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The role of platelet thrombin receptors PAR1 and PAR4 in platelet activation

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To Per and Moa

ABSTRACT:

Platelets play a pivotal role in coagulation and haemostasis. Their most prominent task is to seal damaged blood vessels by the formation of a platelet plug at the damaged area. Once the injury is covered, platelets retract the coagulum to close the wound and allow the blood to flow freely in the vessel. Platelets are strongly activated by the essential enzyme thrombin, formed in the coagulation cascade. Activation of the platelet thrombin receptors PAR1 and PAR4 leads to shape change, secretion of granule content, and aggregation, all of which can be accomplished by each receptor individually. However more and more findings indicate that there are differences between the receptors and that they have different physiological functions.

This thesis presents studies performed to elucidate the relative role of PAR1 and PAR4 in platelet activation and coagulation.

We have studied the effects on platelet activation and coagulation, and revealed a possible physiological role for PAR4 in the stabilisation of the coagulum. We also investigated the relative role of PAR1 and PAR4 in the cross-talk between thrombin and epinephrine with and without inhibition of COX-1. We demonstrated that PAR4 interacts with adrenergic receptors and causes an aggregation of platelets dependent on released ATP and its receptor $P2X_1$, thereby circumventing the inhibition by aspirin. Not only is this an interesting specific role for PAR4, but it may also be of clinical importance considering that COX-1 inhibition is the most common treatment for patients with cardiovascular disease to prevent thrombosis.

We show that the number of PAR1 receptors varied between donors and that this variation was correlated to the response on receptor activation. The number of PAR1 receptors on the platelet surface was decreased after PAR1 stimulation but increased after stimulation of other receptors.

In a final attempt to elucidate the nature of PAR1 and PAR4 we used mathematics to evaluate the effect of co-stimulation of the receptors. We found a strong synergistic effect for both platelet activation and aggregation. This indicates that PAR1 and PAR4 interact in a yet unknown way to regulate or amplify the effect of each other rather than merely transmitting the incoming signal the same way.

SVENSK SAMMANFATTNING

Trombocyter (blodplättarna) spelar en viktig roll för den initiala koagulationen (blodlevringen), där de snabbt upptäcker en kärlskada och börjar täppa till den. Trombin är ett nyckelenzym i koagulationskaskaden (den process där ett flertal enzym aktiverar varandra vilket till slut leder till att blodet levrar sig) och börjar bildas i små mängder redan tidigt i koagulationsprocessen. Trombin är den starkaste kroppsegna trombocytaktivatorn. Trombocyterna har många olika typer av receptorer (mottagare) som på olika sätt reagerar på stimuli, t.ex. har de två trombinreceptorer som benämns PAR1 och PAR4. Stimulering av dessa receptorer leder till trombocytaktivering och aggregation (ihopklumpning av trombocyterna) men deras respektive egenskaper och uppgifter är ännu inte klarlagda.

Vi har presenterat resultat som visade att maximal aktivering av PAR4 inducerar mer fibrinogenbindning än maximal aktivering av PAR1 (vilket är viktigt för att trombocyterna ska kunna klumpa ihop sig, aggregera). Både koagulationstiderna och starten på trombinbildningen påverkades mest av PAR4-hämning. Detta indikerar att PAR4 spelar en viktig roll tidigt under koagulationsskedet då trombinkoncentrationen fortfarande är låg. Blodkoaglets elasticitet sjönk signifikant när PAR4 hämmades. Detta betyder att stabiliteten hos koaglet försämrades då PAR4 hämmandes vilket skulle kunna vara relaterat till att PAR4 inducerade mer fibrinogenbindning än PAR1. En fysiologisk funktion för PAR4 skulle alltså kunna vara att stabilisera koaglet, såväl mekaniskt som mot upplösning.

Vi har också studerat PAR1 och PAR4 och deras relativa roll för effekten av trombin i kombination med adrenalin samt vikten av att ADP/ATP utsöndras från trombocyten vid aktivering. Våra resultat visade att PAR4 samspelar med adrenalins α_{2A}-receptor för att ge en stark aggregation samt att ATP var en viktig aktör i samspelet mellan PAR4 och adrenalin. Dessa fynd, liksom de ovan nämnda, antyder att PAR4 spelar en viktig och unik roll för trombocytsignalering och aktivering.

I nästa arbete ville vi knyta responsen av aktiverade PAR1-receptorer till antalet PAR1-receptorer per trombocyt. Vi ville också se om antalet receptorer sjönk

efter stimulering med olika aktivatorer vilket skulle kunna tyda på att receptorerna internaliserades (försvinner in i cellen efter aktivering). Vi fann att det var en stor skillnad i antalet receptorer mellan olika individer och att den skillnaden kunde knytas till den respons vi fick när PAR1 stimulerades. Vi såg också en signifikant nedgång i PAR1-receptorer på trombocytytan efter stimulering av PAR1. Efter stimulering av andra receptorer ökade antalet PAR1 receptorer.

I en nyligen genomförd studie konstaterade vi en stark samverkan (synergi) mellan PAR1 och PAR4 vid samtidig stimulering. För att bekräfta detta beräknades synergin matematiskt. Då alla våra resultat; genomförda såväl med flödescytometri som med aggregometri; gav en mycket stark synergi drar vi slutsatsen att PAR1 och PAR4 troligtvis samarbetar på ett intrikat sätt för att förstärka varandras effekt vid mycket låga trombinkoncentrationer.

PREFACE

Paper I: K Vretenbrant, S Ramström, M Bjerke, T.L Lindahl. Platelet activation via PAR4 is involved in the initiation of thrombin generation and in clot elasticity development.
Thromb Haemost. 2007;97. 417-24

Paper II: S Ramström, K Vretenbrant Öberg, F Åkerström, C Enström, T.L Lindahl. Platelet PAR1 receptor density- Correlation to platelet activation response and changes in exposure after platelet activation.
Thromb Res. 2008:121. 681-688.

Paper III: M Grenegård, K Vretenbrant Öberg, M Nylander, S Desiléts, E Lindström, A Larsson, I Ramström, S Ramström, T.L Lindahl.
The ATP-gated P2X1 receptor plays a pivotal role in activation of aspirin-treated platelets by thrombin and epinephrine.
J Biol Chem. 2008 Jul 4;283(27):18493-504.

Paper IV: K Vretenbrant Öberg, N Boknäs, S Kalsum, S Ramström, T.L Lindahl. *Platelet thrombin receptors PAR1 and PAR4 act in a synergistic manner.*
In manuscript.

TABLE OF CONTENTS

ABBREVIATIONS

ADP/ATP:	adenosine di/tri phosphate
APC:	activated protein C
CRP:	collagen-related peptide
DHPLC:	denaturating high performance liquid chromatography
DTS:	dense tubular system
FITC:	fluorescein isothiocyanate
FOR:	free oscillation rheometry
FS:	forward scatter
FX/FXa	factor X/activated factor X
G':	Elasticity modulus
GP:	glycoprotein
GTP:	guanosin tri phosphate
MFI:	mean fluorescence index
OCS:	open canalicular system
PAR:	protease activated receptor
PCR:	polymerase chain reaction
PI3K:	phosphatidyl inositol 3 kinase
PKA/PKC:	protein kinase A/C
PLC:	phospholipase C
PPP	platelet poor plasma
PRP:	platelet rich plasma
SNP:	single nucleotide polymorphism
SS:	side scatter
TF:	tissue factor
VWF:	von Willebrand Factor

INTRODUCTION

To maintain life we need an intact and functional circulatory system to supply the rest of the body with life-supporting agents such as oxygen and nutrients. In addition, it provides a defence system against hostile organisms. Haemostasis (from the Greek "haimi" for blood and "stasis" for stop) is the harmony that regulates the blood flow in case of vessel injury. Haemostasis is dependent on a fine balance between mortal bleeding and mortal blood coagulation and platelet aggregation, thrombosis.

Platelets are vital for haemostasis, as are other blood cells, plasma proteins and the vessel wall. They search the vascular system for damage at all times, and as soon as they find a vessel wall injury they immediately adhere to the site of injury to prevent blood loss. The adhesion causes the platelets to become activated and to release proteins and molecules which participate in the propagation and stabilisation of the platelet plug formed over the injury. The adhered platelets recruit more platelets which will aggregate and, with fibrin supplied by the coagulation, form an armoured plug covering the injury.

To prevent unwanted platelet activation and coagulation the endothelial cells lining the vessel wall continuously release inhibitory substances that control the quite explosive haemostatic system.

In a delicate balance such as this, mistakes may occur, leading to events such as heart attack and stroke or bleeding.

By learning more about platelets and coagulation, many of these events may be prevented in the future by better understanding the consequences of our lifestyle and through better medical treatment. My contribution to the science of platelets and coagulation will be discussed in this thesis.

HAEMOSTASIS

PRIMARY HAEMOSTASIS

The platelet adhesion and aggregation at the site of injury is referred to as the primary haemostasis. Both of these involve platelet activation which can be divided into three overlapping stages: initiation, propagation and perpetuation, (reviewed in [1]) see Figure 1.

