

# Signaling pathways in the activation of platelets



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# Preface

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Because the course “Haemostase en Trombose” in the second year of my bachelor interested me very much, I asked the course coordinator Mark Roest if it was possible to do my Bachelor thesis at his laboratory. I was lucky that he told me that there were possibilities for me, where after he proposed a subject for my project: intracellular signaling pathways in platelets. This thesis represents my ten week long Bachelor’s project at the department of clinical chemistry and haematology of UMC Utrecht.

Using this opportunity, I would like to thank Mark Roest, who gave me support during the literature part of my researchproject in particular. Besides, I would like to thank Thijs van Holten, who helped me during the experiments at the laboratory.

Iemke Klein Koerkamp  
Wijhe, June 2011

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# Abbreviations

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ADP	adenosine diphosphate	PRD	proline rich domain
Calpain	calcium dependent protease	PS	phosphatidylserine
DAG	1,2-diacylglycerol	PSP	platelet sec1/munc18
DGK	diacylglycerol kinase	PTB	phosphotyrosine-binding
ECM	extracellular matrix	RA	Ras association
ER	endoplasmatic reticulum	RGS	regulator of G protein signaling
FA	focal adhesion	SFKs	Src family tyrosine kinases
FERM	4-point-one/ezrin/radixin/moesin	SLP-76	Src homology 2-containing leucocyte protein of 76 kDa
GAPs	GTPase activating proteins	SNAPs	N-ethylmaleimide-sensitive factor attachment protein
GDI	GDP dissociation inhibitor	SNARE	soluble N-ethylmaleimide sensitive factor attachment protein receptor
GEF	guanosine exchange factor	SRF	serum response factor
GPCR	G-protein coupled receptor	Syk	spleen tyrosine kinase
GPVI	glycoprotein VI	TF	tissue factor
ILK	integrin-linked kinase	TM	transmembrane
IP3	inositol triphosphate	TxA2	thromboxane A2
LAT	linker for activation of T-cells	vWf	von Willebrand factor
MD	membrane distal		
MLC	myosin light chain		
MP	membrane proximal		
NSF	N-ethylmaleimide-sensitive factor		
PA	phosphatic acid		
PAR	protease activated receptor		
PH	pleckstrin homology		
IP	inositol phospholipids		
IP3	inositol 1,4,5-triphosphate		
PI	phosphatidylinositol		
PI3K	phosphoinositide 3-kinase		
PIP	phosphatidyl inositol monophosphate		
PIP2	phosphatidyl inositol 3,5-diphosphate		
PIP3	phosphatidyl inositol 3,4,5-triphosphate		
PKB/Akt	protein kinase B		
PKC	protein kinase C		
PKD	protein kinase D		
PLC	phospholipase C		
PM	plasma membrane		
PR	proline rich		

# Abstract

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*Platelets are the major players in haemostasis. They respond to the binding of a broad selection of agonists and adhesive proteins, after which they aggregate and form a haemostatic plug.*

*Upon vessel wall injury, collagen in the sub endothelium becomes exposed to the bloodstream, which leads to the binding of the platelet receptor GPVI to collagen. Binding of collagen to GPVI on platelets activates them. This activation involves activation of PLC $\gamma$ , PKC and PI3K. Activated platelets can spread and form pseudopods on their surface, which facilitate binding and aggregation of platelets.*

*Binding of an agonist to its receptor on the platelet membrane activates platelets. Most of the platelet agonists transduce signals via seven transmembrane domain receptors that are coupled to heterotrimeric G-proteins. Upon stimulation of the heterotrimeric receptor, the  $\alpha$ -subunit is released from the  $\beta\gamma$ -complex. The liberated subunits are second messengers and may exert either a stimulatory or inhibitory role, depending on the receptor they were bound to. A major effector that is activated via a heterotrimeric receptor is PLC. PLC cleaves PIP<sub>2</sub> in the membrane to form DAG and IP<sub>3</sub>. DAG remains in the membrane, but IP<sub>3</sub> enters the cytosol and binds to the IP<sub>3</sub>-receptor on the ER. This results in the release of Ca<sup>2+</sup> from the ER, and subsequently an increase in the cytosolic calcium concentration. The presence of both DAG and Ca<sup>2+</sup> leads to the activation of calcium-dependent isoforms of PKC. PKC phosphorylates its downstream targets, which finally results in granule secretion, activation of ion-exchangers and regulation of the integrin  $\alpha$ IIb $\beta$ 3 affinity.*

*Vessel injury also initiates the coagulation cascade, resulting in the conversion of prothrombin into thrombin. Thrombin converts fibrinogen into fibrin, which in turn stabilizes the thrombus. Thrombin is in addition to collagen a second platelet agonist. One of its receptors is PAR-1. This receptor is cleaved by thrombin and the new amino acid terminus acts as a tethered ligand, which auto-activates the PAR-1 receptor. This platelet activation results in activation of PLC $\beta$ .*

*Platelet activation via GPVI and PAR-1 results in platelet granule release. Platelets contain dense granules,  $\alpha$ -granules and lysosomes. The dense granules contain nucleotides (e.g. ADP). ADP can activate platelets via two receptors, P<sub>2</sub>Y<sub>1</sub> and P<sub>2</sub>Y<sub>12</sub>. Stimulation of P<sub>2</sub>Y<sub>12</sub> with ADP leads to activation of PLC $\beta$  and PKC.*

*The final result of all platelet stimulations is the release of granule contents and the activation of GPIIb-IIIa. These processes are strictly regulated. Granule secretion is mediated by the SNARE 1 complex, formed by three membrane associated proteins: the syntaxins, the vesicle-associated membrane proteins (VAMPs) and the SNAP family proteins. Rab GTPases are involved in granule secretion also, they regulate alpha granule release.*

