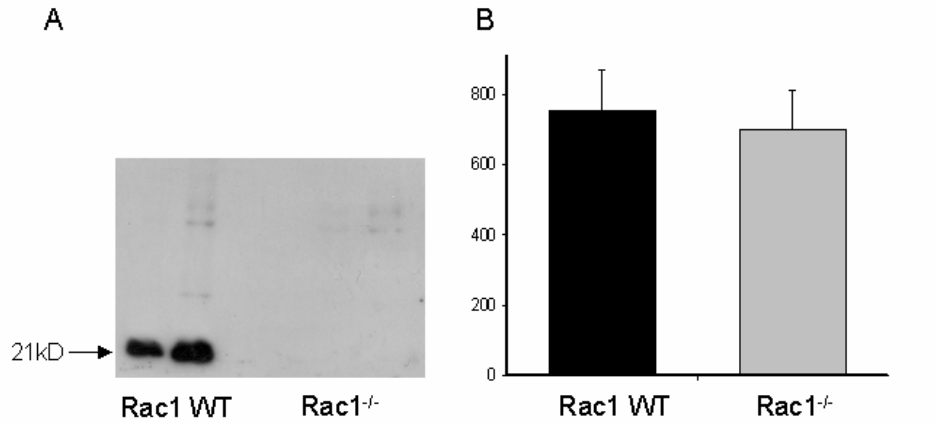
C 4. Rac1 is an effector of the GPVI signaling cascade and involved in adhesion and thrombus formation *in vivo*

Little is known about Rac1 function downstream of tyrosine kinase signaling in platelets, especially downstream of the main collagen receptor GPVI. Although several studies have demonstrated Rac1 activation in response to GPVI stimulation, this has been proposed to be dependent on the action of released G-protein coupled agonists and the functional relevance of the observation is not entirely clear (143).

In the present project, conditional Rac1-deficient mice were used to study the role of the small GTPase in platelet collagen interactions and thrombus formation *in vitro* and *in vivo*. It is demonstrated here that cellular activation downstream of GPVI is severely defective in the absence of Rac1. This defect leads to a dramatically reduced ability of the cells to form stable thrombi on collagen *in vitro* and in arterial injury models *in vivo*.

4.1. Conditional Rac1-deficient mice display normal platelet count and normal platelet glycoprotein expression

Deletion of the Rac1 gene in Rac1$^{flox/flox}$ Cre+ mice was induced by repeated injections of polyIC which induces Cre expression in the hematopoietic system and leads to efficient gene deletion in megakaryocytes and, consequently, protein deficiency in platelets (48). Rac1$^{flox/flox}$ mice not carrying Mx-Cre were used as wild-type controls (Rac1 WT). The absence of Rac1 in platelets from Rac1$^{flox/flox}$ Cre+ (furtheron referred to as Rac1-deficient or Rac1$^{-/-}$ mice) was confirmed by Western blot analysis of whole cell lysates whereas the expression level of the protein was not affected in the controls (Fig. 22A). Deletion of Rac1 had no significant effect on peripheral platelet counts or the expression of prominent surface receptors including integrins αIIbβ3 and α2β1 as well as GPIb und GPVI (Fig. 22B and C). These results demonstrated that Rac1 is not essential for megakaryocyte development and platelet production and that it can efficiently be deleted from the circulating platelet population due to the short life-time of these cells in mice, which is approximately 5 days (14).



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Mean FL1	α2	β1	GPIb	GPV	GPVI	GPIX	CD9
Rac1 WT	81 ± 32	133 ± 19	428 ± 82	192 ± 11	53 ± 7	328 ± 27	1175 ± 186
Rac1$^{-/-}$	82 ± 16	131 ± 56	442 ± 56	177 ± 13	50 ± 7	279 ± 41	1137 ± 122

Fig. 22: Rac1-deficient mice have normal platelet count and display normal levels of prominent surface glycoproteins. **(A)** Quantification of Rac1 in wild-type and Rac1-deficient platelets 10 days after the first polyIC injection. Washed platelets were lysed, proteins separated on 15 % SDS-PAGE under reducing conditions and transferred onto PVDF membrane. The membrane was blotted with mouse anti-mouse Rac1 antibdoy followed by rat anti-mouse IgG-HRP and proteins detected with ECL-solution. **(B, C)** Platelet count and glycoprotein expression of wild type and Rac1-deficient platelets. Whole blood was washed twice, resuspended in PBS and glycoproteins were detected with fluorescently labeled antibodies in flow cytometry **(C).** The amount of platelets was counted in events per second **(B)**.

4.2. Defective spreading of Rac1-deficient platelets on fibrinogen

Stimulation of G protein coupled receptors in platelets, e.g. with thrombin leads to rapid Rac1 activation followed by actin assembly (141;143). Rac1 may also be activated upon platelet binding to immobilized fibrinogen through clustering of αIIbβ3 integrins and subsequent outside-in signaling which is thought to be essential for the formation of lamellipodia and spreading (145). To determine the role of Rac1 in lamellipodia formation, Rac1-deficient and wild type platelets were washed and allowed to adhere to immobilized fibrinogen for 25 min under static conditions. It is known, that mouse platelets in contrast to human platelets do not fully spread on fibrinogen without exogenous stimulation (129). Therefore, in order to increase the efficiency of adhesion and spreading, the cells were preactivated with 1U/ml thrombin. In accordance with the proposed function of Rac1, Rac1-deficient platelets failed to form lamellipodia and to spread on the fibrinogen surface, but retained the ability to adhere and form filopodia as visualized by staining of actin with alexa fluor® phalloidin (Fig. 23). In contrast, wild-type control platelets spread normally and efficiently formed lamellipodia at their ventral side. These results confirmed findings recently reported by others (129) demonstrating that Rac1 is required for platelet spreading and efficient lamellipodia formation on surface-bound fibrinogen.

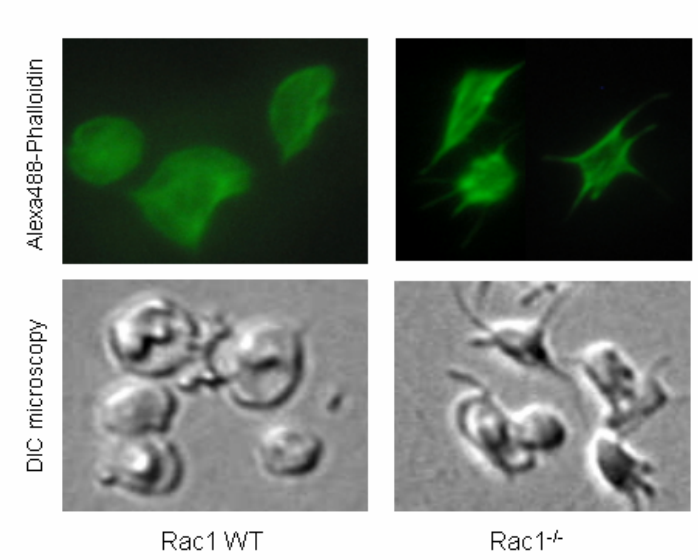

Fig. 23: Rac1-deficient platelets fail to form lamellipodia on fibrinogen coated surfaces.
Rac1 wild-type (left) and Rac1-deficient (right) platelets were washed, diluted 1:10 in tyrode´s buffer and plated onto fibrinogen (1 mg/ml) coated cover-slips. Prior to seeding platelets were activated with 1 U/ml thrombin in the presence of 2 U/ml apyrase and 10 µM indomethacin. Platelets were allowed to adhere for 25 min, then fixed with 3,7 % formaldhyde and analyzed with differential interference contrast microscopy (x100/ 1,4 oil DIC, lower panel). For staining of the actin cytoskeleton (upper panel), fixed platelets were permeabilized with Triton X-100 prior to incubation with alexa fluor® phalloidin (see section B 2.18). Samples were visualized by fluorescence microscopy (x100/ 1,4 oil DIC). Images are representative for at least three independent experiments.

4.3. Rac1-deficient platelets show diminished collagen responses

The response of Rac1-deficient platelets to different agonists was tested by standard aggregometry. In response to thrombin, ADP, and the stable TxA2 analog U46619 (not shown), Rac1-deficient platelets aggregated normally at all tested concentrations (Fig. 24A), although a slightly faster reversion of ADP induced aggregation was consistently observed with mutant platelets as compared to the wild-type controls. Very unexpectedly, however, Rac1-deficient platelets showed a clear reduction in their reactivity towards collagen which was most evident at low and intermediate concentrations of the agonist (Fig. 24B). Since platelet activation by collagen is mediated by GPVI, next the possible defect in this signaling pathway by the GPVI-specific agonist CRP was tested. Indeed, similar to the effect seen with collagen, Rac1-deficient platelets displayed a markedly reduced reactivity towards this agonist that was most evident at low and intermediate concentrations (Fig. 24B).