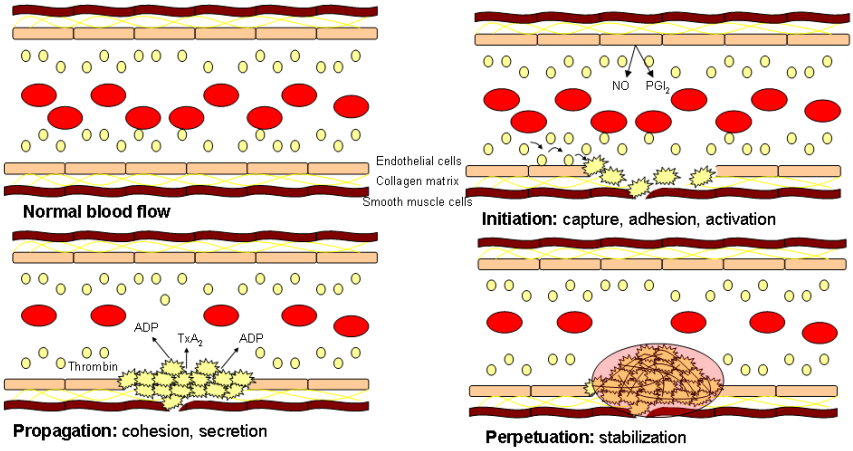


Figure 1: A schematic illustration of haemostasis. First, platelets identify a vessel injury and start to adhere to the damaged vessel wall, which also leads to platelet activation. Activated platelets attract more platelets, which soon form a platelet plug over the injury. The plug will subsequently be armoured by a fibrinogen network.

Initiation may occur because circulating platelets are captured and activated by exposed collagen and von Willebrand factor (VWF), forming a monolayer that supports thrombin generation and the following platelet activation. Key receptors in this event are the receptors that bind to collagen (integrin α2β1 and glycoprotein VI (GPVI)) and those that bind to VWF (GPIbα and α2bβ3, also named GPIIb/IIIa). Binding to, and activation of, these receptors leads to

intracellular signalling. In thrombotic and inflammatory disorders, platelet activation will be initiated by thrombin activating the protease-activated receptors (PAR) 1 and 4 without prior activation of other receptors. Collagen receptors support the capture of slowly flowing platelets to the site of injury and hereby cause a weak activation of the captured platelets (reviewed in [2, 3]). The activation stimulates cytoskeletal reorganisation, which allows the platelets to change shape from discoid to spherical, to develop pseudopodia and finally to spread, flatten and adhere more closely to the exposed vessel wall. This process is initiated by a rise in cytosolic calcium, which is mediated by the platelet-collagen interaction or by soluble agonists such as thrombin and ADP. GPVI and GPIb are able to bind collagen and VWF, respectively, without prior activation. Once the activation begins, α2β1 and GPIIb/IIIa are able to bind their respective agonists as well. As a consequence of the shape change the granules inside the platelet are centralised and prepared for release via exocytosis. Dense granules, which are released first, require the lowest energy consumption, followed by the α-granules and λ-granules [4].

The formation of a platelet monolayer is sufficient to initiate platelet plug formation but not to stop bleeding. Extension takes place as additional platelets are recruited to the site of injury and in their activated state stick on top of each other, accumulating on the initial layer of platelets. Agonists such as thrombin, secreted ADP and thromboxane A_2 play an important role in this stage as they activate phospholipase C (PLC) in platelets, causing an increase in cytosolic Ca^{2+} concentration. The signal supports the activation of integrin GPIIb/IIIa, which facilitates the coherent interactions between the platelets that are critical to the formation of the haemostatic plug. Between 40,000 and 80,000 copies of GPIIb/IIIa are expressed on resting platelets, and additional pools of receptors are stored in α-granules which are exposed on the platelet surface upon activation [5, 6]. Activation of GPIIb/IIIa not only promotes platelet adhesion and aggregation but also induces outside-in signalling that results in amplification of platelet activation (reviewed in [7]). As the platelet activation starts, the degranulation and release of secondary platelet agonists such as ADP and serotonin in addition to thrombin generation will accelerate the aggregation process by recruiting and activating new platelets to the area. Platelet aggregation at the injury site results in the formation of a fragile platelet plug which will subse-

quently be stabilised by a fibrin network formed as the result of the coagulation cascade [8].

Perpetuation refers to the late phase of the platelet plug formation when the intense signalling has faded. These events stabilise the platelet plug and prevent disaggregation by close contacts between platelets and outside-in signalling through integrins. This step coincides with the final step of the secondary haemostasis, clot retraction. Clot retraction depends on the interaction between actin/myosin complexes and GPIIb/IIIa as well as the binding of fibrinogen or VWF [9].

SECONDARY HAEMOSTASIS – PLASMA COAGULATION

Coagulation involves the transformation of flowing blood into a stable gel and is generally initiated when the blood comes in contact with extravascular surfaces. The process is based on complex enzymatic mechanisms and ends in a fibrin-fibre armoured coagulum, see Figure 2.

The coagulation is divided into two pathways: intrinsic and extrinsic. The names originate from the fact that all functional components of the intrinsic pathway are present in the blood, whereas the triggering component of the extrinsic pathway, tissue factor, is found in extravascular tissue.

The extrinsic/intrinsic pathways were first proposed by investigators in Oxford in the 1950s and included two ways of thrombin generation. One way, the extrinsic pathway was dependent on tissue factor, whereas the intrinsic pathway was not. In 1964 the coagulation was first described as a sequence of reactions in which each proenzyme is converted to its active form by the action of the previous factor in the sequence [10, 11]. Despite the differences in the enzymatic cascades both pathways converge into the common pathway leading to fibrin formation. In present time it has been revealed that the pathways are highly interconnected and, not least, that the platelet surface is of great importance to the proper functioning of the coagulation. (Reviewed in [12])

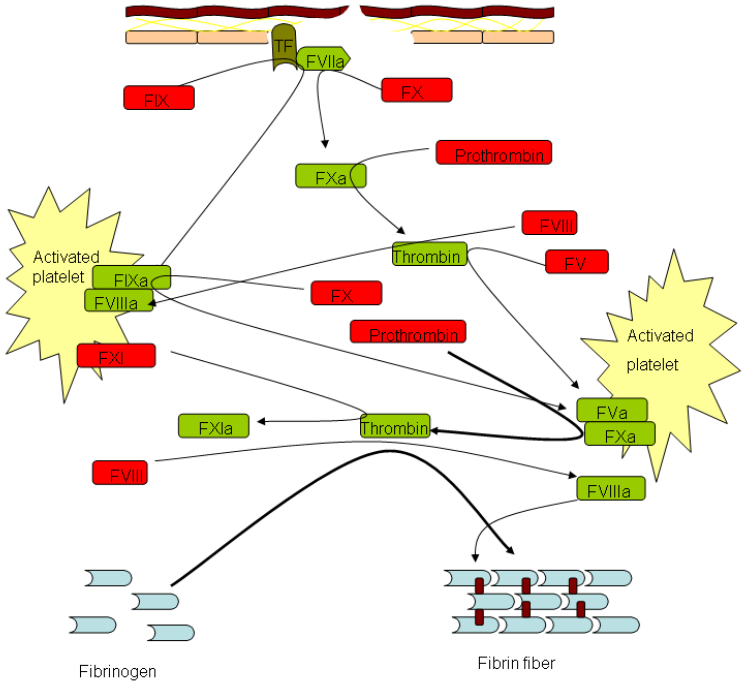


Figure 2: The coagulation cascade.

EXTRINSIC PATHWAY – TISSUE FACTOR PATHWAY

This pathway is initiated by tissue (TF) found in extra vascular tissue [13, 14]. On its own TF has no enzymatic function but serves as a cofactor and rapidly activates factor VII (FVIIa)[15]. Approximately 1% of factor VII normally circulates in its activated form [16]. The TF/FVIIa complex (also called extrinsic Xase) can activate factor X (FX) and factor IX (FIX). Moreover, as part of an initial amplification loop, both FX and FIX contribute to further initiation of coagulation by feedback activation of FVII to FVIIa [17, 18]. Small amounts of FXa are initially formed, which leads to a limited conversion of prothrombin (FII) to the enzymatically active thrombin. The very low concentrations of thrombin formed in this step is not enough for fibrin formation, although it suffices to activate platelet PAR receptors [19, 20] and to convert factor V (FV) and factor VIII (FVIII) in the intrinsic pathway to their active state [21]. FXI can also be activated by thrombin, which is facilitated if FXI is bound to acti-

vated platelets via GPIbα [22, 23]. FXIa will activate more FIX [24], which together with FVIIIa forms a complex on the procoagulant surface of activated platelets. This complex is a potent activator of FX, and the formation of FXa accelerates dramatically. Activated platelets in this stage will facilitate the final burst of thrombin generation as they provide the surface for forming the prothrombinase complex, FXa and FVa, which convert large amounts of FII (prothrombin) into thrombin [21]. The high local concentration of thrombin will induce clot formation by cleaving fibrinogen [8]. The fibrin network is finally covalently cross-linked and stabilised by FXIIIa, which is also activated by thrombin [25]. Thrombin-activatable fibrinolysis inhibitor (TAFI) is slowly activated by thrombin but both activated and inactivated by plasmin. Its inhibitory effect is based on the fact that it strongly reduces the activation of plasminogen on the fibrin surface and thereby inhibits fibrinolysis (reviewed in [26]).

INTRINSIC PATHWAY – CONTACT ACTIVATION PATHWAY

This activation system is regulated by three plasma proteins: factor XII, prekallikrein and high molecular weight kininogen.