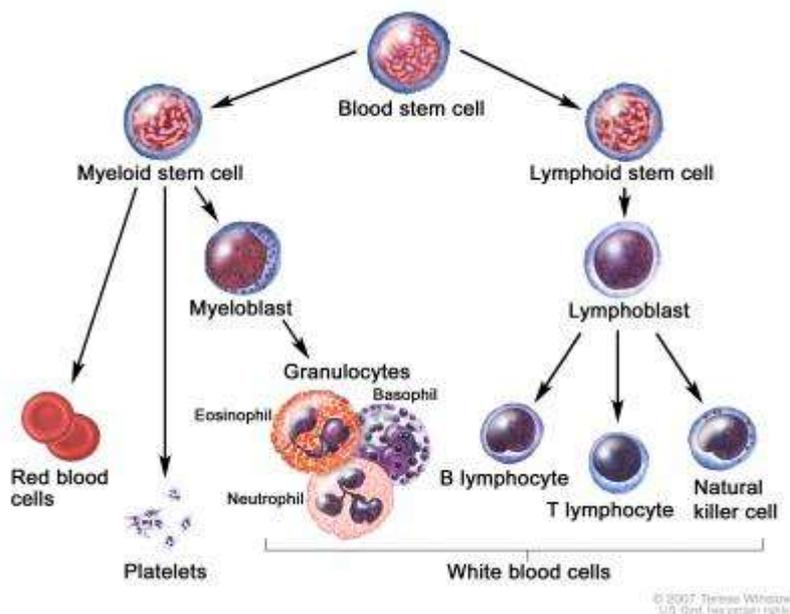
*GPIIb-IIIa activation involves the binding of talin and kindlins to the integrin tails and the subsequent conformational change from a low to a high affinity state for ligand binding.*

# Chapter 1: Introduction

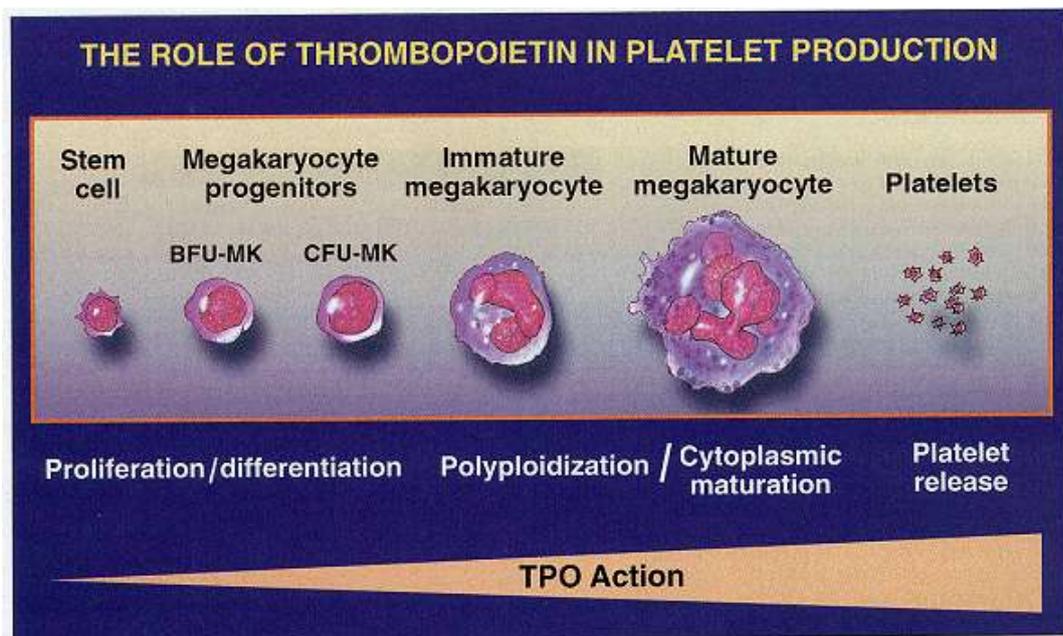
Haemostasis is the mechanism that becomes activated upon vessel wall damage in order to prevent blood loss and restore vessel integrity. The major players in this process are platelets. Platelets, also known as thrombocytes, are small (diameter of 1-2 $\mu$ m), regularly shaped clear cell fragments without a nucleus. They respond to the binding of a broad selection of agonists and adhesive proteins, after which they aggregate and form a haemostatic plug.<sup>1</sup>

## Platelet development

Platelets are formed by fragmentation of the cytoplasm of megakaryocytes. Megakaryocytes are big polyploidy cells, with a lobed nucleus, that can grow up to 50 $\mu$ m or more. The cytoplasm of these cells contains many basophilic granules and cytoplasmic organelles. The maturation of platelets takes approximately 10 days in human. Multipotent haematopoietic stem cells in the bone marrow give rise to all circulating blood cells. These cells differentiate into CFU-GEMM (colony-forming unit granulocyte, erythrocyte, monocyte, megakaryocyte). This is the common precursor cell that can differentiate into granulocytes, erythrocytes, monocytes and megakaryocytes. Megakaryocytes mature under the influence of thrombopoietin (TPO), a glycoprotein. The plasma volume increases and the amount of nuclei grows to about 8 nuclei. This all happens without division of the cell. When the cell contains about 8 nuclei, the cytoplasm becomes granular and the cell fragments, releasing 2000-3000 platelets into the bloodstream. Platelets have a life-span of 7-10 days.<sup>1,2</sup>



**Figure 1.** Hematopoiesis. Source: <http://www.cancer.umn.edu>, consulted 6 June 2011



**Figure 2.** Regulation of megakaryocytopoiesis by TPO. TPO acts at multiple levels, including proliferation and differentiation of megakaryocyte progenitors and maturation of megakaryocytes into large polyploidy cells capable of producing platelets. Source: <http://www.cancernetwork.com>, consulted 6 June 2011.

### Quiescent ready state

Platelets circulate in the blood at a relative high concentration of  $1,5-4,5 \times 10^9$  per ml in a quiescent state ready to form a haemostatic plug at sites of vascular damage. Their shape, small size and the physics of laminar flow in blood vessels, whereby red blood cells cause the platelets to marginate to the vessel wall, means they are ideally placed to detect injury to the vessel wall. By exposure to sub endothelial matrix proteins, platelets are activated. They are also activated by positive feedback mechanisms from the platelets themselves and through the generation of thrombin.<sup>1,2</sup>

In order to know how platelets become activated the main question is: **What are the most important intracellular signaling pathways in the activation of platelets?** To answer this question the following sub questions rise: What are the most important platelet receptors? How are these receptors activated? How are the signals from these receptors transduced intracellularly? How will lead stimulation of platelet receptors to platelet granule release and GPIIb-IIIa activation? How are platelet granule release and GPIIb-IIIa activation regulated?

These questions are as well as possible answered in this thesis. In the first three chapters, the three most important receptors and their intracellular signaling pathways will be discussed. Then the signal transduction to platelet granule release (chapter 4) and GPIIb-IIIa activation (chapter 5) and their regulation will be discussed. The last part of this thesis contains experiments concerning activation and inhibition of platelets via the P2Y<sub>12</sub> receptor and via GPVI. In these experiments, the importance of ADP will be shown.