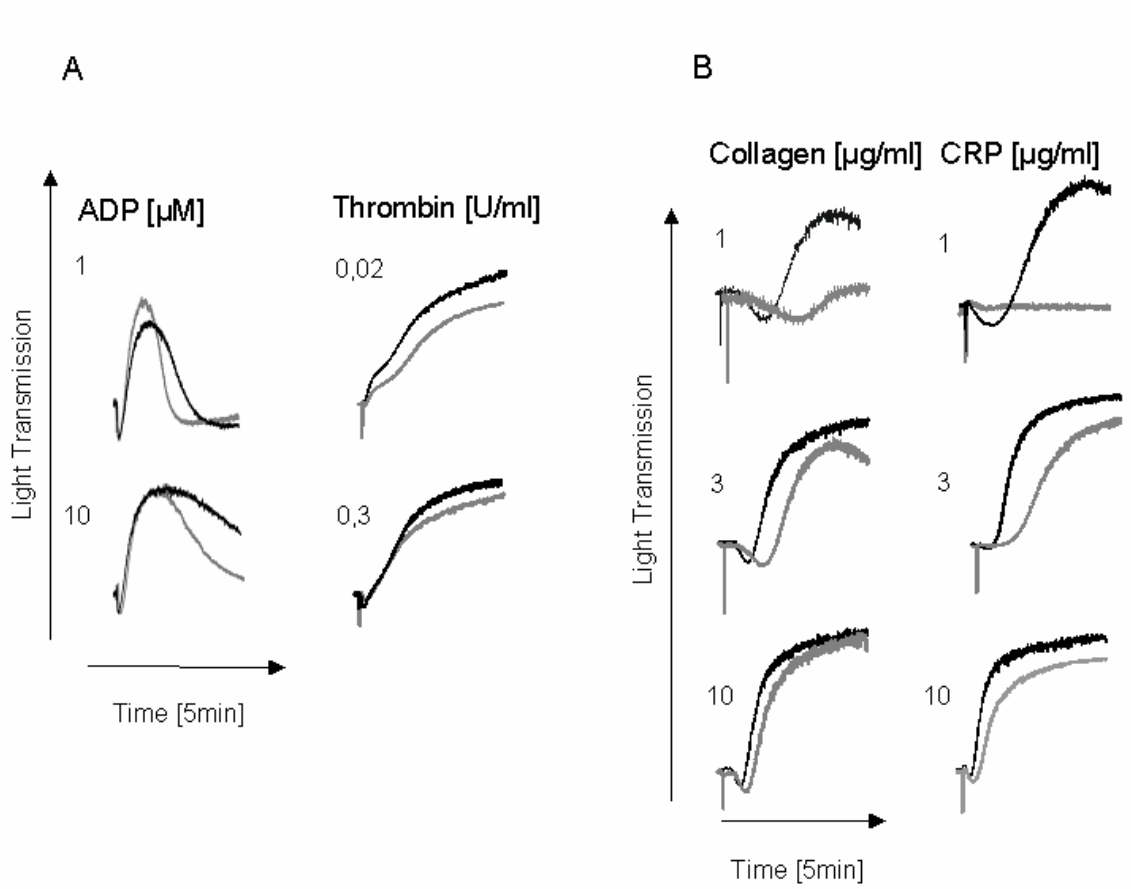

Fig. 24: Rac1-deficient platelets show impaired aggregation responses to GPVI agonists. Heparinized prp from Rac1 wild-type (black line) and Rac1 $^{-/-}$ (grey line) mice was activated with ADP, collagen and CRP and washed platelets were activated with thrombin at various concentrations. Aggregation was recorded as % of light transmission. **(A)** Representative aggregation curves of ADP- and thrombin- stimulated prp or washed platelets, respectively. In the latter case exogenous fibrinogen was added to allow aggregation. **(B)** prp was activated with collagen or CRP.

It is well known that under conditions of standard aggregometry, aggregation in response to collagen, and to a much lower extent also CRP, is dependent on the action of released "second-wave" mediators, such as ADP and TxA2 (50). Therefore, the observed defect in the aggregation response to collagen or CRP might either be based on defective collagen receptor signaling or inappropiate responses of the cells to the secondarily acting agonists. In order to discriminate between these two possibilities, flow cytometric analysis of single platelets in diluted suspensions, i.e. under experimental conditions that largely exclude the accumulation of released mediators (199), were performed. Washed platelets were stimulated with increasing concentrations of CRP and the activation of integrin αIIbβ3 (JON/A-PE, (200)) as well as degranulation-dependent surface exposure of P-selectin (α P-selectin-FITC) was determined. As shown in Fig. 25, strong αIIbβ3 activation was observed in Rac1-deficient platelets in response to thrombin and ADP, respectively (Fig. 25A upper panel and middle). Furthermore, thrombin-induced P-selectin expression was indistinguishable between wild-type and Rac1-deficient platelets whereas ADP, as expected, failed to induce significant P-selectin expression in both groups (Fig. 25A middle). In contrast, Rac1-deficient platelets almost completely failed to respond to CRP as shown by the only very low levels of αIIbβ3 activation and P-selectin expression even at very high concentrations of the agonist (Fig. 25A lower panel). Statistic analysis further proved the significance of the defect of Rac1-deficient platelets towards CRP stimulation at three different concentrations (Fig. 25B). Together, these results demonstrated a pronounced defect in GPVI-dependent platelet activation but normal responses to G-protein coupled receptor agonists in the absence of Rac1.