The pathway is initiated by factor XII binding to a negatively charged surface where it undergoes a spontaneous activation [27]. Activated factor XII converts factor XI and prekallikrein to factor XIa and kallikrein, respectively. Factor XI is also activated by thrombin, which is considered to be the most relevant pathway physiologically. Factor XIa subsequently activates the vitamin K-dependent factor IX [28]. Factor IX activates factor X, which is the factor that connects the extrinsic and the intrinsic pathways. Activated factor X can then convert prothrombin into thrombin, but this step is limited by the lack of co-factors for factor IXa and factor Xa (factor VIIIa and factor Va, respectively). Thrombin, as well as factor X, is able to convert the pro-cofactors into active cofactors, which facilitates the formation of FIXa/FVIIIa and the FXa/FVa complexes on a phospholipid surface [29, 30]. As the complexes become functional, large amounts of thrombin are generated, converting fibrinogen into fibrin and ultimately resulting in fibrin network formation.

CLOT RETRACTION

When clot formation is completed, clot retraction occurs. This is assumed to be of importance in concentrating the plug to the damaged area and possibly to closing the wound and opening occluded vessels [31]. Clot retraction is mediated by the platelets and is dependent on binding to the polymerising fibrin strands [32]. The binding is in part mediated by the GPIIb/IIIa receptor, which forms a link between the fibrin strands and the network of actin and myosin of the platelet cytoskeleton.

INHIBITORS OF THE COAGULATION SYSTEM

The amplification mechanisms of the coagulation and thrombus formation ensure a fast enzymatic route that prevents blood loss at a vessel injury. An explosive system like this needs tight regulation to stop the thrombus growth from spreading far away from the site of injury. Thrombin works not only as fuel for the coagulation but also inhibits the process by activating protein C (APC) via thrombomodulin on the surface of intact endothelial cells. APC and its cofactor, protein S, function as inhibitors of coagulation by inhibiting factor Va and factor VIIIa [33]. Tissue factor pathway inhibitor is another inhibitor of the coagulation. It inhibits factor Xa and the TF/VIIa complex [34]. Antithrombin is a protein that can inhibit thrombin's enzymatic action as well as inactivate factor Xa, IXa, VIIa/TF by complex formation [35].

FIBRINOLYSIS

As the final step in the life of a thrombus, it needs to be removed to maintain haemostatic harmony. This task is carried out by the fibrinolytic system and involves the conversion of plasminogen into active plasmin. The reaction is catalysed by tissue plasminogen activator or urokinase-plasminogen activator. Plasmin is an enzyme that degrades fibrin into fibrin degradation products, which in turn dissolve the clot [36].

Thrombin is truly a multi-functional enzyme that is important for haemostasis in many ways. Thrombin has direct effects on coagulation through the activation of platelets, the formation of fibrin clots and the activation of various cofactors in the coagulation cascade. Thrombin activity extends from the coagulation process to anticoagulation and stimulation of the fibrinolytic processes. Its role also continues into the tissue repair and remodelling phase necessary to regenerate damaged vascular tissue [37].

THE PLATELET

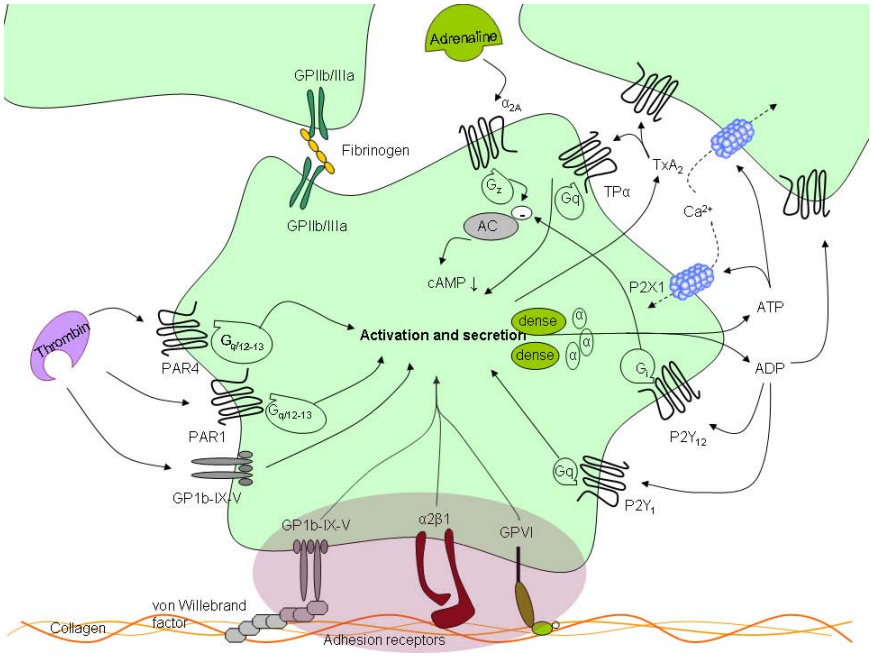


Figure 3: Platelets and an overview of the platelet receptors.

Platelets are not cells by definition but rather cell fragments originating from megakaryocytes. They have neither a nucleus nor ribosomes, which makes them almost unable to synthesise proteins. However they are capable of oxidative phosphorylation and synthesis of fatty acids. Platelets circulate in the blood for an average of approximately 10 days before removal by macrophages. The normal range of platelet number in the blood is 150,000 – 450,000/μL blood; the mean number is approximately 200,000/μL [38]. Resting platelets have a discoid shape of 0.5 – 3 μm. In connection with the microtubule system, which composes the foundation of the cytoskeleton, lies the open canalicular system (OCS) and the dense tubular system (DTS). The OCS is a membrane system which connects the cytosol with the surrounding medium and is believed to be involved in degranulation of platelet granules. DTS stores important metabolic enzymes (reviewed in [39]).

Platelets contain at least three different granules with different contents. The release of granule content is both specific and selective [40, 41].

The dense granule contains calcium, serotonin, ATP/ADP (ratio 2/3) and adrenaline among other substances.

The α-granule contains proteins synthesised by the megakaryocyte as well as proteins taken up from the surrounding plasma. After secretion, these proteins act as procoagulants (fibrinogen, von Willebrand factor, platelet factor 4, β-thrombomodulin) or growth-promoting factors and mitogens (platelet-derived growth factor, thrombospondin). The α-granule also contains receptors such as CD62P (P-selectin), GPIb-IX-V and GPIIb/IIIa as well as coagulation factor V.

Lysosomes contain hydrolases (such as cathepsins D and E, membrane proteins LAMP-1, -2 and -3) that can eliminate the circulating platelet aggregate.

The platelet cytoskeleton serves as a system of molecular struts and girders that defines the discoid shape of a resting platelet. Critical components of this system are a spectrin-based skeleton, a microtubule coil and a rigid network of cross-linked actin filaments (reviewed in [9]). The first event observed as the platelet makes contact with a surface is that the discoid form is lost and it becomes more spherical. Next, finger-like projections grow from the cell periphery, and the platelet surface flattens. As the platelet flattens, granules and organelles are squeezed into the centre of the cell being organised for coming degranulation.

Platelets are the force-generating components of the clot retraction. After activation, GPIIb/IIIa becomes tethered to the underlying actin filaments and, once tethered to actin, cytoplasmic myosin is the molecular motor that applies the contractile force on actin filaments.

PLATELET RECEPTORS

Platelets possess a great number of receptors (see Figure 3), which are concisely reviewed in [42]. In the present thesis I will focus on the receptors involved in my scientific work.

Thrombin receptors

Thrombin is an essential enzyme in the coagulation system, but it is also the strongest endogenous platelet agonist [43]. Thrombin's effect on platelets is mainly mediated by two G-protein coupled receptors, PAR1 and PAR4. However, GPIbα within the GPIb-IX-V complex is also thought to contribute [44], but this interaction is not completely understood. See more in "Role of GPIbα" below.

PAR receptors

PAR1 was the first protease-activated receptor (PAR) to be identified [45]. Today four PARs have been identified, of which only PAR1 and PAR4 are expressed on human platelets [46]. PARs have a unique activation mechanism. Thrombin binds to the receptor and cleaves the extracellular N-terminal at a specific site, and the new N-terminal acts as a tethered ligand binding to the active site of the receptor [46-48]. Thus the PAR1 and PAR4 carry their own hidden ligand. Synthesised peptides mimicking the new N-terminals (SFLLRN for PAR1 [45] and GYPGQV for PAR4 [49]) are useful tools in the study of the receptors. However, the mouse sequence AYPGKF is more potent for PAR4 activation [50].

Shut-off and internalisation of PAR1 depends on phosphorylation of its carboxyl tail on receptor activation. In contrast, phosphorylation of PAR4 has not been detected, and the activation-dependent internalisation was much slower than that seen for PAR1 [51].

Both PAR1 and PAR4 couple to $G\alpha_q$ to activate PLCβ, which in turn leads to increased cytosolic calcium concentration and PKC (protein kinase C) activation (see Figure 4). In addition, both receptors are also coupled to $G\alpha_{12/13}$, which leads to calcium independent shape change on stimulation [52, 53] (see Figure 5). PAR1 activation induces a rapid increase in intracellular calcium, whereas PAR4 triggers a more prolonged response. Results from our group and others indicate that PAR1 and PAR4 do not signal through the same signalling cascade [54-57] and that the outcomes of PAR1 and PAR4 activation differ [58, 59].