## Chapter 2: Glycoprotein VI (GPVI)

In case of vessel wall injury, collagen in the sub endothelium becomes exposed to the bloodstream, which leads to the association of plasma Von Willebrand Factor (vWF) to collagen. vWf is produced by and stored in endothelial cells. Platelet binding to vWF, which is a reversible process, slows down platelets so, that strong binding to collagen is possible. This contact triggers the activation of platelets and thereby platelet aggregation and the formation of a haemostatic plug.<sup>3,4</sup>

There are several collagen receptors on platelets including integrin  $\alpha_2\beta_1$  and glycoprotein VI (GPVI), an immunoglobulin (Ig) superfamily member. On resting platelets, GPVI binds insoluble fibrous collagen. Soluble collagen does not bind to resting platelets, but only to the other collagen receptor, integrin  $\alpha_2\beta_1$ , on activated platelets. Collagen responses in low-GPVI platelets are mediated by the integrin  $\alpha_2\beta_1$  and suggest that as GPVI levels increase the role of integrin  $\alpha_2\beta_1$  becomes increasingly unnecessary.<sup>3,5</sup>

GPVI is a 60 kDa glycoprotein receptor composed of 319 amino acids residues. Human GPVI has two Ig-C2-like extracellular domains formed by disulfide bonds, that contain the collagen binding domain<sup>1</sup>, a single transmembrane domain and a cytosolic tail of about 51 amino acids.<sup>6</sup> GPVI is expressed on platelets in complex with a signaling adaptor, the Fc receptor (FcR) gamma chain.<sup>7,8,9,10</sup> GPVI has a positively charged arginine in its transmembrane region which is required for association with the FcR gamma chain.<sup>9</sup>

The cytosolic tail of GPVI contains sequence motifs for binding to calmodulin. This tail also has a proline-rich (PR) domain (PRD), immediately C-terminal of the calmodulin-binding site, that is a consensus motif for binding to SH3 domains of Src family tyrosine kinases (SFKs) including Fyn and Lyn. This binding directly activates these tyrosine kinases. Moreover, research has shown that loss of this PR domain does not change sustained GPVI signaling in platelets, but that the domain is required for rapid platelet activation by GPVI and loss of this domain delays GPVI signal transduction. GPVI PRD is also required for efficient platelet adhesion to collagen under flow.<sup>9,10</sup>

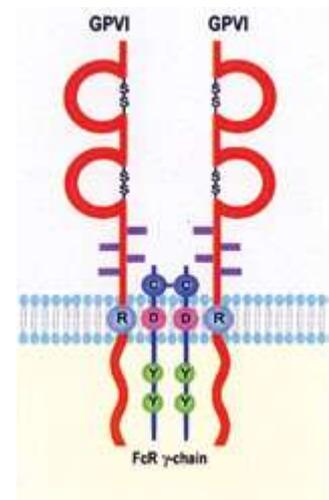


Figure 3. Two GPVI's in complex with two FcR  $\gamma$ -chains{{24 Moroi,M. 2004}}

The FcR gamma chain is essential for expression of GPVI on platelets, because it is required for GPVI to reach the platelet surface. It contains an immunoreceptor tyrosine based activation motif (ITAM) that is phosphorylated by Fyn or Lyn after cross linking of GPVI by ligand binding.<sup>9,10,11</sup> This phosphorylation will lead to a tandem phosphotyrosine motif recognized by the SH2 domains of the tyrosine kinase Syk (spleen tyrosine kinase).<sup>7, 12</sup> After association, Syk undergoes autophosphorylation and phosphorylation by Src kinases Fyn and Lyn and it will transduce the

activation signal to other proteins in the downstream signaling cascade.<sup>7,10</sup> This signaling cascade involves a signalosome, which will be discussed now.

### **Signalosome**

The GPVI signaling pathway involves a signalosome, which contains several adaptor and effector proteins. The adaptor proteins act as an intracellular scaffold, which regulates and targets effector proteins to correct regions of the cell. Thus, the effector proteins are brought into contact with their molecular substrates via the adaptor proteins of the signalosome. The core of the signalosome contains the transmembrane adaptor LAT (linker for activation of T-cells) and two cytosolic adaptors SLP-76 (Src homology 2-containing leucocyte protein of 76 kDa) and Gads.<sup>9</sup> One of the major effector proteins in the GPVI signaling pathway is phospholipase C. This protein will now be discussed, followed by the different proteins of the signalosome.

### **Phospholipase C**

Phospholipase C is a class of cytosolic and membrane-bound enzymes that cleave phospholipids. There are seven phospholipase C isozymes belonging to the PLC  $\beta$ ,  $\gamma$  and  $\delta$  families, the major isoforms are PLC $\beta$ 2, PLC $\beta$ 3 and PLC $\gamma$ 2. Several platelet agonists including ADP, thrombin and thromboxane A<sub>2</sub> activate PLC $\beta$  isozymes through  $G\alpha_q$  protein-coupled receptors. The agonist collagen and immune complexes bind to GPVI and activate the PLC $\gamma$ 2 isoform.<sup>13</sup> The regulation of the activation of PLC will be discussed later in this chapter.

Activated PLC $\gamma$ 2 (or PLC $\beta$ ) in the plasma membrane hydrolyzes phosphatidylinositol 4,5-triphosphate (PIP<sub>2</sub>) to generate the second messengers inositol 1,4,5-triphosphate (IP<sub>3</sub>) and 1,2-diacylglycerol (DAG).<sup>14,15</sup> IP<sub>3</sub> acts as a small intracellular mediator, it leaves the plasma membrane (PM) and diffuses rapidly through the cytosol. It can bind to IP<sub>3</sub>-gated Ca<sup>2+</sup> channels in the ER membrane. As a result these channels will open and calcium stored in the ER is released in the cytosol through these open channels. This will cause a raise in the cytosolic calcium concentration and results in the activation of calcium-responsive proteins.<sup>15,16</sup> The other cleavage product of PIP<sub>2</sub>, DAG, exerts different effects at the same time that IP<sub>3</sub> is increasing the intracellular calcium concentration. DAG also acts as a small intracellular mediator, but it remains fixed in the plasma membrane (PM), where it has several signaling roles. One role is to activate protein kinase C (PKC). Because DAG remains fixed in the PM, PKC has to translocate to the PM for activation by DAG. The rise in the intracellular calcium concentration induced by IP<sub>3</sub> alters PKC so that it translocates to the PM. PKC is activated on the PM by the combination of calcium, DAG, and the negatively charged membrane phospholipid phosphatidylserine (PS). When PKC is activated, it will phosphorylate and activate its downstream target proteins, which will be discussed afterward.<sup>15</sup>