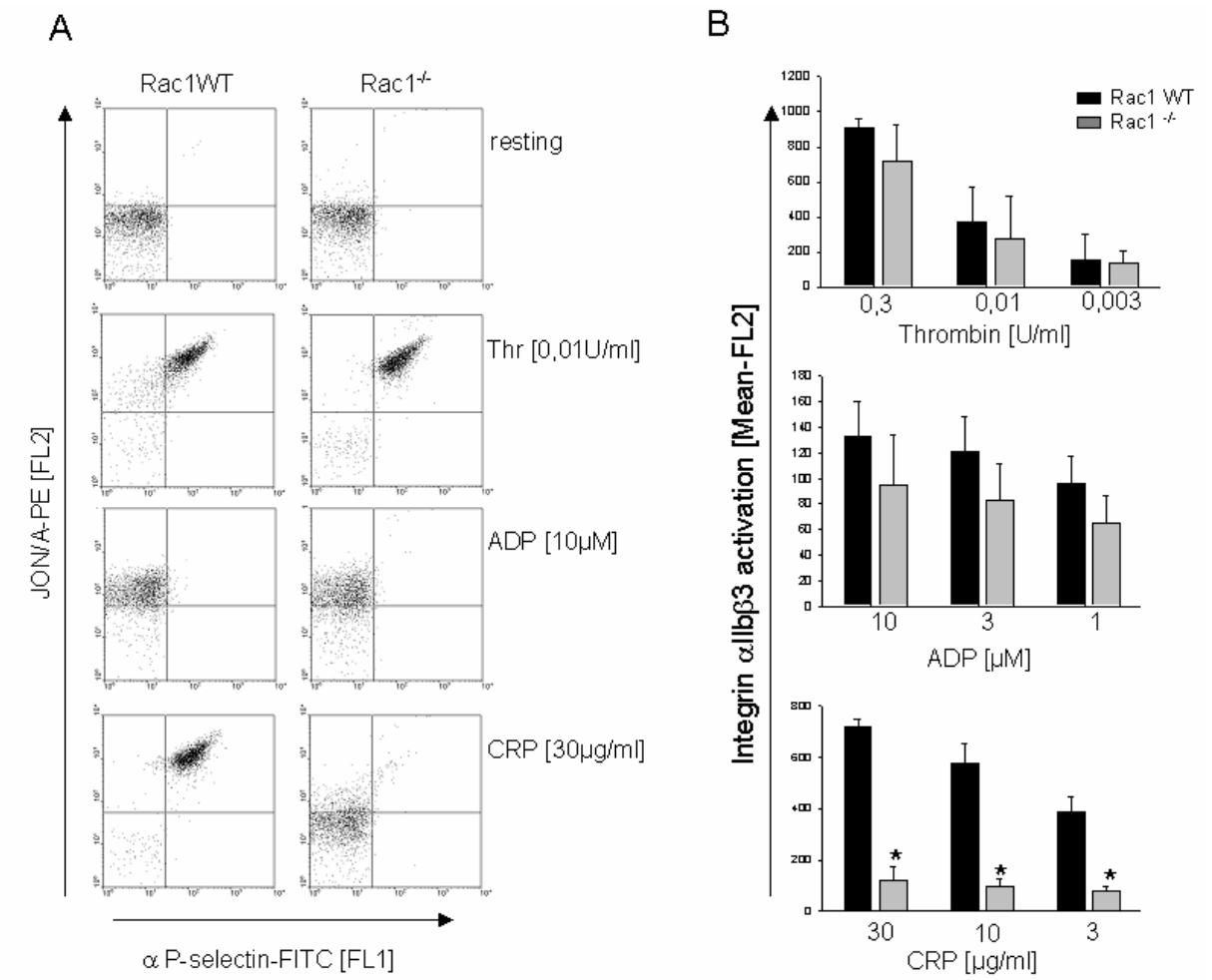

Fig. 25: Rac1-deficient platelets display impaired integrin activation and secretion in response to GPVI activation. Washed blood from wild-type and Rac1-deficient mice was incubated for 10 min at 37 °C with the indicated agonists and concentrations in the presence of PE labeled rat anti–mouse integrin αIIbβ3 (JON/A)- and FITC labeled rat anti–mouse P-selectin-antibody. Incubation was stopped by addition of 500 µl PBS and samples immediately analyzed on FACScalibur. **(A)** Dot plots of representative experiments **(B)** Statistic evaluation of integrin activation. Black bars: wild-type, grey bars: Rac1-deficient samples. Data shown are the mean ± s.d of at least three separate experiments, $p \leq 0.05$.

4.4. Defective spreading of Rac1-deficient platelets on CRP

At sites of vascular injury, subendothelial collagens are a major trigger of thrombus formation as they provide a direct and indirect (via vWF) adhesion substrate for platelets and very efficiently activate the cells. GPVI is the central collagen receptor in these cells and strictly required for efficient adhesion and thrombus growth on the matrix protein (48;59). Since the above described results had revealed an involvement of Rac1 in GPVI-induced platelet responses, spreading of Rac1-deficient platelets on CRP was studied under static conditions. To exclude the effects of released ADP and/or TxA2, the experiments were performed in the presence of apyrase (2 U/ml) and indomethacin (10 µM). Washed platelets from Rac1-deficient and wild-type mice were plated onto CRP-coated coverslips and allowed to adhere

for 30 min and analyzed by fluorescent and DIC microscopy (Fig. 26). Indeed, while wild-type platelets adhered normally on CRP and formed lamellipodia, Rac1-deficient platelets failed to form lamellipodia and, consequently, did not fully spread (Fig. 26). These data strongly indicate that Rac1 is indispensable for GPVI mediated platelet spreading in the absence of GPCR signaling.

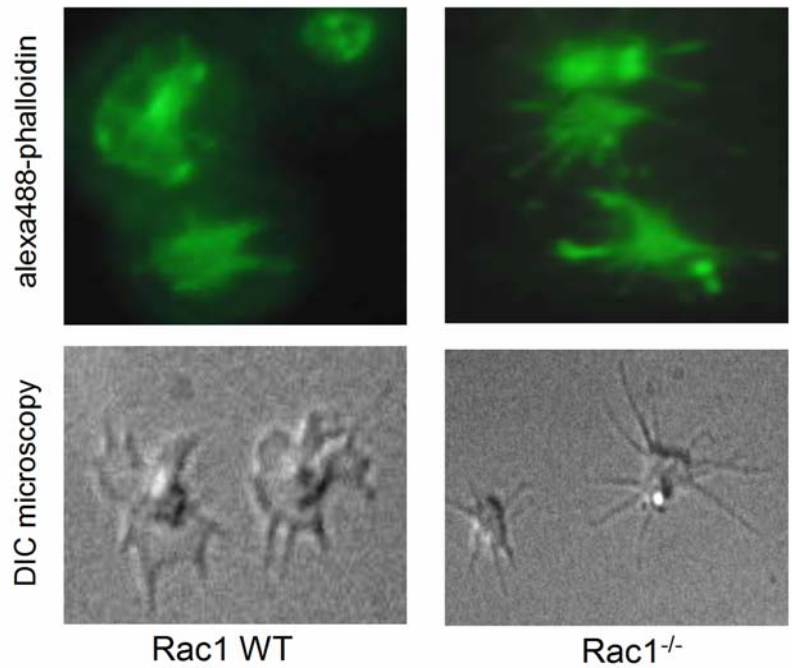

Fig. 26: Rac1–deficient platelets fail to fully spread on CRP coated surfaces.
Washed platelets of wild-type or Rac1-deficient mice were allowed to spread on CRP-coated cover slips for 25 min in the presence of 2 U/ml apyrase and 10 µM indomethacin, fixed with 3,7 % formaldehyde and analyzed using DIC microscopy (lower panel). Pictures were taken using a Cool Snap Camera (Visitron Systems GmbH). For staining of cytoskeletal actin, samples were incubted with alexa fluor® phalloidin and analyzed by fluorescence microscopy (upper panel). Pictures were taken using a digital camera.

4.5. Defective adhesion of Rac1-deficient platelets on collagen under flow

Next, platelet adhesion and thrombus formation on collagen was studied under flow conditions using a whole blood perfusion system (201). Under both high (1000 s^{-1}, 4 min) and low (150 s^{-1}, 10 min) shear conditions, wild-type platelets rapidly adhered to the collagen surface and recruited additional platelets from the blood stream resulting in the formation of stable three-dimensional thrombi (Fig. 27A, upper panel). In sharp contrast, adhesion of Rac1-deficient platelets was significantly reduced as compared to the control and the formation of stable three-dimesional thrombi was virtually abrogated (Fig. 27A, lower panel). As a result, the surface area covered by platelets at the end of the perfusion period was markedly reduced for Rac1-deficient blood as compared to the wild-type and consisted almost completely of a single platelet layer (Fig. 27). These results demonstrated that Rac1 is essential for the formation of stable three-dimensional platelet thrombi on collagen under flow conditions (Fig. 27A and C).

To test whether the observed defect is due to the failure to secrete second wave mediators ADP and TXA2 and can be overcome by addition of these mediators, ADP and U46619 were co-infused to Rac1-deficient blood directly before it entered the flow chamber (196). Under these experimental conditions, large thrombi formed within 2 min under high (Fig. 27B) and low (not shown) shear conditions. These thrombi were stable and did not detach from the collagen-coated surface or shed emboli, demonstrating that Rac1-deficient platelets can adhere and form shear-resistant thrombi on collagen when stimulated appropriately (Fig. 27B and C). These results suggest that the defect in GPVI-dependent cellular activation rather than the inability to form lamellipodia is the major cause for the markedly reduced thrombus stability observed with Rac1-deficient platelets.

