

# Transient and persistent aspects of human platelet activation

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## **Summary**

Blood platelets establish the formation and development of arterial thrombi and orchestrate normal hemostasis. Current antiplatelet agents prescribed for the prevention of (secondary) thrombotic events are associated with bleeding side effects. It is considered that improved drugs will arise from a better understanding of the complex mechanisms of platelet activation. Human platelets can be stimulated via multiple receptors, with clear (patho)physiological importance of the signaling collagen receptor, glycoprotein VI (GPVI), the protease-activated receptors for thrombin, PAR1 and PAR4, the receptors for ADP, P2Y<sub>1</sub> and P2Y<sub>12</sub>, and the thromboxane A<sub>2</sub> receptor TP. Crucial outcomes of the platelet activation process are a change in the fibrin(ogen) receptor integrin αIIbβ3 from the inactive to the active, ligand-binding state; the secretion of storage granules (assessed as P-selectin expression); and the development of procoagulant activity. In this thesis, I examined the intrinsic reversibility of integrin  $\alpha IIb\beta 3$  activation, such in contrast to the irreversible secretion process. Furthermore, I studied the transiency in Ca<sup>2+</sup> signaling of activated platelets, and I compared the development of procoagulant activity of platelets with that of red blood cells. The thesis thereby focuses on key signaling pathways contributing to transient state of platelet activation.

Chapter 1 provides a brief, general background on the roles of key platelet receptors in thrombosis and hemostasis. Particular attention is paid to GPVI and PAR1/4, with as downstream signaling modes the activation of protein tyrosine kinases and protein kinase C (PKC). The chapter furthermore introduces how, next to PKC isoforms, elevated cytosolic  $Ca^{2+}$  can be placed in the center of the platelet activation process, also mentioning the main  $Ca^{2+}$  transporters in store-regulated  $Ca^{2+}$  entry, including the Orai1 channel. Furthermore, I describe the blood coagulation as a process that can be monitored by determining the generation and inactivation of thrombin. **Chapter 2** provides an in-depth review, describing the current knowledge on platelet agonists and conditions that lead to a reversible platelet aggregation as a result of transient ligand binding to integrin  $\alpha$ IIb $\beta$ 3. Highlighted herein are the roles of the platelet ADP receptors, and the signaling pathways downstream of these via phosphoinositide 3-kinase (PI3K) isoforms. Argued is how a transient P2Y1/12 receptor function and PI3K activity relate to the observed GPVI- and PAR-dependent reversibility of integrin activation.

To better understand how other signaling pathways contribute to a transient platelet integrin  $\alpha IIb\beta 3$  activation, in **Chapter 3**, we used a panel of pharmacological inhibitors to pre-treat or post-treat platelets activated via the GPVI, PAR, or P2Y receptors. These inhibitors targeted PKC or PI3K isoforms,

glycogen synthase kinase-3 (GSK3), Ras-related proteins or  $\beta$ -arrestins. The most responsive platelet receptors in terms of transiency appeared to be PAR1 (triggered by the peptide TRAP6), P2Y12 (triggered by ADP) and GPVI (triggered by collagen-related peptide). We established that platelet pre-treatment with the inhibitors decreased GPVI- and PAR-induced  $\alpha$ IIb $\beta$ 3 activation and P-selectin expression in the order of PKC > GSK3 >  $\beta$ -arrestin > PI3K. In addition, the post-treatment with inhibitors revealed secondary  $\alpha$ IIb $\beta$ 3 inactivation (not P-selectin expression), in the same order, in which case the reversibility was confined to GPVI and PAR1 agonists. It appeared that a combined inhibition of the so-called conventional and novel PKC isoforms was more effective for integrin closure than the use of isoform-specific inhibitors alone. Platelet spreading assays revealed that either PKC or P2Y12 inhibition provoked a partial conversion from lamellipods to a more discoid platelet shape. We concluded that PKC isoforms and autocrine ADP contribute to the persistent integrin  $\alpha$ IIb $\beta$ 3 activation state and hence a stabilized platelet response.