Most knowledge about the role of PLC $\gamma$ 2 in platelet aggregation came from studies with PLC $\gamma$ 2 deficient mice. These mice responded normally to agonists acting through G protein coupled receptors but they had a prolonged bleeding time. The residual activation was found to be dependent on both GPVI and integrin  $\alpha_2\beta_1$  and, through activation of PI3K and GPIIb-IIIa to lead to an ADP/TXA<sub>2</sub>-dependent response.<sup>13</sup>

The main finding of the study was the existence of a PLC $\gamma$ 2 independent response to collagen in platelets. The study showed that collagen-induced aggregation was severely impaired in PLC $\gamma$ 2-deficient platelets in mice, but some remaining activation still occurred. It was also shown that

agonists acting through G-protein coupled receptors activate platelets independently of the PLC $\gamma$ 2 pathway. Selective stimulation of GPVI completely failed to induce platelet activation in PLC $\gamma$ 2 deficient mice. But there was a low level of aggregation in response to collagen, which was not activated via GPVI or integrin  $\alpha$ 2 $\beta$ 1, but in particular via TxA2 production and ADP secretion. Agonists acting through G protein-coupled receptors were also found to activate platelets independently of the PLC $\gamma$ 2 pathway.<sup>13</sup>

### **Protein kinase C (PKC)**

As said above, DAG, the product of PIP2 hydrolysis by PLC, activates protein kinase C (PKC). This protein will be discussed now. PKC plays a role in several signaling pathways in platelets. The first one involves dense and  $\alpha$ -granule release in synergy with calcium (which will be discussed later), and GPIIb-IIIa activation. Secretion of ADP, fibrinogen, and other compounds from the platelet granules enhance the integrin activation process. PKC promotes conformational changes of GPIIb-IIIa by phosphorylation, but activation of  $\alpha$ Ib $\beta$ 3 occurs also in the absence of PKC. Thus, there are two pathways of integrin activation, only one is PKC-dependent. The other signaling pathway in which PKC is involved is the inhibition of the calcium-dependent phosphatidylserine (PS) exposure and the coagulation process. Via these two pathways, PKC simultaneously increases the pro-aggregatory activity of platelets and suppresses the procoagulant properties of platelets.<sup>17</sup> It is still not entirely understood how PKC activation results in platelet secretion. However, there is a possible role for protein kinase D (PKD), which will be discussed now.

### **A role for protein kinase D (PKD)**

A recent study showed that two isoforms of PKC, PKC $\alpha$  and PKC $\beta$ , are positive regulators of protein kinase D and that another isoform, PKC $\theta$ , is a negative regulator of PKD. The PKD family of serine/threonine kinases consists of three members, PKD1, PKD2 and PKD3. PKDs contain a tandem repeat of zinc-finger-like cysteine-rich motifs at their N-termini, which is highly homologous to domains found in DAG activated PKCs and other signaling proteins regulated by DAG. However, a difference between PKCs and PKDs is that PKDs lack the C2 domain, which is responsible for the calcium sensitivity of classical PKCs. PKDs have an autoinhibitory PH domain and a catalytic domain. The catalytic domain of PKD has low homology with the conserved kinase domain of the PKCs.<sup>18</sup> PKC directly interacts with the PH domain of PKD and transphosphorylates its activation loop at S744 and 748, which lead to PKD activation.<sup>19</sup>

Although both PKD2 and PKD3 are expressed in platelets, only PKD2 becomes activated after agonist stimulation. PKD2 is said to regulate dense but not  $\alpha$ -granule release.<sup>18</sup>

### **LAT (linker for activation of T-cells)**

Linker for activation of T-cells (LAT) is the major protein of the signalosome. It is a transmembrane protein that has many tyrosine residues that can be phosphorylated by protein kinases.<sup>16,12</sup> LAT is essential for aggregation and secretion.<sup>20</sup> Together with the other two core proteins, it associates with several signaling molecules to regulate one of the major effector enzymes in the GPVI signaling cascade, PLC $\gamma$ 2.<sup>7</sup> By using LAT-deficient platelets it is revealed that LAT tyrosine phosphorylation occurs downstream of Syk and upstream of PLC $\gamma$ 2 phosphorylation and activation.<sup>5,12</sup> Phosphorylated LAT binds to the SH2 domain of PLC $\gamma$ 2, localizes PLC $\gamma$ 2 near the plasma membrane where PLC $\gamma$ 2 is activated through phosphorylation by the Src family tyrosine kinases Fyn and Lyn.<sup>12,21,22</sup>

Phosphorylated LAT also binds to the SH2 and/or other domains of many other proteins, such as PI3K<sup>12</sup>, Gads, Btk (Bruton's tyrosine kinase, a Tec family protein tyrosine kinase) and small G protein exchange factors Vav1 and Vav3. These proteins play important roles in the activation of PLC $\gamma$ 2 downstream of Syk in platelets.<sup>21</sup>

### **SLP-76**

The cytosolic protein Src homology 2-containing leucocyte protein of 76 kDa (SLP-76) is critical for the activation of PLC $\gamma$  by GPVI.<sup>20</sup> SLP-76 is composed of four domains: a C-terminal SH2 domain, a proline-rich region, a sterile  $\alpha$  motif domain and an N-terminal acidic domain, which contains three tyrosine phosphorylation motifs (Y<sup>112</sup>, Y<sup>128</sup> and Y<sup>145</sup>). All three tyrosines are required for  $\alpha$ -granule release and  $\alpha$ IIb $\beta$ 3 activation, especially Y<sub>145</sub>, which is critical for GPVI-induced aggregation. The tyrosines are also critical for GPVI induced phosphorylation of Btk and PLC $\gamma$ 2.<sup>23</sup>

SLP-76 is a substrate for Syk and is essential for tyrosine phosphorylation of PLC $\gamma$ 2.<sup>12</sup> The fact that SLP-76 has a role in the GPVI-dependent activation pathway between Syk and PLC $\gamma$ 2 is also shown in SLP-76 deficient platelets, where Syk was tyrosine phosphorylated, while there was almost no phosphorylation of PLC $\gamma$ 2 after collagen activation.<sup>5</sup>

### **Gads**

In platelets, SLP-76 is constitutively associated with the adapter Gads, which also binds to tyrosine phosphorylated LAT, thereby providing a potential pathway of regulation of SLP-76. Gads undergoes tyrosine phosphorylation upon stimulation of GPVI. Mutation of the Gads binding site on SLP-76 impairs GPVI-induced platelet secretion.<sup>20</sup>

Gads is required for efficient aggregation and secretion in response to weak stimulation of GPVI. Gads is not essential for activation following stronger stimulation of GPVI. It is also not essential for spreading induced through GPIIb-IIIa or the GPIb-IX-V complex. These findings suggest the presence of a Gads-independent pathway of platelet activation downstream of LAT.<sup>20</sup>

There are also other proteins involved in the GPVI-induced signaling pathway including PI3K, which will be discussed now.