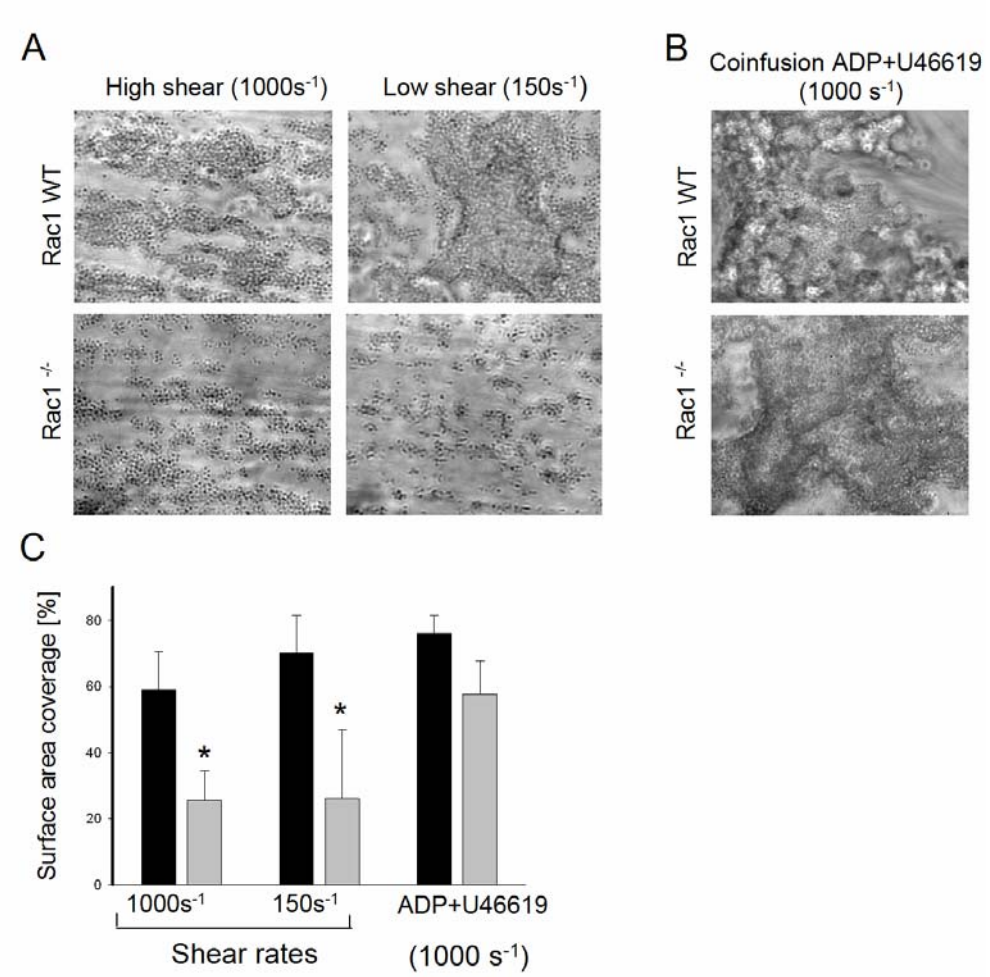

Fig. 27: Rac1 deficient platelets are unable to adhere and to form stable thrombi on a collagen coated surface. (A) Whole blood of Rac1-deficient and wild-type mice collected in heparin was perfused over a collagen coated cover slip for 4 min or 10 min at high (1000 s^{-1}) or low (150 s^{-1}) shear rates respectively. **(B)** Blood was coinfused with 10 µM ADP and 1 µM U46619 shortly before entering the chamber. **(C)** The percentage of the covered area was calculated using Metaview software. Five windows of three independent experiments were analyzed. Shown data are the mean ± s.d, $p < 0.05$.

4.6. Defective arterial thrombus formation in Rac1-deficient mice *in vivo*

The above described experiments indicated that Rac1 deficiency results in a marked GPVI signaling defect and an inability of the platelet to spread on different substrates. To test the significance of this defect in arterial thrombus formation *in vivo*, two different well established thrombosis models were used. In the first model, platelet adhesion at the injured carotid artery was analyzed by *in vivo* fluorescence microscopy. This model allows the visualization and quantitation of platelet adhesion on the exposed subendothelial matrix which has been shown to be a largely GPVI-dependent process (63). Platelets purified from donor mice of the same genotype were fluorescently labeled and injected into recipient mice. Vascular injury was induced by vigorous ligation of the carotid artery, a process that consistently causes disruption of the endothelial layer and frequent breaching of the internal elastic lamina, followed by rapid collagen-dependent platelet adhesion and aggregate formation. In wild-type mice, numerous platelets adhered to the site of injury within the first minutes after injury and virtually all platelets establishing an initial contact with the subendothelium remained firmly adherent (2579,4 ±218,86/mm^2, t=5 min). In contrast, in Rac1-deficient mice, the number of adherent platelets was markedly reduced (43 %) as compared to the control (1120,7 ±74,6/mm^2, t=5 min). An even more pronounced reduction in adhesion was observed in mice, in which GPVI had been depleted by treatment with anti-GPVI antibody, JAQ1 (54) which served as a positive control (Fig. 28A, right). Counting the adherent cells 10 min after injury validated the significant difference between wild type and Rac1-deficient mice (Fig. 28A left and middle), the latter showing a similar phenotype than GPVI deficient mice (Fig. 28B). These results confirmed the importance of GPVI-collagen interactions for platelet recruitment in this arterial injury model and revealed a central role of Rac1 in this process.

The significance of Rac1 for arterial thrombus formation was confirmed in a second model, where injury is mechnically induced in the aorta, and blood flow monitored with an ultrasonic flow probe. After a transient increase directly after injury, blood flow progressively decreased for several minutes in all animals. In all wild-type mice (7/7), this decrease resulted in complete and irreversible occlusion of the vessel with maximally 7 min thereafter (mean occlusion time 3.0 ± 2.3 min, Fig. 28D. In contrast, while a progressive reduction in blood flow was observed during the first minutes after injury in Rac1-deficient-mice, blood flow increased again to normal and 7 of 8 mice displayed essentially normal flow rates through the injured vessel at the end of the observation period (45 min) (Fig. 28D). Fig. 28C shows represetatve flow measurements. Similar results were obtained with GPVI-depleted mice, confirming earlier results (202) (not shown).

In summary the present data strongly support an essential role for Rac1 in GPVI-mediated platelet adhesion, spreading, integrin activation and secretion both *in vitro* and *in vivo*.

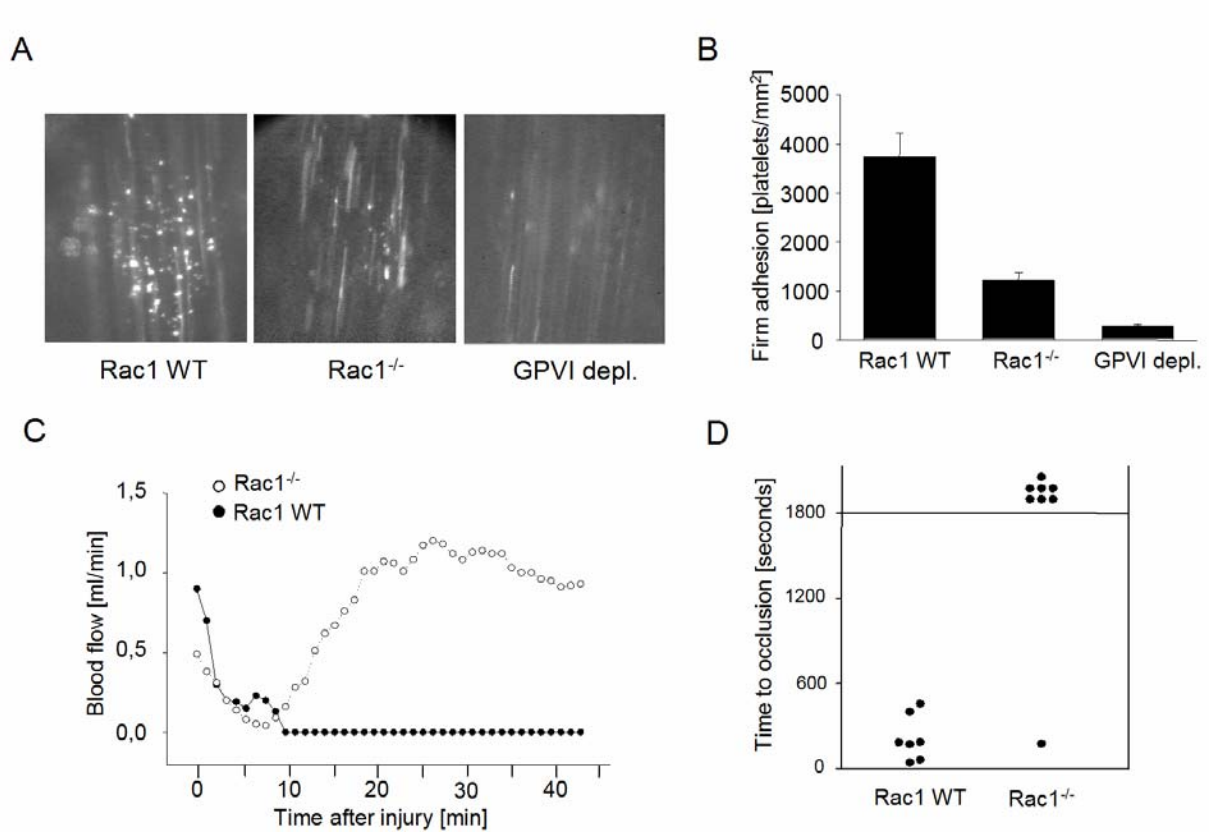

Fig. 28: Rac1 deficient mice display a severe adhesion defect *in vivo* and do not form stable thrombi. (A) Platelet adhesion to the carotid artery 10 min after injury. CFSE labeled platelets of the same genotype/phenotype were injected into anesthesized wild-type, Rac1-deficient and GPVI depleted mice. The carotid artery was injured through ligation using a surgical filament and adhesion of platelets to the vessel wall was monitored over 10 min by fluorescence microscopy *in vivo*. **(B)** Statistical evaluation showing the adhesion of platelets to the vessel wall per mm^2. Shown are ± SEM of at least three independent experiments. **(C)** Aorta occlusion model. Representative graph of blood flow of a wild type and Rac1-deficient mouse after mechanical injury of the aorta [at time 0]. **(D)** Occlusion time after injury of the aorta. The aorta of all control mice occluded whereas in 7 out of 8 Rac1-deficient mice the blood flow of the aorta was unaffected during 45 min observation time.