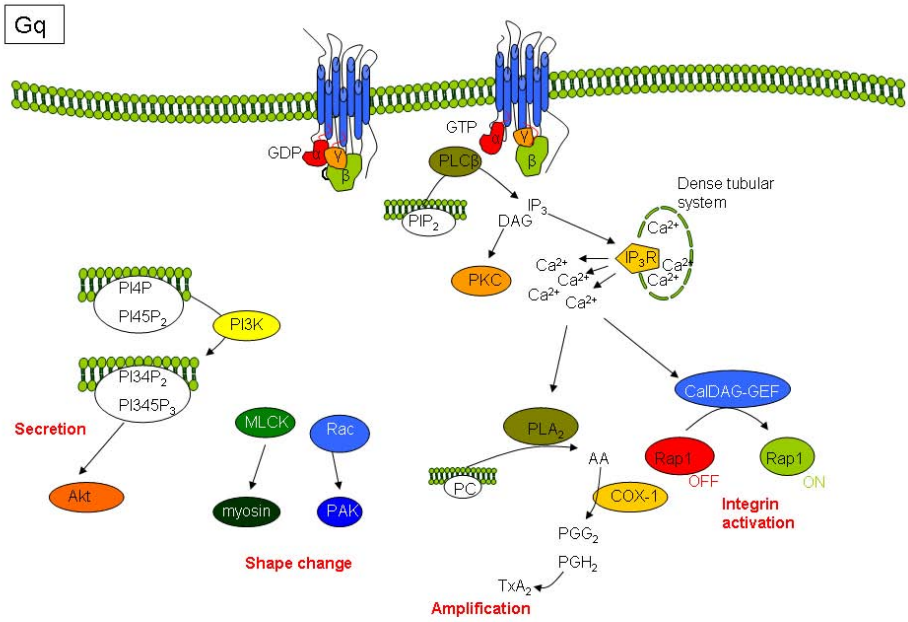


Figure 4: $G\alpha_q$ receptor signalling.

In 2002, Kim et al concluded that inhibition of adenylyl cyclase induced by thrombin and thrombin-receptor activating peptide exclusively depended on secreted ADP stimulating G_i signalling pathways and that thrombin and activating peptides induced platelet aggregation independently of G_i signalling [60]. In contrast to this, Bilodeau et al reported in 2007 a difference between PAR1 and PAR4. They showed that inhibition of PAR4 is dependent on the regulation of both calcium mobilisation and dense granule release, whereas PAR1 is dependent predominantly on the regulation of dense granule release [61]. The same year, Voss's group showed that PAR1, but not PAR4, is G_i coupled and activates PI3K. By inhibiting PI3K they found that PAR1-induced activation of GPIIb/IIIa and aggregation were eliminated, whereas the PAR4-induced response was not affected. However, whether this was a direct effect of PAR1 coupled to G_i or an indirect effect of secreted ADP acting on its G_i receptor was not elucidated.

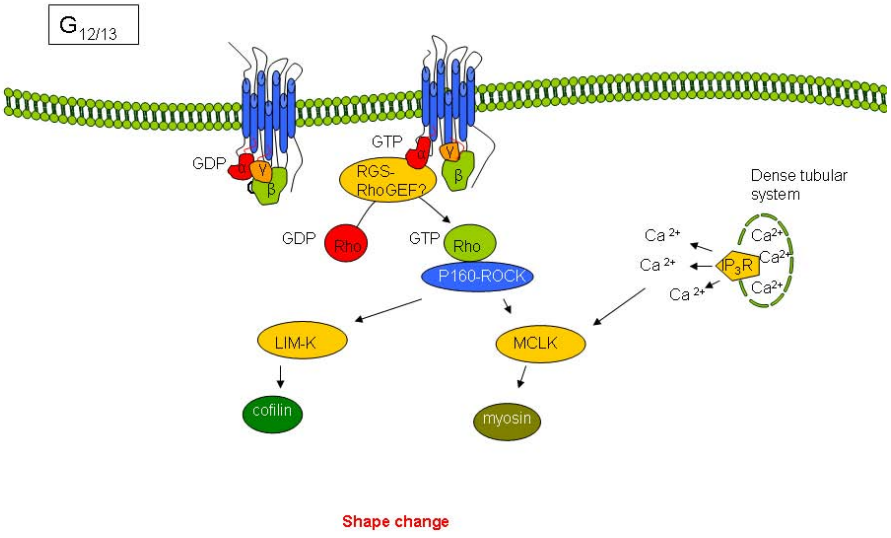


Figure 5: $G\alpha_{12/13}$ receptor signalling.

They also suggest that PLCβ is not required downstream of PAR1 and PAR4 for GPIIb/IIIa activation [57]. Kim's group further demonstrated that $G_{12/13}$, but not G_q, may contribute to thrombin-induced Akt-phosphorylation in platelets. $G_{12/13}$ may mediate its potentiating effect through Src activation. G_q, on the other hand, contributes indirectly to Akt phosphorylation by inducing secretion of ADP acting on its G_i receptor [62]. On the same subject, Reséndiz et al published a paper in 2007 in which they report that Akt is phosphorylated downstream of both PAR1 and PAR4. They saw that rapid Akt phosphorylation occurred downstream of PLC, whereas the Akt phosphorylation was maintained by a positive feedback pathway mediated by PI3K. For PAR1 but not PAR4, this pathway required released ADP acting on its $P2Y_{12}$ receptor [63]. In 2006 Holinstat et al reported that at high concentrations of thrombin, PAR4 induced platelet aggregation via Ca^{2+} mobilisation and synergistic $P2Y_{12}$ activation, whereas PAR1 was insensitive to inhibition of these pathways [55]. In 2007 the same group reported that PAR1, but not PAR4, required a phospholipase D-mediated phosphatidic acid signalling pathway. This was not de-

pendent on differences in granule secretion induced by PAR1 and PAR4 [54]. Recently Holinstat also presented results in concert with earlier results indicating that irreversible platelet activation requires PAR1-induced signalling involving phosphatidylinositol kinases, phosphatidylinositol bisphosphate and Rap1 activation independently of $P2Y_{12}$ [64]. However, there is no final conclusion to this issue.

It has also been proposed that the outcome of receptor activation differs between PAR1 and PAR4.

Our own results indicate that PAR4 activation may be of importance to the clotting time and in stabilising the coagulum [58]. Covic et al have shown that PAR4 stabilises platelet-platelet aggregates and that this effect was not dependent on ADP activation of $P2Y_{12}$ receptor [59]. It has also been proposed that platelets need PAR4 activation for full spreading at a fibrinogen matrix [65].

Role of GPIbα

The specific role of GPIbα in thrombin activation is still unclear. However, in 2001 Soslau et al concluded that GPIb, PAR1 and PAR4 are most likely the three receptors acting upon thrombin stimulation on platelets [66]. The same year it was also proposed that GPIb, at least in part, mediated the thrombin-induced platelet activation that leads to procoagulant activity [67]. In 2003, Adam et al presented a study in which they concluded that proteolytically inactive thrombin immobilised on a surface induced platelet adhesion, spreading, dense granule secretion and GPIIb/IIIa-dependent platelet-platelet interactions. They maintained that this must be dependent on GPIb because no adhesion was seen in patients with Bernard Soulier syndrome, whose platelets are deficient in GPIb [68]. The same year, Dubois et al reported the same phenomenon, namely that thrombin binding to GPIb could induce fibrin binding to GPIIb/IIIa, leading to platelet aggregation [69]. It has also been proposed that GPIb may be a cofactor facilitating cleavage of PAR1 [70, 71].

ADP/ATP-receptors

Platelets possess two ADP receptors, $P2Y_1$ and $P2Y_{12}$. Both of them are G-protein coupled receptors but $P2Y_1$ is Gq-coupled, whereas $P2Y_{12}$ is G_i-

coupled. The PAR receptors' dependence upon secreted ADP acting on $P2Y_1$ and $P2Y_{12}$ has been widely debated.

In 2002, Chung et al concluded that PAR4-induced aggregation was entirely ADP-dependent in contrast to PAR1-induced aggregation. In an article by Nylander et al, our research group showed that only $P2Y_{12}$ inhibition but not $P2Y_1$ inhibition had effect on PAR1- or PAR4-induced platelet activation. However, they, as well as Covic et al [59], speculate that only PAR1-induced ADP release is of physiological relevance because all ADP should already be released before PAR4 is cleaved and activated [72]. As more diverse roles for PAR1 and PAR4 have been proposed, there have also been new suggestions concerning ADP dependence. The findings of Holinstat et al indicate that both PAR1 and PAR4 are influenced by $P2Y_{12}$ activation, but not $P2Y_1$ activation, and that activation of $P2Y_{12}$ may be required for PAR-mediated platelet aggregation [55]. However, Resendiz et al find that $P2Y_{12}$ inhibition abolishes the Akt phosphorylation induced by PAR1-activating peptide (AP) but not PAR4-AP or thrombin, indicating that the $P2Y_{12}$ receptor is required for PAR1 to induce Akt phosphorylation and aggregation, whereas PAR4 and thrombin bypasses $P2Y_{12}$ [63]. On the other hand, Holinstat et al reported in a later publication that PAR1 induces stable platelet aggregates independently of $P2Y_{12}$. More examples are presented in the section about PAR receptors. The discrepancies in these results may be due to the use of different techniques, different parameters in the focus of the study and other yet unknown factors.

P2X receptors are Ca^{2+} permeable ligand-gated non-selective cation channels. The relevance of $P2X_1$ receptors to platelet function has been questioned because they rapidly desensitize [73] and their selective activation *in vitro* evokes a transient shape change without significant aggregation [74]. However, murine models demonstrate an important contribution of this receptor, particularly in small arteries [75, 76]. One explanation for the major contribution of $P2X_1$ to platelet activation *in vivo* is that ATP released from dense granules contributes to signaling events following initial stimulation by other agonists [77]. In support of this, human and murine studies have shown a role for $P2X_1$ in responses to low doses of collagen and thrombin [75]. Whilst much work has been conducted to characterize $P2X_1$ involvement in downstream platelet func-

tion, the extent to which $P2X_1$ acts independently or in synergy with other secondary mediators during the early stages of platelet activation remains unclear.