### **PI3K**

Phosphoinositide 3-kinase (PI3K) is a plasma membrane bound enzyme, which phosphorylates inositol phospholipids. Phosphatidylinositol (PI), a membrane lipid, has several phosphorylation sites on its inositol head group. Phosphorylation of PI, which is a reversible process, produces a variety of phosphorylated PI lipids called phosphoinositides. These phosphoinositides include PIP, PIP2 and PIP3 (phosphatidylinositol 3,4,5-triphosphate). As said before, PIP2 is hydrolyzed by PLC $\beta$  or PLC $\gamma$  to generate the second messengers IP3 and DAG. PIP3 is not cleaved by PLC; it is made from PIP2 and then remains in the plasma membrane until specific phosphoinositide phosphatases dephosphorylate it.<sup>15</sup>

The p85 regulatory subunit (with SH2, SH3 and proline-rich (PH) domains) of PI3K binds to a phosphorylated tyrosine (phosphorylated by Syk) of LAT. The p85 subunit becomes phosphorylated by tyrosine kinases, which activates PI3K.<sup>24</sup> The binding to LAT is required for platelet aggregation and activation in response to collagen, without the p85 subunit there is decreased platelet aggregation and activation in response to collagen.<sup>5, 21, 24</sup>

PI3K is also required for maximal activation of PLC $\gamma$ 2 downstream of Syk in platelets. The p110 catalytic subunits of PI3K catalyze the formation of PIP3, which influences recruitment and activation of PLC $\gamma$ 2.<sup>16</sup> The p110 subunits are pulled to the membrane by the p85 subunit.<sup>4, 9</sup> PLC $\gamma$ 2 has PH (pleckstrin homology) domains that bind to PIP3. These bindings result in recruitment of PLC $\gamma$ 2 to the plasma membrane. Tec family kinases also have these PH domains for binding to PIP3. These bindings also results in recruitment to the plasma membrane.<sup>14</sup>

#### *PI3K isoforms*

There are various types of PI3Ks. Those activated by GPCRs belong to class Ib. Class I PI3Ks are heterodimers composed of a common catalytic subunit and different regulatory subunits. Class Ib PI3Ks have a regulatory subunit that binds to the  $\beta\gamma$  subunit of an activated trimeric G protein when GPCRs are activated by their extracellular ligand.<sup>15</sup>

Intracellular signaling proteins binds to PIP3 produced by activated PI3K via a specific interaction domain, such as a pleckstrin homology (PH) domain. PH domains function mainly as protein-protein interaction domains.<sup>15</sup>

#### *PI3K targets*

The most important PH-domain containing protein is protein kinase B (PKB)/Akt, a serine/threonine protein kinase. There are 3 isoforms of Akt, including Akt1, Akt2 and Akt3. Akt1 and Akt2 are present in platelets and play a role in platelet activation. Akt regulates platelet function by phosphorylating and inhibiting GSK beta. Thus, Akt is important for platelet function and thrombus formation.<sup>16,25</sup>

Next to the PI3K mediated activation of Akt, there is also an PLC $\beta$ 2 dependent pathway via a calcium dependent PKC subtype that increases the activity of Akt1 by thrombin.<sup>26</sup> Activation of PKC leads to the PI3K independent increase in Akt1 activity by selective phosphorylation of Ser473, although this is not sufficient for full PKB activation.<sup>26</sup>

Other targets of PI3K via its phosphoinositide products are other PH-domain containing effector molecules. These include Tec family tyrosine kinases and guanosine diphosphate/guanosine triphosphate (GDP/GTP) exchange factor (GEF) families such as Vav.<sup>24</sup>

PI3K $\gamma$  also activates and regulates several other kinases including PKC, Map kinase kinase (MEKK1) , Src family tyrosine kinases and many others.<sup>27</sup>

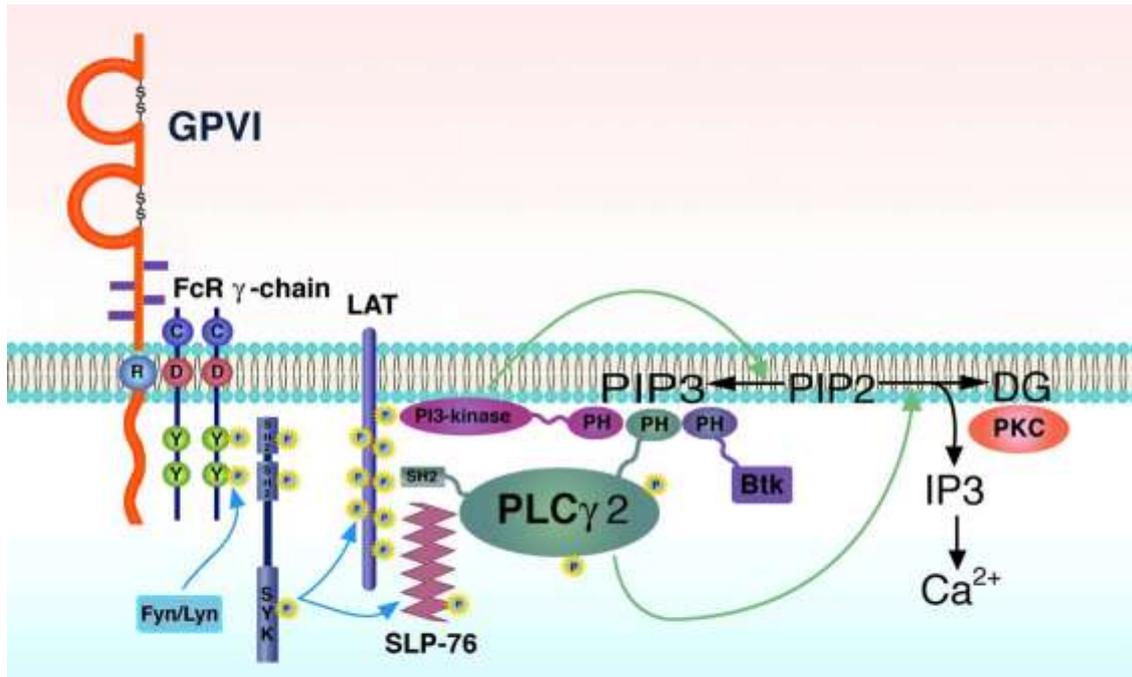


Figure 4. Model of the activation pathways induced by GPVI. In this figure, the blue arrows indicate tyrosine phosphorylation, and the green arrows indicate enzyme-catalyzed conversion.<sup>5</sup>

#### Short chapter summary

GPVI activation results in several actions including the intracellular mobilization of calcium via IP3 and activation of PKC and PI3K, this is also shown in figure 2. These actions will finally result in platelet shape change, platelet degranulation, integrin activation and platelet aggregation. The regulations of these actions will be discussed in the following chapters.