4.7. Rac1-deficient platelets show decreased calcium release

As Rac1 was reported to stimulate PLCγ2 (153) which in turn is required for calcium release, in the next set of experiments the increase of cytosolic Ca^{2+} in Rac1-deficient mouse platelets and controls upon platelet stimulation with different agonists was tested in flow cytometry.

Platelets were loaded with Fluo4-AM and activated with the GPCR agonist thrombin or the GPVI agonist CRP immediately before measure. In addition to these agonists, a snake venom protein, rhodocytin, was used, that is known to bind to the recently characterized receptor Clec2 on platelets and to stimulate a similar signaling cascade as GPVI (203). The time course of changes in cytosolic Ca^{2+} were monitored by an increase in mean fluorescence intensity and could be followed in real time. Interestingly in response to both, rhodocytin and thrombin, Rac1-deficient platelets showed similar increase in cytosolic Ca^{2+} as compared to controls (Fig. 29A). In contrast, when platelets were stimulated with CRP, reduced changes in cytosolic Ca^{2+} were observed in Rac1-deficient platelets as compared to

wild-type cells (Fig. 29A and B). Together, these results show that lack of Rac1 results in decreased Ca^{2+} mobilization in response to GPVI stimulation, but not in response to stimulation of GPCR or Clec2. Consequently, this strongly indicates that Rac1 plays an important role in the GPVI mediated signaling pathway, where it is involved in signaling upstream of Ca^{2+} mobilization.

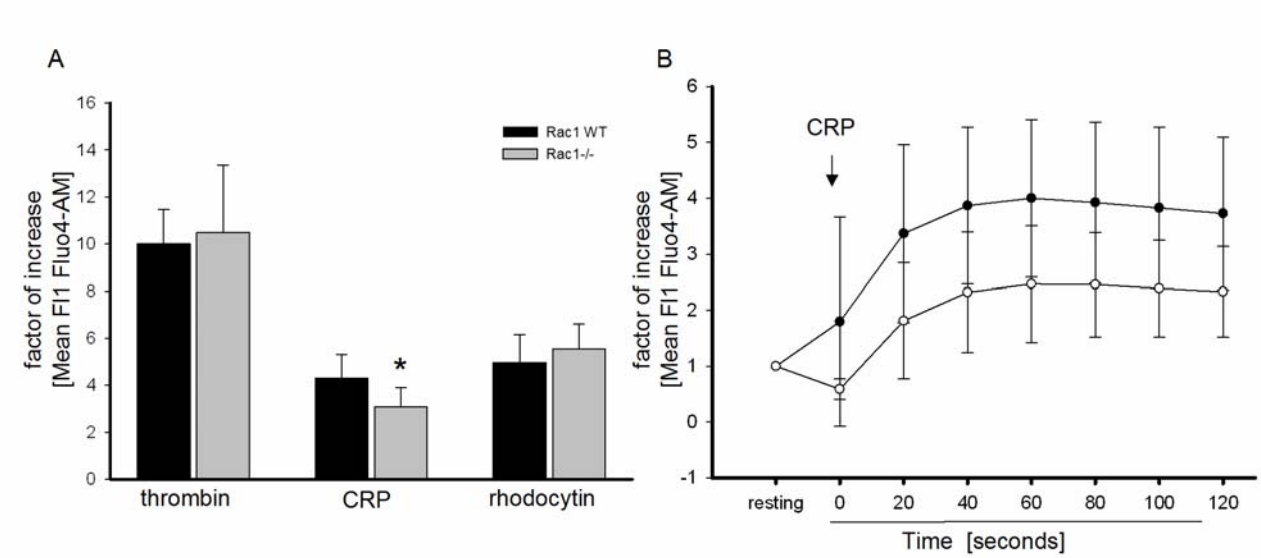

Fig. 29: Rac1-deficient platelets show reduced intracellular calcium release in response to CRP. Heparinized prp was prepared, centrifuged once and the pellet resuspended in Ca^{2+} free tyrode´s buffer. Platelets were loaded with Fluo4-AM for 10 min, diluted 1:50 in Ca^{2+} free tyrode´s buffer, activated with the indicated agonist and immediately analyzed by flow cytometry. **(A)** Increase of cytosolic Ca^{2+} concentration of wild-type and Rac1-deficient platelets after activation with indicated agonists (thrombin 1 U/ml, CRP 10 µg/ml, rhodocytin 1 µg/ml), ± s.d., $p<0.05$. **(B)** Time course of intracellular calcium release of Rac1 wild-type (●) and Rac1-deficient platelets (○) in response to CRP. Data shown are the ± s.d. of at least three idependently performed experiments.

D) Discussion

D 1. Regulation of the cell surface receptors GPVI and GPV by metalloproteinases

In this study evidence is provided for two distinct shedding mechanism of the platelet glycoprotein receptors GPV and GPVI. First, in collaboration with Bergmeier et al. it is reported here that GPVI is cleaved from the surface upon mitochondrial damage of platelets with CCCP, to release a 55 kDa fragment. It is demonstrated here, that this cleavage is independent of platelet activation which stands in contrast to cleavage of GPV described in this work (see below) and GPIbα, reported earlier (171;172). Both, GPV and GVI cleavage, however, could be inhibited by a broad range metalloproteinase inhibitor.
This illustrates that proteolytic cleavage of platelet surface receptors is strongly regulated and involves distinct signaling events and metalloproteinases. The protease responsible for GPVI shedding needs further investigation, in addition the pathway that induces metalloproteinase activation upon mitochondrial injury remains to be elucidated.

Importantly, the present study shows that the metalloproteinase ADAM17 is expressed in platelets and provides evidence that it mediates ectodomain shedding of GPV. Similar to other ADAM17 substrates, like CD40 ligand on platelets and L-selectin on endothelial cells (104;109.), GPV is cleaved near the transmembrane region, leading to the release of an intact GPV ectodomain. The function of soluble GPV is still unknown, but its generation during platelet activation has been used to develop a specific ELISA for the receptor that may become useful for monitoring platelet activation under conditions of clinical thrombosis (92). This approach was initially based on the hypothesis that thrombin is the major protease that cleaves GPV from the platelet surface, therefore making soluble GPV a specific indicator of intravascular thrombin activity. However, the results show that platelet activation in the absence of thrombin results in ADAM17-mediated ectodomain shedding of GPV. The generated fragment is bigger than GPVf1 (~82 vs. ~69 kDa) and should therefore also be detected by the ELISA system. In the course of the current study, a similar ELISA for mouse GPV was developed and it was shown that it detects both fragments with similar efficacy (Fig. 13B). In order to discriminate between thrombin-induced platelet activation events and those occurring independently of the coagulation protease, an assay will be required which detects the ADAM17-generated fragment, but not GPVf1. Through the discovery of the metalloproteinase responsible for the release of the bigger fragment and the identification of the size of the fragment the generation of such an ELISA detection system is facilitated. Thus, for such an assay, antibodies against the ~13 kDa C-terminal portion of the GPV ectodomain will be required.