GPIIb/IIIa (α_{2b}/β_3)

GPIIb/IIIa is a fibrinogen-binding integrin that needs calcium to function properly. Stimulation of the integrin is induced by several platelet agonists and results in a conformational change leading to binding of fibrinogen and VWF, and thereby platelet aggregation and clot retraction [78, 79]. Activation of the receptor also leads to cytoskeletal reorganisation, allowing full aggregation, granule secretion and platelet spreading with the help of collagen receptors and VWF.

CD62P (P-selectin)

CD62P (P-selectin, GMP140) is a transmembrane protein normally localised in the membranes of the α-granule. On platelet activation the granule content is released, and the receptor is translocated to the cell surface. CD62P plays an important part in the rolling of leucocytes on activated endothelial cells and platelets. The expression of CD62P will also induce newly recruited platelets to roll through interactions with their GPIb-V-IX complex [80].

Adrenaline receptor

Adrenaline (referred to as 'epinephrine' in the U.S.A.) is a catecholamine released during a sympathetic activation such as myocardial infarction and physical exercise. Adrenaline acts by binding to the G_z-coupled α_{2A} adrenergic receptor and by inhibiting adenylyl cyclase and the production of cAMP [81].

INTRACELLULAR SIGNALLING

Considering that perhaps only a minority of the proteins involved in platelet signalling are characterised, it is important to remember that models of platelet signalling are only models that are based on current knowledge. The current understanding of platelet signalling includes a few main activating and inhibiting pathways. These will be discussed in this section.

Overall, the main platelet stimuli can be divided into adhesion and GPCR-dependent activation, see Figure 6.

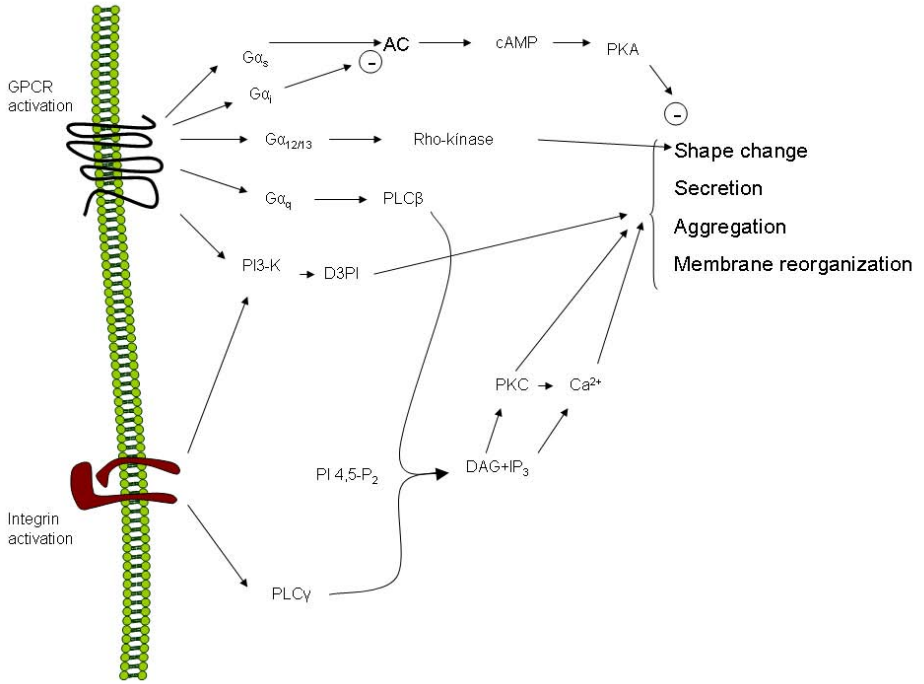


Figure 6: Simplified scheme of platelet signalling.

Integrin-dependent activation has been described as relying on adhesion-induced clustering of the receptors involved (reviewed in [2, 3, 7, 82, 83]. This is possible due to the versatile nature of the ligands, VWF, fibrinogen and fibrin and collagen. Integrin signalling leads to activation of PLCγ [2, 3, 82]. Activation of GPCRs can lead to both activation and inhibition of the platelets by signals mediated via different Gα subtypes. $G\alpha_{12/13}$ mediates a calcium-independent shape change that involves Rho-kinases [84, 85]. GPRCs coupled to $G\alpha_q$ activate PLCβ. Both PLCγ and PLCβ hydrolyses phosphatidylinositol 4,5 bisphosphate (PI 4,5-P_2) into inositol trisphophate (IP_3) and diacylglycerol (DAG) [86]. IP_3 will induce the release of calcium stored in DTS into the cytoplasm, and DAG combined with calcium will activate protein kinase C (PKC). Multiple isoforms of PKC are expressed in platelets, and evidence are emerging that different isoforms play distinct roles in the platelet activation process [87]. PKC signalling has been suggested to play a role in Ca^{2+} entry [88, 89], granule secretion [90], GPIIb/IIIa activation [91] and outside-in signalling

[92, 93]. PKC is also involved in negative regulation of platelet function and has been reported to be involved in receptor desensitisation [94], extrusion of intracellular Ca^{2+} [95], secretion and actin-mediated filopodia formation [96]. Calcium is released from the DTS or via influx through ion channels in the plasma membrane. An increase in cytosolic calcium induces several processes, including the initiation or acceleration of membrane reorganisation, shape change, secretion, Thromboxane A_2 production, aggregation. PKC activates a number of substrates which are involved in processes contributing to platelet activation [97]. Finally, secondary agonists such as ADP, ATP, serotonin and chemokines secreted from granules on activation amplify the platelet activation by activating their respective platelet receptors. GPCRs coupled to $G\alpha_s$ provide a negative regulation of platelet activation by activating adenylyl cyclase (AC) resulting in increased synthesis of cyclic adenosine monophosphate (cAMP). cAMP serves as a negative regulator of platelet function by activating PKA, which phosphorylates a number of substrates that lead to decreased concentrations of cytosolic calcium. PKA may also conclude GPCR signalling [97]. $G\alpha_i$ signalling will counteract $G\alpha_s$ as it inhibits AC and thereby reduces the cAMP concentration. Several subtypes of PI3-kinases are present in platelets and can be differentially activated by both adhesion and GPCRs [3, 98, 99]. PI3-kinases specifically phosphorylate the D3 group of the inositol ring of PI. D3PI acts as a potent second messenger promoting platelet activation [99]. The three isoforms if PI3K, α, β,γ, seems to have distinct functions but all appears to be needed to stabilise growing thrombi and prevent their dissolution [100].

AIMS OF THE THESIS:

* To study the role of platelet PAR1 and PAR4 in thrombin-induced platelet activation and coagulation

* To study the role of PAR1 and PAR4 in the cross-talk of thrombin and adrenaline.

* To study the variation in PAR1 receptor number and how the variation affects the response on activation.

* To study the platelet response to activation of PAR1 and PAR4 individually and co-stimulated.

METHODOLOGY:

FLOW CYTOMETRY

Flow cytometry can be defined as simultaneous measurements of multiple specific characteristics on single cells in a moving fluid (reviewed in [101]). The analysis is based on the differences in the light-scattering properties of the cells and on fluorescent signals. The sample is arranged in a stream of single cells in front of a laser beam. Traditionally, an argon laser with a 488 nm wavelength has been used, but today there is a broad variety of lasers and wavelengths to choose between. The cells are carried by a sheath fluid into the flow chamber in which the light is scattered by the aligned cells. The light scattered in the extension of the incoming laser is referred to as forward scatter (FS). The FS detector converts the FS light to a voltage pulse that is related to the cell size. The side scatter (SS) light can also be detected and reflects the cytoplasmic complexity/granularity of the cell. Fluorescence will occur when the wavelength of the laser beam can cause excitation of the fluorochromes bound to or incorporated with the studied cells. Exited fluorochromes will then emit light at higher wavelengths, which is collected by the optic and electronic system of the cytometer.

PLATELET ACTIVATION MEASURED BY FLOW CYTOMETRY

Platelet activation can be measured by flow cytometry in a number of ways [102-105]. In three of my four papers platelet activation was measured as platelet-bound fibrinogen and CD62P expression on the platelet surface utilising flow cytometry. A typical protocol looks as follows. Well-mixed anticoagulated whole blood was transferred to plastic tubes containing FITC-conjugated antibodies against human fibrinogen or anti-CD62P and HEPES buffer. An agonist was added to the samples at exact time intervals. After exactly 10 minutes, the reaction was stopped by the addition of 1mL of HEPES buffer. The samples were diluted 1:5 in HEPES buffer before analysis on the flow cytometer. All steps were performed at room temperature and without stirring the samples. The platelet population was identified using its forward light scatter and side light scatter; 5000 particles were collected. A cut-off was pre-set in the

FITC fluorescence channel to divide the platelet population containing non-binding antibody with corresponding fluorescence intensity into two fractions: one that contained 98.5 – 99.5% of the platelets and the other containing the brightest 0.5 – 1.5% of the platelets. Platelets with fluorescence intensity higher than the pre-set cut-off were identified as fibrinogen-binding or CD62P-positive cells. All samples were run in duplicate. In the same flow cytometry experiment the mean intensity of the fluorescence (MFI) for the whole platelet population was also analysed. The MFI value, an arbitrary unit which depends on the brand of the flow cytometer, shows the mean fluorescence signal from all individual platelets. This correlates to the number of fluorescent antibodies that have bound to the platelet surface.