# Chapter 3: Protease activated receptor 1 (PAR-1)

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Platelets could also be activated by thrombin, a serine protease which can bind to and cleaves protease activated receptors (PARs) on platelets.<sup>27</sup> Thrombin is not always present in the blood. It is present in its non-active form as prothrombin. Prothrombin can be converted to thrombin in case of vessel wall injury via the coagulation cascade. Exposure of tissue factor (TF) by TF expressing cells (including sub endothelial cells) during vessel wall injury initiates the coagulation cascade. Via this cascade prothrombin is cleaved twice to generate thrombin.<sup>28</sup>

The cleavage of PARs by thrombin is irreversible and reveals a new N-terminus that acts as a tethered ligand. This ligand binds to the receptor and initiates intracellular signaling pathways, which finally result in platelet shape changes, dense granule secretion and GPIIb/IIIa receptor activation.<sup>27</sup>

PARs are G-protein coupled receptors (GPCRs) on the surface of platelets. There are four different isoforms of PARs characterized, from which PAR1 and PAR4 are present on the surface of human platelets. Platelets are known to contain four different G-proteins involved in intracellular signaling pathways. These include  $G_s$ ,  $G_i$ ,  $G_q$  and  $G_{12/13}$ . These G proteins have amino acid sequence similarities and comparable functions. Both PAR1 and PAR4 couple to  $G_q$  and  $G_{12/13}$  in human platelets and cause fibrinogen receptor activation upon activation by a ligand. The  $G_q$  pathway involves phospholipase C activation and simultaneous intraplatelet calcium mobilization and PKC activation. The  $G_{12/13}$  pathway involves Rho/Rho kinase activation and actin remodeling, which causes platelet shape change.<sup>29</sup>

First, the G-proteins in common will be discussed, then the different G-proteins involved in platelet signaling will be discussed.

## **G-proteins in common**

When an extracellular signal molecule binds to a GPCR, the receptor undergoes a conformational change that enables it to activate a trimeric GTP-binding protein (G protein). The G protein is connected to the cytoplasmic face of the plasma membrane, where it functionally couples the receptor to enzymes or ion channels in the membrane. In some cases, the G protein is physically associated with the receptor before the receptor is activated, whereas in others it binds only after receptor activation. As said before, there are various types of G proteins, each specific for a particular set of GPCRs and for a particular set of target proteins in the plasma membrane.<sup>15</sup>

All G proteins are made up of three protein subunits,  $\alpha$ ,  $\beta$  and  $\gamma$ . In the unstimulated state, the  $\alpha$  subunit has GDP bound and the G protein is inactive. When a ligand activates the GPCR, it induces a conformational change in the receptor that allows the receptor to function as a guanine nucleotide exchange factor (GEF) that induces the  $\alpha$  subunit to release its bound GDP and bind GTP in its place. This exchange triggers, although not always, the dissociation of the  $\alpha$  subunit, bound to GTP, from the  $\beta\gamma$  dimer and the receptor. Both the GTP-bound  $\alpha$  subunit and the  $\beta\gamma$  subunit then activate different signaling cascades or second messenger pathways and effector proteins. The receptor is able to activate another G protein.<sup>15</sup>

The  $\alpha$  subunit of a G protein is a GTPase; when it hydrolyzes its bound GTP to GDP it becomes inactive. The time for which the G protein remains active depends on how quickly the  $\alpha$  subunit hydrolyzes its bound GTP. This time is usually short because the GTPase activity is greatly enhanced by the binding of the  $\alpha$  subunit to a second protein, which can be either the target protein or a specific regulator of G protein signaling (RGS). RGS proteins act as  $\alpha$  subunit specific GTPase-activating proteins (GAPs), and they help shut off G protein-mediated responses.<sup>15</sup>

There are also "small" G proteins (20-25kDa) that belong to the Ras superfamily of small GTPases. These proteins are homologous to the  $\alpha$  subunit found in heterotrimeric G proteins, and are in fact monomeric. However, they also bind GTP and GDP and are involved in signal transduction. These proteins will be discussed later.

### **G<sub>q</sub> pathway**

Cleavage of PAR1 by thrombin and binding of the tethered ligand to the receptor stimulate G<sub>q</sub> $\alpha$  to activate PLC $\beta$ .<sup>30</sup> Human platelets contain four isoforms of PLC $\beta$  that are activated by G<sub>q</sub> $\alpha$  coupled receptors.

As said in the chapter GPVI, PLC hydrolyzes PIP<sub>2</sub> to generate the second messengers IP<sub>3</sub> and DAG. These second messenger molecules function in the mobilization of calcium from intracellular stores (via IP<sub>3</sub>) and activation of PKC (via DAG), which lead to platelet secretion and aggregation.<sup>16</sup>

Next to hydrolysis of PIP<sub>2</sub>, PIP<sub>2</sub> becomes also phosphorylated by PI3K $\gamma$  to generate PIP<sub>3</sub>.<sup>31</sup> PIP<sub>3</sub> has several functions, among which the activation of PKB/Akt in platelets.<sup>32</sup>

### **G<sub>12/13</sub> pathway**

Next to the activation of G<sub>q</sub>, there is another G-protein stimulated after PAR1 stimulation. This is the G<sub>12/13</sub> protein. G<sub>12/13</sub> $\alpha$  activates Rho/Rho kinase signaling, which in turn regulates the cytoskeleton. Active GTPase RhoA activates Rho kinase, which then activates and regulates various downstream target proteins. One of them is ROCK (regulator of G protein signaling), a serine/threonine kinase, which translocate from the cytosol to the PM in the presence of active RhoA. ROCK mediates cell contraction through phosphorylation and inhibition of myosin light chain (MLC) phosphatase, thus enhancing MLC phosphorylation and MLC dependent contraction. It also increases the serum response factor (SRF)-dependent gene transcription<sup>27,33</sup> The inhibition of MLC phosphatase will result in a calcium independent platelet shape change and is thus RhoA/ROCK dependent.<sup>27</sup>