ADAM 17 is a well-known sheddase that cleaves a variety of adhesion receptors, including L-selectin, CD40-ligand and VCAM-1 (204-206). Similar to the regulation of these two adhesion molecules, the metalloproteinase-dependent release of GPV from the platelet surface can be stimulated by the phorbol ester PMA, that directly activates the PKC pathway and subsequently ADAM17. The induction of ADAM17 appears to be conserved across multiple cell types. This is also supported by the observation that calmodulin plays a central role in the regulation of the shedding of GPV and L-selectin. Calmodulin is known to bind to the cytoplasmic domain of both receptors, and prevention of such interaction by calmodulin inhibitors stimulates ectodomain shedding by ADAM17 as shown in this study (Fig. 12). This is in line with previous findings (207;208). Very recent evidence suggests that similar mechanisms may be involved in the cellular regulation of the major platelet collagen receptor, GPVI (186). Besides its interaction with receptors, calmodulin also acts as a cellular intermediate of multiple Ca^{2+} actions; binding of four calcium ions induces conformational changes in calmodulin and its subsequent binding to its downstream targets. Calmodulin is known to interfere with the PKC pathway as calmodulin binding sides on substrates also contain PKC phosphorylation sites (209;210). Thus, one can speculate that dissociation of calmodulin from the intracellular domains of GPV provides PKC phosphorylation sites which contribute to or facilitate metalloproteinase dependent receptor shedding. However, although PKC itself is a potent activator of ADAM17, also PKC-independent pathways of ADAM17 activation have been reported (211;212). It remains to be elucidated how ADAM17 becomes activated and what exactly determines the substrate specificity of the enzyme (213). Although the pathways leading to ADAM17 activation may be conserved, i.e. calmodulin inhibition and/or PKC activation, the sequences cleaved by the enzyme in different proteins are highly variable, suggesting that it recognizes more complex motifs and/or sequences distal to the cleavage site.

Further, the function of GPV shedding is still unknown. In earlier years P-selectin and its subsequent shedding by an yet unknown protease was always considered as marker for platelet activation and inflammation. At the beginning of this century, eventually, it was dicovered by Wagner and colleagues that soluble P-selectin contributes to platelet procoagulant activity and accelerates hemostasis (215). In addition, soluble CD40L released from the surface by ADAM17 upon platelet stimulation was a couple of years ago shown to trigger itself platelet activation by binding to integrin αIIbβ3 and subsequent induction of outside-in signaling (104). Similar, soluble GPV might not only be used as indicator for acute ischiamic stroke but have proper physiological functions. Having characterized with this study the signaling pathway - PKC and Calmodulin mediated - that lead to GPV release is an important step forward in clarifying platelet receptor regulation. Furthermore the identification

of the responsible metalloproteinase with this work may open up the way for therapeutic treatments, once the role of GPV regulation is completely understood.

D 2. Dual role for PKC in platelets

The present results provide a first unifying concept to understand the seemingly contradictory functions of PKC isoforms in platelets. They indicate that in platelets PKC phosphorylation has a dual role in controlling collagen-induced thrombus formation: On one hand, inhibition of the classical PKC isoforms with RO31 or GF10 inhibits secretion and incompletely down-regulates αIIbβ3 activation which results in decreased aggregate formation, under conditions when secretion is a limiting factor, i.e. in washed platelets, when no second wave mediators are present. Importantly, inhibition of PKC with RO31 or GF10 in whole blood also abrogated thrombus formation under conditions of flow. On the other hand, PKC inhibition increases agonist (GPVI)-induced Ca^{2+} responses and Ca^{2+}-dependent PS exposure with, as a result, increased coagulation activity, as was established in thrombin generation experiments. Accordingly, this study first identified the only known signaling pathway in murine and human thrombus formation that can balance the two principal roles of platelets, i.e. aggregate formation and coagulation stimulation. Here for the first time it is demonstrated, that PKC simultaneously increases the proaggregatory activity and suppresses the procoagulant properties of platelets (Fig. 30). This dual role of PKC was still apparent, when most of the autocrine ADP- and thromboxane-induced thrombus formation, via $P2Y_1$ and TP (and PAR) receptor signaling was not functional in platelets lacking the Gqα subunit, strongly indicating that the observed effect is an intrinsic PKC characteristic.

The two structurally different inhibitors used in this work, GF10 and RO31, were selected because of their high activity on platelets in the presence of plasma or whole blood. They bind with high affinity to the classical, Ca^{2+}-dependent isoforms PKCα, $\beta_{I/II}$ (γ is absent in platelets) and with somewhat lower affinity to other isoforms (193). Since Ca^{2+}-dependent PKC activity mediates a significant part of the (collagen-induced) αIIbβ3 activation and secretion (120;195) it is expected that the other, non-classical isoforms δ, θ, η' and ζ (lacking the Ca^{2+}-binding C2 region) are less markedly involved in these processes. However, it should be realized that only very little is known about the functions of the latter isoforms in platelets.

Both, in human and mouse flow experiments, a reduced deposition on collagen with PKC-inhibited platelets was shown, which was largely a consequence of greatly diminished aggregate formation. Close examination of the recorded video indicated that also the frequency of stable platelet adhesion was diminished under this condition. This is in agreement with the earlier reported stabilizing effect of PKC on platelet adhesion to collagen, which is mechanistically still unexplained but was found to be independent of Syk, and focal

adhesion kinase, Fak, which get phosphorylated rapidly upon platelet binding to collagen via α2β1 or GPVI even so PKC isoforms are blocked by specific inhibitors (123). Therefore it was considered that PKC does not contribute to the initial attachment of platelets to the collagen matrix but that it is responsible for stabilizing adherent platelets in later stages of platelet adhesion. It was also suggested that PKC initiates inside-out signaling leading to activation of β1 integrins. In this work evidence is provided, that PKC importantly contributes to β3 integrin activation under flow (Fig. 18C). Thus the observed increased platelet detachment from the collagen matrix in the present study is due to decreased GPVI-induced αIIbβ3 activation in the absence of PKC, which reduces irreversible platelet binding to collagen bound von Willebrand factor through αIIbβ3. Others have observed that activated αIIbβ3 (e.g. by von Willebrand factor-induced GPIb signaling) in turn stimulates PKC, which points to the importance of positive feedback loops between αIIbβ3 and PKC (216).

Both in the absence and presence of flow, not all platelet aggregation was abolished in the absence of PKC activity. This is in agreement with optical aggregation measurements from others, and clearly points to PKC-independent signaling mechanisms in the regulation of αIIbβ3 activation (195). Recently, evidence has been obtained for the existence of another diacylglycerol-dependent (PKC-independent) pathway, involving the Ras family-regulating protein, CalDAG-GEFI, as a modulator of integrin activation and platelet aggregation (197). In platelets, it likely acts as a regulator of Rap1b, which has also been implicated in integrin activation (217). In the absence of PKC activity, receptor signaling via both Gq and GPVI still ensures phospholipase C activity, diacylglycerol formation and Ca^{2+} release, which allows CalDAG-GEFI activation and subsequent activation of integrin (197) (and see Fig. 30). The present data, however, show dramatically diminished aggregation and integrin activation in the absence of PKC under static and under flow conditions where secretion is the limiting factor (Fig. 14 and 18, respectively), suggest that in GPVI-induced thrombus formation this alternative pathway is of only limited importance. Hence, the present study establishes an important role for PKC in positively regulating the proaggregatory activity of platelets.