In **Chapter 4**, we examined the time-dependency of effects of consecutive addition of agonists of the GPVI and PAR1/4 receptors. Outcome measurements were platelet aggregation and cytosolic Ca2+ rises. We confirmed that an iloprostinduced elevation of cAMP level, either before or simultaneously with GPVI or PAR1 stimulation, completely eliminated the aggregation responses. However, PAR signaling demonstrated a longer time sensitivity for post inhibition than GPVI signaling. Furthermore, also tirofiban (integrin αIIbβ3 blocker) and Syk kinase inhibition sub-acutely modulated platelet aggregation, in other words halted this process even when applied after an agonist. We also examined whether platelets are capable to react to sequential stimulation. After initial activation via PAR, the cells still responded to GPVI, but they failed to respond to repeated PAR stimulation. Cytosolic Ca<sup>2+</sup> measurements indicated that, in comparison to prior PAR stimulation, prior GPVI stimulation induced a more persistent, priming activation state, which influenced the response to a next agent. Overall, our work revealed a high degree of versatility of platelets to rapidly react to a second receptor agonist, with a longer-term signal memory effect after GPVI than after PAR stimulation.

Similarly to other cells, cytosolic  $Ca^{2+}$  is in platelets an important second messenger that regulates the majority of functional responses. **Chapter 5** describes time-dependent effects of GPVI and PAR1/4 stimulation on intracellular  $Ca^{2+}$  mobilization and the accompanied extracellular  $Ca^{2+}$  entry, with a 96-well plate-based method also allowing calculation of the  $Ca^{2+}$  entry ratios. We established that, when compared to CRP and thrombin, collagen and TRAP6 act as

weaker  $Ca^{2+}$ -rising agonists that are also more dependent on the secondary mediators ADP and thromboxane  $A_2$ . We also investigated the effect of the compound thapsigargin, which blocks the back-pumping of cytosolic  $Ca^{2+}$  by  $Ca^{2+}$ -ATPases. When CRP or thrombin were combined with thapsigargin, the  $Ca^{2+}$  entry ratios greatly increased to 400 (GPVI stimulation) or 40 (PAR stimulation). This pointed to a major role of the so-called store-operated  $Ca^{2+}$  entry. By using a panel of pharmacological inhibitors, we could establish that the main  $Ca^{2+}$  carriers that control both the GPVI- and PAR-induced  $Ca^{2+}$  entry into platelets are the channel Orai1 and  $Na^+/Ca^{2+}$  exchange proteins. Much less important were the ATP receptor  $P2X_1$  (only initial curve value), and the channels TRPC6 and Piezo-2.

In the connected **Chapter 6**, we investigated the impact of novel and conventional PKC isoforms on store-operated Ca<sup>2+</sup> entry. We found a redundant contribution by both types of isoforms in the suppression of this process. Key involvement of the Orai1 channel was confirmed using platelets from patients with a mutation in the *ORAI1* gene. GPVI activation through both PKC isoforms had a significant suppressive effect on the Ca<sup>2+</sup> entry process. When checking for an important mediator of the Orai1-STIM1 interactions, namely the adapter protein BIN2, we identified by label-free analysis of the platelet phosphoproteome 45 regulated phospho-sites in BIN2 and 18 in STIM1, of which four had the characteristics of PKC multi-isoform substrates. Functional platelet testing indicated that the negative PKC-dependent regulation of Ca<sup>2+</sup> entry was accompanied by a downregulation of GPVI-dependent phosphatidylserine exposure and a suppressed thrombin generation in the presence of plasma.

In addition to platelets, red blood cells also play a role in the modulation of thrombus stability, and hence contribute to thrombosis and clot formation. In **Chapter 7**, we describe a novel whole-blood fluorogenic assay to elucidate the complementary roles of platelets and red blood cells in the process of thrombin enhancement of thrombin generation generation. We find an phosphatidylserine-exposing red blood cells, independent of the coagulant trigger. Blockage of the exposed phosphatidylserine with annexin A5 appeared to enhance the GPVI-induced procoagulant activity of platelets. In patients with (hemolytic) anemia or erythrocytosis, cluster analysis revealed both high and low whole-blood thrombin generation profiles. We concluded that better determination of the whole blood hypo-or hyper-coagulant activity may help to characterize a bleeding or thrombosis risk. **Chapter 8** discusses the main findings and conclusions of this thesis in the light of relevant literature. Foreseen is that the current analysis of transient aspects of platelet activation will lead to the development of improved antiplatelet medications, limiting the current bleeding risk.