There is also a calcium dependent platelet shape change after PAR1 activation. The calcium release after PAR1 activation has been shown to enhance MLC kinase activity, because MLC kinase is calcium/calmodulin-dependent. This enhancement results in increased MLC phosphorylation, the interaction of myosin with actin filaments to form stress fibers and thus platelet shape change, which will be discussed now.<sup>30</sup>

### **Platelet shape change**

A shape change in platelets is an extremely rapid dynamic process based on the reorganization of the cytoskeleton. It is the initial response of platelets to activators such as thrombin, ADP, or TxA<sub>2</sub> and it includes contractile events and regulation of a circumferential microtubule coil. During the shape

change, new actin filaments are formed and polymerization of actin results in the formation of filopodia. Besides, actomyosin (the contractile protein of platelets)-based contractile processes are stimulated, which results in the centralization of dense and  $\alpha$ -granules. Finally, the circumferential microtubule coil depolymerizes, which result in a change from a disc-shape to a more spherical shape. Platelet shape change can be induced by agonist concentrations lower than those required for granule secretion and aggregation. Platelet shape change is a prerequisite for efficient secretion of granule contents and greatly facilitate adhesion of platelets to each other and to components of the ECM.

Thus, the  $G_{12/13}$  pathway is required for platelet shape changes, but it is not critical for GPIIb-IIIa activation via PAR1 or PAR4. This is shown by addition of a Rho-kinase inhibitor, which did not significantly affect GPIIb-IIIa activation downstream of PAR1 or PAR4. Thus, Rho kinase is not required downstream of either PAR1 or PAR4 for GPIIb-IIIa activation.<sup>29</sup>

### **Short chapter summary**

PAR1 activation leads to the activation of PLC, which in turn results in an increase in the intracellular calcium concentration via IP<sub>3</sub> and the activation of PKC via DAG. These events finally result in a calcium-dependent and -independent platelet shape change.

# Chapter 4: P2Y12 receptor

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P2Y receptors are G-coupled receptors with a molecular mass of 41 to 53 kDa after glycosylation. Eight P2Y receptors have been identified so far: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14. Human platelets express two P2Y receptors that interact with ADP, the P2Y1 and the P2Y12 receptor.<sup>1</sup> Binding of ADP to these receptors induces platelet activation. P2Y1 is a  $G_q$  coupled receptor, whereas P2Y12 is a  $G_i$  coupled receptor. ADP that binds to P2Y12 or P2Y1 is highly concentrated in dense granules in platelets and is released after platelet stimulation to support complete responses of these cells.<sup>34</sup>

The  $G_q$ -coupled P2Y 1 receptor mediates a transient rise in cytoplasmic  $Ca^{2+}$  and activation of PKC via PLC $\beta$ , platelet shape change, and rapidly reversible aggregation. PLC $\beta$  converts PIP2 into the second messengers DAG and IP3. IP3 opens calcium channels and DAG activates PKC. DAG is after activation phosphorylated to phosphatidic acid (PA) by the enzyme DAG kinase (DGK), which therefore turns off the effects of DAG as second messenger.<sup>35</sup>

Next to  $G_q$  and  $G_{12/13}$ , which are activated via ligand binding to PAR1, there is also a  $G_i$  pathway. This pathway is activated via ligand binding to P2Y12 receptors and will be discussed now.

## **$G_i$ pathway**

The  $G_i$  pathway involves the inhibition of the activity of adenylyl cyclase by the  $\alpha$ -subunit of  $G_i$  and the activation of PI3K $\gamma$  by the  $\beta\gamma$ -subunit of  $G_i$ . Inhibition of adenylyl cyclase results in the reduction of cAMP levels, which regulate platelet activity.<sup>36</sup> cAMP activates protein kinase A (PKA), which in turn phosphorylates its downstream target proteins, including PAR1, PAR3, PAR4, PC $\beta$ , IP3 receptors and several others. So, in this way cAMP inhibits platelet activation. However, after thrombin stimulation, cAMP levels are reduced, so the inhibition of platelet activity by cAMP will also be reduced.<sup>36</sup>

## **PI3K**

As said above, after stimulation of the  $G_i$ PCR, the  $\beta\gamma$  subunit of  $G_i$  activates phosphoinositide 3-kinase. PI3K, discussed earlier in chapter 1, is a plasma membrane bound enzyme, which phosphorylates inositol phospholipids to generate phosphoinositides, including PIP, PIP2 and PIP3.<sup>15</sup> PI3K is required for human platelet GPIIb-IIIa activation and human platelet aggregation. PI3K plays a role in the increase in intraplatelet calcium concentration via calcium from the extracellular milieu.<sup>29</sup> The most important target protein of PI3K is Akt, which regulates platelet function by phosphorylating and inhibiting GSK beta.<sup>25,16,25</sup> PI3K also activates the small GTPase Rap1b, which is a critical regulator of GPIIb-IIIa and platelet aggregation, this process will be discussed in chapter 5.<sup>34,35</sup> The PI3K-Rap1b pathway is responsible for the  $G_i$ -mediated contribution to platelet aggregation, although not all aspects are clearly defined yet.<sup>35</sup>

### **Simultaneous activation of both G<sub>q</sub> and G<sub>i</sub> pathways**

Simultaneous activation of both the G<sub>q</sub> (P2Y1) and G<sub>i</sub> (P2Y12) pathways by ADP is necessary for normal aggregation.<sup>34,35,37</sup> This is shown in platelets by blocking the P2Y12 receptor. Blocking of the P2Y12 receptor resulted in a complete inhibition of the ADP-induced phosphorylation of pleckstrin, the main platelet substrate for PKC. P2Y12 antagonists, such as ATP, also inhibited pleckstrin phosphorylation. Thus, phosphorylation of pleckstrin by PKC, activated via the P2Y1 receptor, requires also activation of the G<sub>i</sub>-coupled P2Y12 receptor.<sup>35</sup>

The P2Y12 receptor contributes to the regulation of PKC through the PI3K-Rap1b-dependent pathway, but not via the inhibition of adenylyl cyclase. P2Y12 antagonists also inhibited Rap1b activation via PI3K and platelet aggregation induced upon G<sub>q</sub> stimulation by the thromboxane A2 analogue U46619. Activation of PLC and intracellular calcium mobilization occurred normally in the presence of these P2Y12 antagonists. Inhibition of DGK restored both PKC and Rap1b activity and caused platelet aggregation. These findings indicate that DAG is a critical messenger for platelet aggregation regulated through G<sub>i</sub>-dependent signaling pathways.<sup>35</sup>

The inhibition of adenylyl cyclase via G<sub>i</sub>α is a key feature of platelet activation by ADP, but there is no causal relationship with platelet aggregation. This suggests that other signaling events downstream of G<sub>i</sub>α are required for activation of GPIIb-IIIa and subsequent platelet aggregation.