MEASUREMENTS OF PAR1 RECEPTOR NUMBER

For the PAR1 density measurements, whole blood was diluted (1:4) with the buffer provided in the Platelet Calibrator kit (Platelet Calibrator, Biocytex, Marseille, France). The diluted blood was incubated with anti-PAR1 (WEDE15) or anti-GPIb mouse antibody or the same concentration and volume of isotypic control. After 10 minutes of incubation, a FITC-labelled secondary polyclonal anti-mouse IgG antibody was added, and after an additional 10 minutes of incubation the mixture was diluted with 1mL of the buffer provided with the kit. Calibration beads were also stained with the secondary antibody and then diluted with 1mL of buffer. All steps were performed according to the manufacturer's instructions. The sample tubes were stored in the dark and analysed with flow cytometry for MFI within 2 hours. On every occasion a calibration curve was elicited, based on the MFI and the known number of antigen sites for the calibration beads. By using the calibration curve, the number of specific PAR1 receptors could be obtained by subtracting the total number of the anti-PAR1 antibody binding sites with the negative isotypic control. To reduce the errors caused by imprecision in the reading of the fluorescence of the calibration beads, we chose to read all samples with the same calibration curve, where the fluorescence values were a mean of 24 measurements performed during the time of the study. A number of samples were also prepared with whole blood, previously activated with different agonists for 10 minutes. Blood samples from some individuals were activated both in the absence and presence of Ro

44-9883 (fibrinogen receptor antagonist, la Roche ltd, Basel, Switzerland) or recombinant hirudin to check for eventual platelet aggregation or thrombin formation in the original experimental setup. As an additional test for platelet aggregation, the time needed for detection of 5000 single platelets during the flow cytometry analysis was also recorded for all samples analysed. All experiments were performed at room temperature and without stirring. All samples were run in duplicate.

RHEOLOGY

Viscosity is a measure of the thickness, or resistance, of a fluid and can be divided into three categories: **Newtonian fluids**, in which the viscosity depends on the temperature but not variations in shear rate or shear stress e.g. water, milk and oil. Cell-free plasma also behaves as a Newtonian fluid; **Non-Newtonian** in which the viscosity depends on both temperature and shear rate. These fluids may be **time-dependent** or **time-independent**. Whole blood is one example of a time-independent fluid in this group (reviewed in [106]). Viscosity measurements in blood can be done in a dynamic instrument where the sample- rotates (shearing) or oscillates (reviewed in [107]). In a low-frequency experiment, the blood will appear to be viscous but in a high-frequency experiment it will appear to be elastic. This elastic nature of the blood is mainly due to the cell properties and, to a large extent, the red blood cells which constitute 99% of the blood cellular volume [107].

In Paper I [58] we used rheology to measure clotting time, clot elasticity and fibrinolysis. For these measurements of rheological changes in whole blood we used a four-channel 10 Hz free oscillation rheometer (FOR) ReoRox®4 [108, 109], disposable gold- plated polypropylene sample cups, gold-plated reaction chambers for bob experiments, and software (ReoRox®4 v. 2.00 and ReoRox®4Viewer v. 2.11k) from MediRox, Nyköping, Sweden. In this rheometer, oscillation is initiated by a forced turn of the sample cup every 2.5 seconds. After a brief hold time, the sample cup is released, allowing rotational oscillation with very low friction around the longitudinal axis. An optic angular sensor records the frequency and damping of the oscillation as a function of time. This is plotted as a curve, from which the time for different curve features may be determined.

By definition, the clotting time is when fibrin fibres have started to form in the sample. This increases the viscosity of the sample, which leads to a decrease in the frequency and an increase in the damping of the oscillation. The "high-sensitivity state detector" was used as a clotting time detector in this study. The clotting time determined by this detector correlates extremely well with the manual reference method of visual clot detection, $r^2=0.97$, as shown by our group [110]. The coagulation process creates fibrin fibres that couple the cup wall to the bob, resulting in an increased frequency and damping of the oscillation as the elasticity of the coagulum rises.

The elasticity measurements were analysed in rh_pro, a computer program developed in-house. In this program the elasticity modulus (G') is plotted as a function of time. From this curve two specific curve features can be determined, ΔG' and G'max. ΔG' (Pa/min) is the maximum change in elasticity achieved during clot development, i.e. where the curve slope reaches its highest value. G'max (Pa) is the G' value read at the point at which the increase has become less than 1 Pascal per second, which is when the elasticity has reached its maximum. Typical curves are shown in a report by Ramström et al [111]. The start and endpoints of the fibrinolysis process were determined using Lysview, a computer program developed in-house. The fibrinolysis start point is when the fibrin fibres in the clot have started to degrade. This decreases the viscosity of the sample, which leads to an increase in frequency and an increase in the damping of the oscillation. The fibrinolysis endpoint is when all fibrin fibres have been degraded and the frequency and damping have returned to the same values as in the sample prior to coagulation (see figure 7).

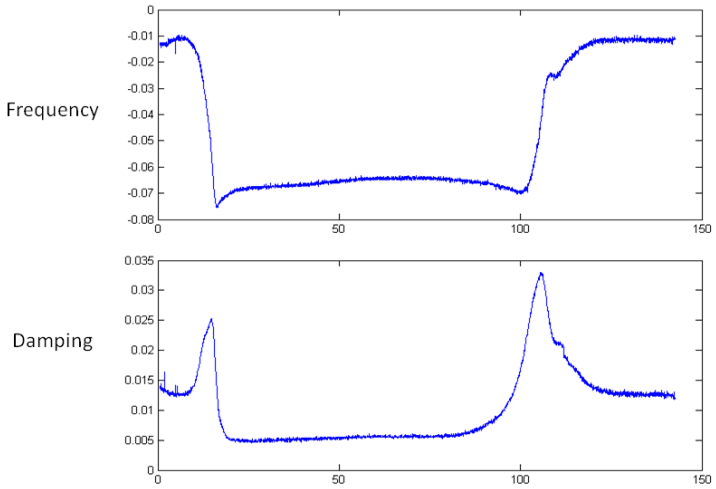


Figure 7: Fibrinolysis curve; frequency and damping.

THROMBIN GENERATION

It should be clear that there is an important difference between the measurements of thrombin generation in an isolated environment in vitro compared with the physiological process in which several components are involved.

Thrombin generation in plasma represents the function of a relevant slice of the in vivo system with all the plasma proteins present, unmodified and near their physiological concentrations. However, the vessel wall is lacking, and in order to simulate its presence the two most important known elements, tissue factor and thrombomodulin, must be added to the plasma.

The thrombin generation test we used is based on a 96-well plate filled with PRP (platelet rich plasma) samples to which a Thrombin Calibrator or PPP (platelet poor plasma)/PRP trigger is added. The PPP reagent contains a mixture of phospholipids and TF, the PRP reagent contains TF, according to the manufacturer (Thrombinoscope BV). The thrombin calibrator is a compound that produces a thrombin-like activity, is not inhibited by plasmatic inhibitors

and does only react with the fluorogenic substrate. The amidolytic activity of this compound can therefore be directly related to the activity of an unknown amount of thrombin in a plasma sample (the Thrombogram Guide by P. Giesen, at Thrombinoscope). The instrument dispenses a mixture of fluorogenic substrate and calcium whereupon the coagulation begins. Finally, the analysis program calculates the parameters of the thrombogram (lagtime i.e. the moment at which thrombin formation starts, ETP (endogenous thrombin potential) i.e. concentration multiplied by time (nanomolar x minute), peak height in nanomolar thrombin, time to peak in minutes, and start tail, which is the time at which thrombin generation has come to an end) and expresses the result as the concentration of thrombin (in nM) at different time points [112]. In Paper I [58] we measured thrombin generation to see possible differences between PAR1 and PAR4 when activated by thrombin concentrations produced by the coagulation itself.

PCR

PCR (polymerase chain reaction) is a technique for multiplying a specific sequence of DNA (reviewed in [113]). Almost all PCR applications employ a heat-stable DNA polymerase. This DNA polymerase enzymatically assembles a new DNA strand from DNA building blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiating DNA synthesis. The vast majority of PCR methods use thermal cycling, i.e. alternately heating and cooling the PCR sample to a defined series of temperature steps. These thermal cycling steps are necessary to physically separate the two strands in a DNA double helix at a high temperature in a process called DNA melting. At a lower temperature, each strand is then used as the template in DNA synthesis by the DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions. In Paper II [114] we used PCR to amplify a fragment of 155 base pairs encompassing the SNP of the PAR1 receptor gene reported to affect the PAR1 expression (IVSn-14 A/T SNP).

DENATURING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Denaturing high performance liquid chromatography (DHPLC) uses reversed-phase HPLC to interrogate SNPs (single nucleotide polymorphisms). Using PCR, two fragments are generated; target DNA containing the SNP polymorphic site and an allele-specific DNA sequence, referred to as the normal DNA fragment. This normal fragment is identical to the target DNA except potentially at the SNP polymorphic site, which is unknown in the target DNA. The fragments are denatured and then allowed to gradually re-anneal. The re-annealed products are added to the DHPLC column. If the SNP allele in the target DNA matches the normal DNA fragment, only identical homoduplexes will form during the re-annealing step. If the target DNA contains a different SNP allele than the normal DNA fragment, heteroduplexes of the target DNA and normal DNA containing a mismatched polymorphic site will form in addition to homoduplexes. The mismatched heteroduplexes will have a different melting temperature than the homoduplexes and will not be retained in the column as long. This generates a chromatograph pattern that is distinct from the pattern that would be generated if the target DNA fragment and normal DNA fragments were identical. The eluted DNA is detected by UV (reviewed in [115]). The IVSn-14 A/T SNP in Paper II [114] was genotyped on an automated DHPLC analysis system (Transgenomic Inc, Omaha, USA). Hetero- and homoduplexes were detected by UV absorbance at 260 nm. Homozygous mutants were detected by mixing samples with a known wild type sample in a 1:1 ratio to induce heteroduplex formation.