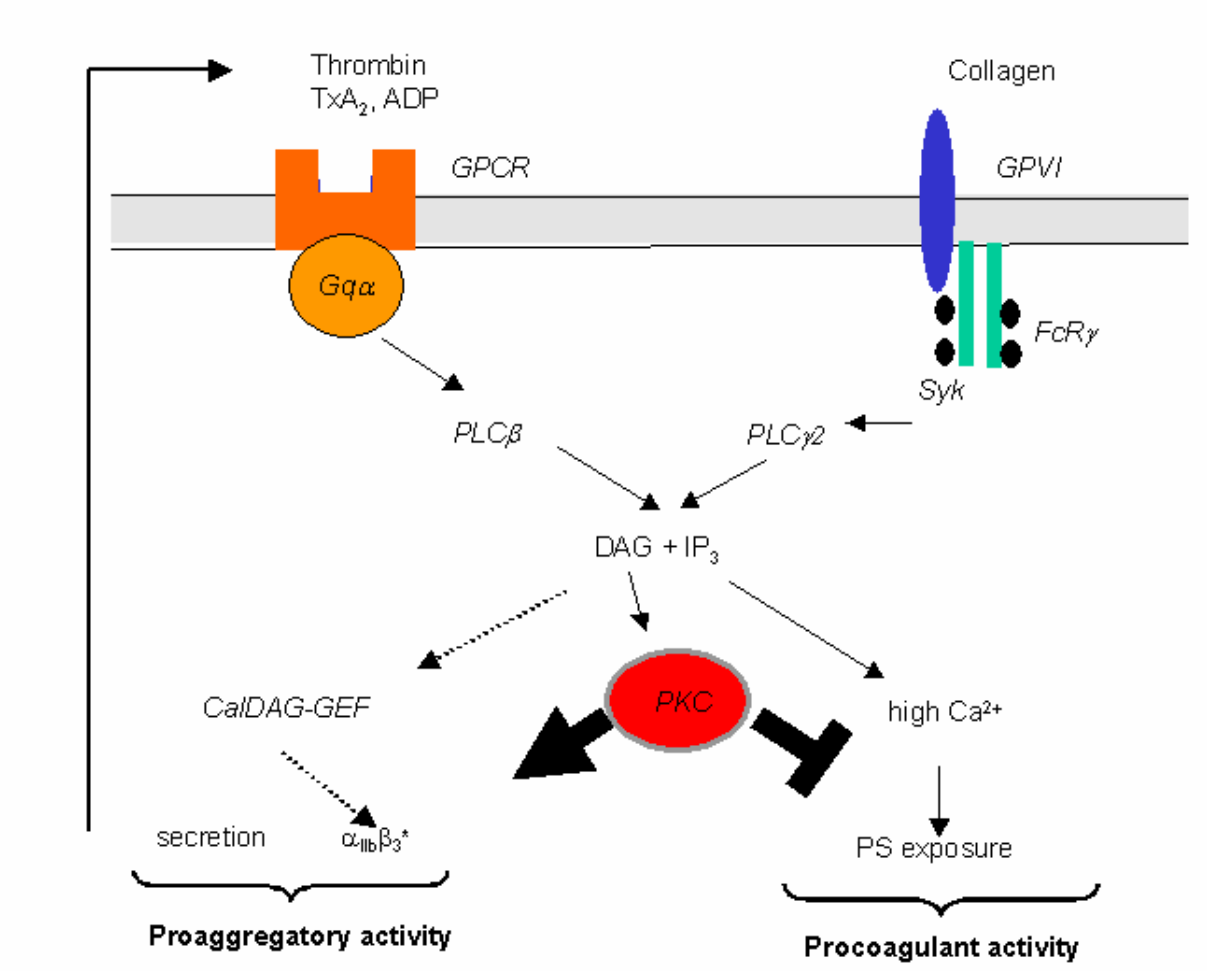

Fig. 30: Dual contribution of PKC to platelet activation in thrombus formation. Schematic representation of PKC activity in stimulating proaggregatory platelet responses (adhesion, integrin activation and release of second wave mediators) and down-regulating procoagulant platelet responses (Ca^{2+} signaling, PS exposure and thrombin generation). Platelet Gqα-protein coupled receptors (GPCR) and GPVI synergize in activation of the classical PKC isoforms via phospholipase C (PLC). PKC inhibition in vivo may thus leads to formation of smaller, but more coagulation-active thrombi.

However, it is intriguing to note that one protein inherits opposite, that is activating (proaggregatory) as well as suppressive (anti-procoagulant) functions in the same cell. Twenty years earlier Watson and Lapentina asked a similar question as they found that PKC activation by thrombin leads to an increase in PIPs production but on the other hand to a decrease in their hydrolysis and thereby to reduced production of second messengers DAG and IP3. They suggested that both actions serve different functions in the cell and are involved in short term or long term responses respectively (218). According to this model hydrolysis of PIPs after platelet stimulation occurs soon after activation. This provides the second messengers DAG and IP3 which in turn lead to PKC activation. Conversely, in longer term responses activated PKC inhibits hydrolysis of PIPs causing their accumulation. The accumulated PIPs could possibly mediate actin rearrangement. Extrapolating these assumptions to the present findings, one can argument that granule secretion and integrin

activation are short term responses while suppression of calcium mobilization and PS exposure occur at long term. Thus PKC itself establishes a negative feed-back mechanism, which finally leads to decreased platelet procoagulant activity and thereby might prevent uncontrolled thrombus growth.

Similarly, in neurons it was reported that PKC mediated phosphorylation of the G-protein coupled D2 dopamine receptor (D2 DAR) results in internalization as well as functional desensitization of this receptor. Such mechanism has been described for various GPCRs and is supposed to fine tune and dampen the response of these receptors in cells that receive various kind of signals (hormonal or neurotransmitter signals) (96). Additionally, in T and B cells PKC was found to suppress calcium mobilization upon T cell receptor (TCR) or B cell receptor (BCR) stimulation (219;220). In B cells inhibition of PKCβ caused an increase in calcium release and decreased activity of the pro-survival proteins Bcl-X_L and Bcl-2 which selectively induced apoptosis of the cells. Thus also in immune cells, PKC is involved in both negative and positive signal-transfer after receptor ligation. This is in line with the observation of a dual function of PKC in platelets, which is reported here. Haemostatis has to be a carefully balanced mechanism, as increased platelet activation can result in vessel occlusion, thrombosis, heart attack and stroke, whereas defective platelet activation can cause massive blood loss through extensive bleeding. To understand platelet signaling processes has therefore become vital for the treatment of thrombosis and haemostatic defects.

In conclusion, the present study reveals for the first time that PKC isoforms have a dual controlling role in thrombus formation by permitting secretion and integrin activation required for platelet aggregation, while suppressing Ca^{2+}-dependent PS exposure and the coagulation process. In addtion, this shows that GPVI activation initiates both processes, as PKC lies downstream of GPVI. The GPVI/PKC pathway balances between two distinct platelet functions, the progaggregatory and procoagulant properties of platelets respectively. PKC inhibition in vivo may therefore potentially lead to the formation of smaller but more coagulation-active thrombi. This might be an important point to consider for the development of therapeutic agents.

D 3. Rac1 is an effector of the GPVI signaling cascade

The involvement of Rac1 in cell cytoskeletal reorganisation and lamellipodia formation in various cell types has been widely discussed in the literature (129;130;133). In platelets and fibroblasts it could be shown that lamellipodia form specifically upon Rac1 activation, after either GPCR stimulation by thrombin (141) or by integrin engagement and signaling initiated through binding of αIIbβ3 to its ligand, immobilized fibrinogen (146).

In this study, however, a significant role for Rac1 in GPVI mediated signaling *in vitro* and *in vivo* is revealed, independent of secondary wave mediators ADP and TxA2.

.

In standard aggregometry, an impaired aggregation of Rac1-deficient platelets to low concentrations of collagen and the GPVI specific agonist CRP was observed, while the response to thrombin and ADP was normal (Fig. 24). It is important to note, that these results had still underestimated the severity of the defect in GPVI-dependent activation, as robust platelet aggregation can be observed even under conditions of extremely low levels of active integrin αIIbβ3 (199). The severity of the defect became clear by the analysis of the activation state on the single cell level by flow cytometry. In addition, in this system, the influence of secondary acting agonists is virtually abolished as the platelet suspension is highly diluted and second wave mediators cannot accumulate efficiently. In agreement with the findings above, ADP and thrombin induced αIIbβ3 activation was not severely affected in Rac1-deficient platelets, while CRP induced activation of this integrin was reduced. In addition, secretion was remarkedly impaired in response to CRP but not in response to thrombin (Fig. 25).

As Rac1 is known to be involved in actin rearrangement the morphology of Rac1-deficient and wild-type platelets after spreading on fibrinogen, collagen and CRP coated surfaces was studied. Confirming the role of Rac1 downstream of the fibrinogen receptor integrin αIIbβ3, platelets were not able to form lamellipodia and did not fully spread on fibrinogen, but still could form filopodia. As expected, a defect in lamellipodia formation upon spreading on CRP-coated surfaces was observed (Fig. 26). To exclude the influence of second wave mediators in the study, the experiment was performed in the presence of apyrase and indomethacin. Thus, the effect observed was exclusively GPVI-dependent, which is in line with the results above, (in aggregometry and flow cytometry) as well as with previous studies by McCarty et al (129), and showing a severe defect of Rac1-deficient platelets in GPVI signaling.

The defect in GPVI signaling was further studied under flow conditions at different shear rates. Whole blood of wild type and knock out mice was perfused over a collagen coated surface at low (150 s^{-1}) and high (1000 s^{-1}) shear rates (Fig. 27A). Platelets of Rac1-deficient mice failed to form stable thrombi and much less area was covered compared to the controls. The adhesion defect of Rac1-deficient platelets on collagen at both shear rates (not shown) could be overcome by ADP and U46619 Co-infusion that activate GPVI-independent pathways. This shows that the observed defect is rather due to defective GPVI signaling than due to defective G-protein coupled signaling mediated by second wave messengers as assumed by McCarty *et al* (129).

Previously, a crucial role of GPVI for platelet recruitment to the injured vessel wall *in vivo* has been reported (50). *In vivo* studies on the carotid artery in GPVI depleted mice showed that virtually no platelet adhesion to the injured vessel wall occurred in absence of functional

GPVI. With regard to this experiment and in respect to the findings above, which all indicate a GPVI defect in Rac1-deficient mice, the described (221) carotid artery model with Rac1-deficient mice in comparison to control mice and GPVI depleted mice was performed (162). In this model, platelet adhesion to the vessel wall of the injured carotid artery was studied by *in vivo* fluorescence microscopy. Rac1-deficient mice showed strongly reduced platelet adhesion to the vessel wall compared to wild-type mice. A slightly stronger defect was evident in GPVI-deficient mice, indicating that in Rac1–deficient another pathway independent of Rac1 may be able to partially bypass the defective signaling downstream of GPVI and lead to adhesion. A similar effect was also observed in the aorta occlusion model (Fig. 28C and D). Taken together these results provide strong evidence for the involvement of Rac1 in the GPVI signaling cascade that is independent of the defects in cytoskeletal rearrangement.

In addition, the results presented here indicate that Rac1 is also involved in Ca^{2+} mobilization downstream of GPVI (Fig. 29). This is consistent with previous findings by Piechulek *et al*., who reported an involvement of Rac1 in PLCγ2 activation (153). Although PLCγ2 activation is known to be essential for Ca^{2+} release downstream of GPVI, this is most likely not responsible for the dramatic integrin activation- and granule secretion– defect, reported here as Ca^{2+} release is only slightly reduced in Rac1-deficient mice compared to the controls (Fig. 29A and B), thus the Rac1 pathway seems not vital for Ca^{2+} release downstream of GPVI and one might consider additional functions for Rac1 in the GPVI pathway leading to the pronounced integrin activation-, granule secretion- and platelet adhesion- defect. Therefore it can be suggested that an yet unknown pathway might exists that might be independent of PLCγ2 signaling. This would be in line with earlier findings by Rabie T. in this laboratory, who observed a PLCγ2 independent (and Lat independent) pathway for GPVI downregulation following receptor ligation (Rabie T., 2006, submitted for publication). Consequently, Rac1 might participate in this pathways and lead to integrin activation and secretion independent of PLCγ2. Alternatively, Rac1 might be acting further downstream of Ca^{2+}, where it regulates integrin activation and granule secretion. Future work will by required to identify Rac1 binding partners and to clarify the exact level of involvement of Rac1 in platelet signaling. To conclude, the present data demonstrate a vital role for Rac1 in the GPVI mediated signaling pathway *in vitro* and *in vivo*. The findings further elucidate the regulation and signal transmission of GPVI, which has been proposed as a new anthithrombotic target.

E) Conclusion and Perspectives

With the present work three main aspects in platelet biology were investigated:

1. It could be shown that GPVI is shed from the platelet surface upon mitochondrial injury and it was demonstrated that GPV is cleaved from the surface upon platelet activation with CRP and PMA. The metalloproteinase responsible for GPV shedding, ADAM17, was identified. Receptor regulation is a highly controlled mechanism and takes place, as demonstrated here, in physiological stress situation (mitochondrial injury in the case of GPVI) as well as during normal platelet activation (in response to CRP and PMA in case of GPV) and can be blocked with metalloproteinase inhibitors (both for GPVI and GPV shedding). Future work will be needed to address the question if GPVI shedding is an aging process to clear platelet from the blood stream or if the shed fragment may exert an intrinsic function and contribute to platelet signaling or thombus stabilization. Latter is a potential function for the shed GPV fragment. Analyzing the role of the cleaved fragment could be of extreme significance for treatment of arterial thrombosis and all connected diseases, such as heart attack and stroke.
2. Another aspect addressed here was the role of PKC in platelet activation and platelet procoagulant activity, downstream of GPVI. As firstly proven here, PKC has a dual role in platelet signaling. On one hand it stimulates integrin activation and platelet aggregation and on the other hand it suppresses calcium mobilization and thereby PS exposure. It will be of great interest for pharmaceutical treatment to block the platelet proccoagulant response without affecting the hemostatic benefit of PKC function. In addition the newly identified role of PKC may lead to a revised model of platelet signaling downstream of GPVI.
3. The same is true for the third findings reported here. The small GTPase Rac1 is firstly shown to be a crucial part of the GPVI signaling cascade and contributes to integrin activation and secretion *in vitro* and stable thrombus formation *in vivo*. Further work will be required to identify the interaction partners of Rac1 and further contribute to a more complete picture of the GPVI signaling cascade.

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Appendix

Abbreviations

ACD	Acid citrate dextrose solution
ADAM	A disintegrin and metalloproteinase
ADP	Adenosine diphosphate
BSA	Bovine serum albumin
CCCP	Carbonyl 3-chlorophenylhydrazone
CRP	Collagen related peptide
Cvx	Convulxin
DAG	Diacylglycerol
DCF	5-carboxyfluorescein diacetate succinimidyl ester
DMSO	Dimethylsulfoxid
ECL	Enhanced Chemiluminiscence
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmatic reticulum,
FcRγ	Fc receptor γ chain
FITC	Fluorisothiocyanate
FSC/SSC	Forward scatter/Sideward scatter
GAP	GTPase activating protein
GDP	Guanine diphosphate
GDI	GDP dissociation inhibitor
GEF	Guanine nucleotide exchange factors
GF10	GF109203X (PKC inhibitor)
GTP	Guanine nucleotide triphosphate
GTPase	Guanine nucleotide triphosphate binding protein
GPO	Glycine-proline-hydroxyproline
GPV	GlycoproteinV
GPVI	Glycoprotein VI
GPIb-V-IX	Glycoprotein Ib-V-IX complex
GPCR	G-protein coupled receptor
HRP	Horse reddish peroxidase
Ig	immunogloboline
ITAM	Immunoreceptor tyrosine-based activation motive
IP3	Inositoltriphosphate
IP3R	IP3 receptor
LAT	Linker of T cell activation

PAR	Protease-activated receptor (thrombin receptor).
PBS	Phophate buffer saline
PE	Phycoerythrin
Pfp	Platelet free plasma
PGI2	Prostacyclin
PI3k	Phosphatidylinositol 3-Kinase;
PIP2	Phosphatidyl-4,5-bisphosphate;
PIP3	Phosphatidyl-3,4,5-triphosophate;
PKC	Protein kinase C
PLCγ/β	Phospholipase Cβ/γ
PMA	Phorbol 12-myristate 13-acetate
Ppp	Platelet pour plasma
Prp	Platelet rich plasma
PS	Phophatidylserine
RO31	RO318425 (PKC inhibitor)
SH domains	Src homology domains
TACE	TNFα converting enzyme
TxA2	Thromboxane A2
Va	Coagulation factor Va
vWF	von Willebrand factor
Xa	Cofactor factor Xa

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Publication List

Published arcticels

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Manuscript in preparation

Strehl A, Pozgajova M, Brakebusch C, Nieswandt B: Rac1 is an effector of the GPVI signaling cascade and involved in adhesion and thrombus formation in mice *in vivo.*

Oral presentation

Strehl A, Pozgajova M, Brakebusch C, Nieswandt B: Rac1 is an effector of the GPVI signaling cascade and involved in platelet adhesion and thrombus formation. XXth European Platelet Meeting, October 2005, Ede, The Netherlands.

Strehl A, Munnix IC, Marijke J. E. Kuijpers, Bernhard Nieswandt, Johan W. M. Heemskerk: Inhibition of Protein Kinase C Results in Altered Thrombus Phenotype with Suppressed Platelet Aggregation and Increased Platelet Coagulant Activity. XXIth European Platelet Meeting, October 2006, Lutherstadt Wittenberg, Germany.

Poster presentation

Rabie T*, **Strehl A*,** Ludwig A, Nieswandt B, Evidence for a role of ADAM17 (TACE) in the regulation of platelet glycoprotein V. XIXth European Platelet Meeting, Bad Brückenau, Germany.

Strehl A, Munnix IC, Marijke J. E. Kuijpers, Bernhard Nieswandt, Johan W. M. Heemskerk: Inhibition of Protein Kinase C Results in Altered Thrombus Phenotype with Suppressed Platelet Aggregation and Increased Platelet Coagulant Activity. The International Society on Thrombosis and Haemostasis (ISTH), XXth Congress, Sydney, Australia.

Erklärung gemäß §4 Abs. 3 der Promotionsordnung:

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Arbeit selbständig angefertigt und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe.

Diese Dissertation hat weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen.

Ich habe bisher außer den mit dem Zulassungsbesuch urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Amrei Strehl Würzburg, im Dezember 2006