MEASUREMENT OF CYTOSOLIC CA^{2+}

Measurement of intracellular Ca^{2+} levels is used in a variety of cell types (reviewed in [116]). An increase in intracellular Ca^{2+} is a messenger for several signalling pathways in cells, and the study of changes in cytosolic Ca^{2+} is a useful tool to understand cell signalling.

In our case [56] platelets were loaded with fura-2 by incubating PRP with fura-2-acetoxymethylester. The platelets were pelleted and resuspended. Fluorescence signals from platelet suspensions were recorded using a fluorescence spectrofluorometer specially designed for cytosolic free Ca^{2+} concentration

(Hitachi F-2000), $[Ca^{2+}]_i$, measurements. Fluorescence emission was measured at 510 nm with simultaneous excitation at 340 nm and 380 nm. $[Ca^{2+}]_i$ was calculated according to the general equation as described previously [117] $[Ca^{2+}]_i = K_d(R-R_{min})/(R_{max}-R)(F_o/F_s)$. Maximum and minimum ratios were determined by adding 0.1 % Triton X-100 and 25 mM EGTA, respectively. In Paper III [56] we measured cytosolic Ca^{2+} to study differences in PAR1 and PAR4 activation.

MEASUREMENT OF PLATELET AGGREGATION

There are a few different techniques for measuring platelet aggregation (reviewed in [118]). In Paper III [56] all aggregation studies are based on turbidimetric aggregometry, which measures light transmission through platelet rich plasma (PRP) or washed platelets. The PRP solution is initially turbid but allows for increasing transmittance of light as larger and larger aggregates form subsequent to stimulation with an agonist.

MEASUREMENT OF DENSE GRANULE SECRETION

ATP secreted from dense granules binds with luciferin-luciferase producing light that can be measured. In Paper III [56] we used a Chronolog lumi-aggregometer to study differences in dense granule secretion induced by PAR1-AP or PAR4-AP.

The luminescence is proportional to platelet ATP secretion thereby permitting agonist-induced aggregation and dense granule secretion to be monitored simultaneously (reviewed in [119]).

MEASUREMENTS OF PLATELET AGGREGATION ON MICROTITER PLATES

A microtiter plate assay for platelet aggregation measurement is a rapid and time-efficient method in which multiple treatments can be tested simultaneously using only small volumes of PRP.

Agonists are first added to the wells of the microtiter plate followed by PRP, and the measurements are started immediately.

Measurements are conducted in a microplate reader in which the wells are vigorously shaken. The extent of the aggregation is estimated with the equation: Aggregation = $(Abs1_{650} - Abs2_{650})/Abs1_{650}$, where $Abs1_{650}$ is the aggregation end point for the control, and $Abs2_{650}$ is the aggregation end point with aggregation-inducing agonist added. In Paper IV we used PRP and saline as controls. These values are then normalised by comparison with the maximum end point aggregation response, in accordance with [120]. To handle all the samples of the synergy experiments in Paper IV, we chose to use this aggregation assay, which allows a large number of samples and requires only a small volume of PRP.

ANALYSIS OF SERINE PHOSPHORYLATED AKT

To analyse serine^{473} phosphorylated Akt or other proteins, the samples (e.g. fragmented cells) are separated by electrophoresis. The theory of electrophoresis is that by placing the molecules in wells in a gel and applying an electric current, the molecules will move through the matrix at different rates, determined largely by their mass when the charge-to mass-ratio (Z) of all species is uniform, towards the anode if negatively charged or towards the cathode if positively charged (reviewed in [121]). The proteins are then transferred to a membrane where they are probed (detected) using antibodies specific to the target protein. For the detection of phosphorylated Akt in paper III [56], a polyclonal antibody directed towards the serine^{437} position of Akt and a secondary horseradish peroxidase-conjugated antibody were used. After the unbound probes are washed away, the western blot is ready for detection of the probes that are labelled and bound to the protein of interest. This may be performed in different ways depending on the substrate that is used as a probe for the antibodies. Chemiluminescent detection methods depend on incubation of the western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light is then detected by photographic film, and more recently by CCD cameras, which capture a digital image of the western blot. The image may be analysed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density. Newer software allows further data analysis such as molecular weight analysis if appropriate standards are used. In Paper III [56] we used Akt phosphorylation as an indicator of the intracellular signalling pathways that were involved in the cross-talk between thrombin and adrenaline.

RESULTS AND DISCUSSION:

PAPER I:

In this study we wanted to find out more about the relative impact of PAR1 and PAR4 in platelet activation and blood coagulation because little is known about the specific effects of these two thrombin receptors. By activation and/or inhibition of each receptor individually we studied platelet activation, clotting times, clot elasticity, fibrinolysis and thrombin generation. We found that maximum activation of PAR4 induced more fibrinogen binding than maximum activation of PAR1. The CD62P exposure was the same for both receptors. The clotting times were more prolonged when PAR4 was inhibited than when PAR1 was inhibited. Moreover, the start of thrombin generation and the time to peak were more delayed by PAR4 inhibition than PAR1 inhibition. However, the final amount of thrombin generated was not affected. This might indicate that PAR4 plays a more important role early in the coagulation process than it has previously been thought to do. Inhibition of PAR1 significantly increased fibrinolysis time and the time from clotting to the start of fibrinolysis. In contrast, inhibition of PAR4 slightly decreased the fibrinolysis time. Clot elasticity significantly decreased when PAR4 was inhibited. We speculate that this may be related to the fact that PAR4 was able to induce more fibrinogen binding than PAR1 did. Clot elasticity was not significantly affected by PAR1 inhibition. We found a pronounced variation between donors in our rheology experiments.

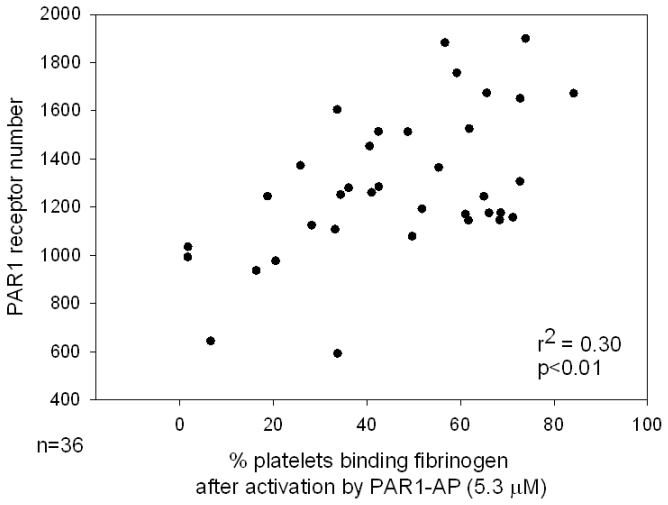
All this led us to conclude that PAR4 most likely is activated by low concentrations of thrombin during the initial phase of thrombin generation and is of importance to the clotting time. We also detected a possible physiological role for PAR4 in the stabilisation of the coagulum.

PAPER II:

In this study we wanted to correlate the response from activated PAR1 to the number of PAR1 receptors on each platelet. We also wanted to evaluate a possible decrease in receptor number after stimulation with different agonists, indicating internalisation of the PAR1 receptor. We detected a large variation in PAR1 receptor number in healthy individuals, a variation that correlated well to the activation response induced by PAR1-AP, see Figure 8a. However, other factors are also likely to exert an influence. The exclusion of the ADP component of the PAR1-AP-induced response did not improve the correlation between PAR1 receptor number and platelet activation response. A portion of the examined individuals were genotyped for the known polymorphism in the intervening sequence (IVSn)-14 A/T. The results showed a trend, although there was no statistically significant difference in PAR1 receptor number and platelet reactivity between A/A individuals and T/A or T/T individuals. We found a significant reduction in PAR1 receptor surface exposure after activation with PAR1-AP, whereas activation with ADP, PAR4-AP or CRP increased the surface exposure of PAR1 receptors, see Figure 8b.

The large inter individual variation may explain a part of the large inter- individual variation found in Paper I.

a.



b.

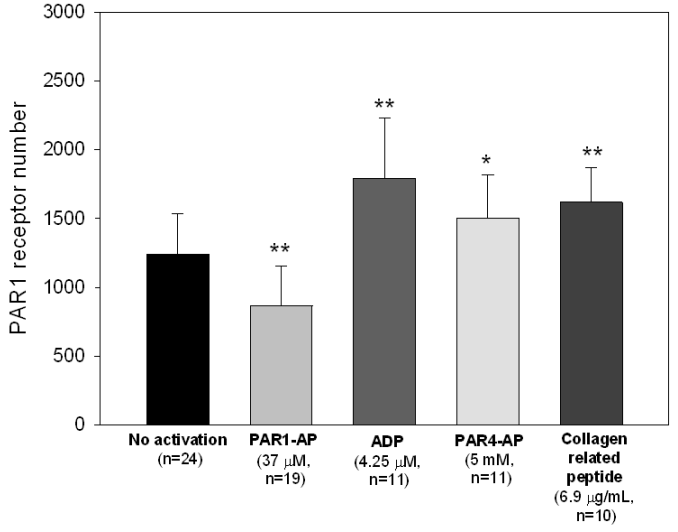


Figure 8: a. Correlation between the number of PAR1 receptors/platelet and the response received on stimulation. b. PAR1 receptor number measured after stimulation with different agonists.

PAPER III:

This project ran over a long period of time and took many turns before final publication, in which only a portion of the experiments was presented.

Our main focus was the respective role of PAR1 and PAR4 in the cross-talk between thrombin and adrenaline and the importance of secondary release of ADP/ATP.

We found that PAR4, but not PAR1, interacted synergistically with the α_{2A}-adrenergic receptor to cause pronounced platelet activation. This was seen in platelets pre-treated with acetylsalicylic acid (ASA, aspirin). Without ASA the response of PAR1 and PAR4 treatment in combination with adrenaline was the same. Considering that ASA is widely used around the world to treat cardiovascular diseases, it should be noted that the synergistic effect of PAR4 and adrenaline is pronounced when COX-1 is inhibited by ASA. We also noted that ATP (and not ADP) via the $P2X_1$ receptor is a key mediator in the cross-talk between PAR4 and adrenaline. Furthermore, we suggest that PAR4-induced activation of PI3-kinase participates in the signalling of this cross-talk, leading to a stronger platelet response. This pathway may be of clinical importance, i.e. treatment failure for patients on aspirin.

All together these findings imply that PAR4 plays an important and unique role in platelet signalling and activation. This is in accordance with our results in Paper I.

PAPER IV:

In this paper we wanted to provide evidence for what we have seen before – that PAR4 is not only a copy of PAR1 activated by higher concentrations of thrombin, but rather plays its own specific role possibly due to a different signalling mechanism or to some yet unknown amplification/regulation mechanism.

Our hypothesis was that if the receptors were coupled to the same intracellular mechanisms, co-stimulating them would lead to an additive effect, i.e. the summarised effect of the receptors activated singularly. However, if the response received from co-stimulation of the receptors was more than additive, we would see a synergistic action; if the response was less than additive, we would have an inhibitory effect.

We saw a strong synergistic effect of co-stimulation of PAR1 and PAR4 both on platelet activation measured as fibrinogen binding and CD62P expression, see Figure 9, and on platelet aggregation. To confirm the synergistic effect we used Berenbaum's equation [122] to calculate the synergy quotas in order to get a value for the effect. A synergy quota higher than 1 indicates synergy; the quotas for all of the measured parameters in this study were higher than 3.2. This leads us to believe that PAR1 and PAR4 may not transmit the incoming signal in the same way, but rather work in an intricate way to strongly support each other at sub-threshold and low concentrations of agonists.

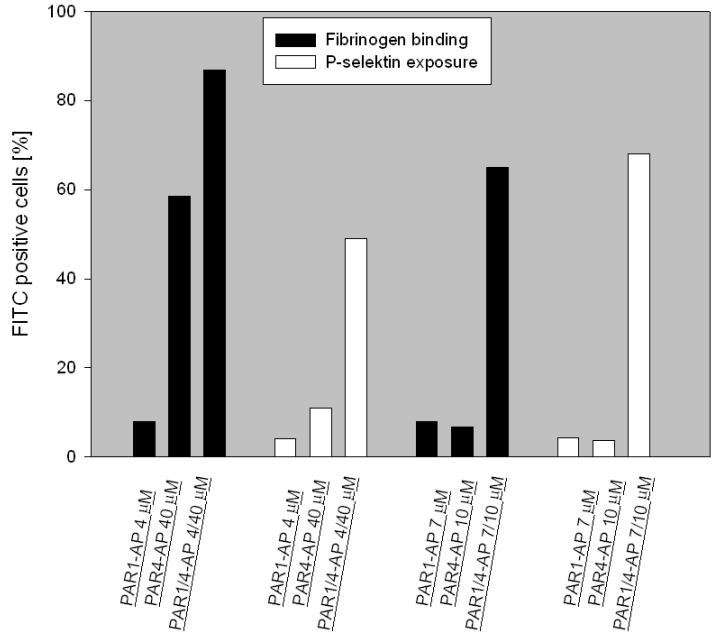


Figure 9: Measurements of platelet activation on one representative donor.

GENERAL DISCUSSION

The connecting thought in these projects has been to learn more about platelet thrombin receptors PAR1 and PAR4 and mainly to elucidate the differences between them. The platelet thrombin receptors are very important for both platelet activation and aggregation but also for the coagulation. However, the relative role of PAR1 and PAR4 is not clear. Our hypothesis was that there were differences between PAR1 and PAR4 activation which might lead to different effects on the platelet function. Our aim was to elucidate these differences and, if possible, to find receptor-specific physiological functions.

In Paper I [58] we used several platelet function analyses to study differences in PAR1 and PAR4 activation. The finding that PAR4 seems to have its own specific role in platelet activation and coagulation was interesting and somewhat in line with other work published at about the same time. Covic et al had seen that PAR4 activation stabilises platelet-platelet aggregates [59]. One other example of a specific role for PAR4 is that only PAR4 activation induces sustained Ca^{2+} mobilisation and full platelet spreading on a fibrinogen matrix [65]. The idea that PAR1 and PAR4 would have different tasks in platelet activation and coagulation seems plausible considering the multi-functional nature of thrombin and the many functions of platelets in the coagulation. The fact that PAR4 seemed to be of importance also at low concentrations of thrombin when only PAR1 is activated may indicate that the main purpose of PAR4 is not to produce the same response that PAR1 does, but rather to initiate other processes not yet fully understood.

Knowing that PAR1 and PAR4 probably have distinct roles in the coagulation we chose to study PAR1 in greater detail in Paper II [114]. We thought that it would be interesting to know the variation in receptor number, especially if it is correlated to the response, to help us interpret our data. The large variation in PAR1 receptor number among healthy individuals found in Paper II and the fact that it was correlated to the platelet response may in part explain the large variation between donors found in Paper I. The finding that the correlation was not improved by excluding the ADP component was perhaps somewhat surprising because it is known that the secretion of, and response to ADP varies between individuals [123]. We have paid a lot of attention to, and spent

many hours, trying to measure PAR4 density on platelets. We have tried to overcome the lack of mouse anti-PAR4 antibody by using a secondary mouse antibody binding to a chicken antibody binding to PAR4. However, this has turned out to be more complex than expected and remains to be achieved.

It would be of great interest to be able to measure both the PAR1 receptor number and the PAR4 receptor number simultaneously and correlate that to the response induced by thrombin. It would also be interesting to measure the receptor number in patients with thrombosis problems to see whether they had a large receptor number compared to healthy individuals.

The well known phenomenon that adrenaline amplifies the response of sub-threshold concentrations of thrombin (and other platelet agonists) [124, 125] was studied in detail in Paper III [56]. To learn more about the differences between PAR1 and PAR4 we wanted to study the relative importance of the receptors in this cross-talk. We found that PAR4 accomplishes this effect in a way that PAR1 could not copy. This is another demonstration of what we saw in Paper I; that PAR4 plays its own specific role in haemostasis. The fact that adrenaline synergistically amplified the effect of low concentrations of PAR4-AP in the absence of COX-1 could be of great interest considering that the treatment with COX-1-inhibitor ASA is widely spread. The finding that ATP rather than ADP played a pivotal role in this interplay was interesting, in addition to knowing that ADP amplifies the response of thrombin and PAR-activating peptides. We also suggest a possible signalling pathway for PAR4 involving PKC, PI3-kinase and Akt phosphorylation. The mechanisms by which PARs signal platelet activation have not been well defined. Although there is evidence that PAR1 and PAR4 signalling pathways are not redundant [55, 126-128], delineating the specific signalling pathways downstream of each receptor has proven to be problematic. It has been shown that Akt is phosphorylated downstream of both human PARs and that rapid Akt phosphorylation occurs downstream of PLC, whereas it is maintained by a positive feedback pathway mediated by PI3K. For PAR1 this pathway requires the binding of released ADP. However, this is not true for PAR4 [63], possibly indicating that the role of PAR4 is to sustain the thrombin effect, in line with the previously discussed results on the PAR4 effect for stabilising the coagulum.

In Paper IV we wanted to pinpoint our hypothesis about PAR1 and PAR4 and the assumption that they differ in regard to outcome of activation. The synergy effect had been previously proposed [129] but never elucidated this way. Many possible roles for PAR1 and PAR4 have been described; for example they have been reported to counter-regulate endostatin and VEGF release from human platelets [40].

Our results from Paper IV do not elucidate any specific role for PAR1 and PAR4 (as we did in Papers I and III) but rather demonstrate that it is highly unlikely that PAR1 and PAR4 act in physiologically the same way. Here we present strong evidence for the synergistic action of PAR1 and PAR4 which indicates an intriguing interplay between the receptors in a way not yet known.

PRINCIPAL FINDINGS

- PAR4 is activated by low concentrations of thrombin during the initial phase of thrombin generation and is also of importance for clotting time.
- PAR4 may play a physiological role in the stabilisation of the coagulum.
- There is a large inter-individual variation in PAR1 receptor number per platelet. The receptor number is correlated to the platelet response upon PAR1 activation.
- PAR4 is responsible for the cross-talk between thrombin and adrenalin in a COX-1 independent way, and ATP is an important co-actor in this cross-talk.
- Co-stimulation of PAR1 and PAR4 with sub-threshold concentrations of activating peptides induces a very strong platelet activation and rapid aggregation. This is due to a true synergy and possibly different mechanisms of action for the two receptors.

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