### **Short chapter summary**

P2Y12 stimulation with ADP results in the activation of the G<sub>i</sub> pathway. This pathway involves inhibition of the activity of adenylyl cyclase and the activation of PI3K.

# Chapter 5: Granule secretion

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The fusion of platelets granules with the plasmamembrane is mediated by the SNARE 1 (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor 1) complex. This complex is formed by three membrane associated proteins: the syntaxins, the vesicle-associated membrane proteins (VAMPs, or synaptobrevins), and the SNAP family proteins. In platelets, the most common complex contains syntaxin 4, platelet VAMP 8 and SNAP-23.<sup>38,39</sup> Platelets also contain other members of the SNAP gene family including SNAP-25 and SNAP-29, but SNAP-23 is the most highly enriched of the three proteins in platelets.<sup>40</sup>

SNARE proteins can be classified as vesicle-associated SNAREs (v-SNAREs), which are located on vesicles or granules, and target-associated SNAREs (t-SNAREs), which are located on target membranes. In the case of platelets, t-SNAREs are located on the cytosolic face of the plasma membrane, because the platelet granules are supposed to fuse with the plasma membrane. The v-SNAREs and the t-SNAREs form a heteromeric complex, which mediates membrane fusion and thus granule release. The human platelet tSNAREs include SNAP-23, SNAP-25 and SNAP29, as well as syntaxin 2, 4, 7 and 11. Human platelet vSNAREs include VAMP-2, -3, -7 and -8.<sup>41</sup>

The SNARE proteins interact with each other through conserved sequences of about 60-70 amino acids, known as the SNARE motif. These sequences include eight heptad repeats, that associate with each other to form hydrophobic coiled-coils.<sup>41</sup>

The SNARE proteins form four  $\alpha$ -helices (one  $\alpha$ -helix from VAMP, one  $\alpha$ -helix from syntaxin and two  $\alpha$ -helices from SNAP) to produce an extremely stable four-helix bundle.<sup>40</sup>

The structure is stabilized by core hydrophobic interactions running the length of the bundle. The parallel arrangement of the helices with all amino termini at one end of the helix bundle should bring the membrane anchors of the SNARE proteins and thus the vesicular and target membranes into close proximity.<sup>40</sup>

The assembly and disassembly of the SNARE complex is modulated by the ATPase N-ethylmaleimide-sensitive factor (NSF), soluble NSF attachment proteins (SNAPs), Rab GTPases and Sec1/Munc18 family proteins.<sup>38,40</sup> Sec1/Munc18 family proteins are cytoplasmic proteins whose binding to syntaxin excludes binding interaction with VAMP and SNAP-25.

## Syntaxins

Syntaxins interact with several other proteins that are believed to modulate exocytosis such as the Sec1/Munc-18 proteins, Munc13, synaptotagmin, Ca<sup>2+</sup> channels, and other. The syntaxins play a key role in SNARE complex formation and exocytosis. Syntaxin 4, a key SNARE protein, is phosphorylated when platelets are activated by thrombin to induce secretion. Syntaxin 4 is a direct substrate for PKC and PKC inhibitors significantly reduce both syntaxin 4 phosphorylation and platelet secretion.<sup>38</sup> Platelet Sec1/Munc18 (PSP) is also phosphorylated by PKC after platelet stimulation.<sup>39,40</sup> Phosphorylation of PSP and syntaxin 4 modulate their interactions with other proteins (e.g. actin cytoskeleton and the membrane skeletal protein, fodrin), and thus may regulate secretion.<sup>40</sup>

Platelets also contain an Sec1 homologue that is likely to modulate platelet secretion through its binding to syntaxin 4.<sup>38</sup>

Another important syntaxin is syntaxin-2, which associates with the filamentous actin cytosolic network following platelet activation. Unlike syntaxin-4, which binds directly to platelet actin both before and after activation, syntaxin-2 binds indirectly to the actin cytoskeleton.<sup>41</sup>

### **VAMP (vesicle-associated membrane proteins)**

Human platelets contain four VAMP proteins including VAMP-2/synaptobrevin, VAMP-3/cellubrevin, VAMP-7/TI-VAMP, and VAMP-8/endobrevin. VAMP-8 is the dominant vSNARE in platelets, required for platelet secretion. VAMP-8 associates with the filamentous actin cytosolic network following platelet activation. The association of VAMP-8 and syntaxin-2 (see above) may be indirect and result in part from activation induced by binding to syntaxin-4 and SNAP-23, respectively.<sup>41</sup>

### **SNAP-23**

Besides phosphorylation of syntaxin 4, platelet stimulation with thrombin also results in the phosphorylation of SNAP-23.<sup>40</sup> Two phosphorylation sites of SNAP-23 has been identified, Ser23/Thr24 and Ser161. They are phosphorylated by PKC after thrombin stimulation and thereby activation of the platelet. The PKC  $\alpha$  isoform is essential for both platelet alpha and dense granule secretion.<sup>40</sup> Phosphorylation of syntaxin 4 and SNAP-23 upon platelet stimulation decreases the binding of the two proteins to each other.<sup>38,40</sup>

### **PKC**

PKC not only regulates platelet secretion through phosphorylation of SNAREs including SNAP-23 and syntaxin 4, but also through phosphorylation of SNARE regulators such as PSP/Munc18c.<sup>40</sup> Although increases in intracellular  $Ca^{2+}$  are sufficient to induce platelet secretion, PKC interacts synergistically with the increasing amounts of intracellular calcium to amplify release of the contents of platelet granules.<sup>38,39</sup>

#### *PKC isoforms*

Platelets contain three classes of PKCs. These are the classic PKCs ( $\alpha$ ,  $\beta$ I,  $\beta$ II), which are regulated by  $Ca^{2+}$  and DAG, the novel PKCs ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ), which are regulated only by DAG, and atypical PKCs ( $\xi$ ,  $\iota/\lambda$ ), which are not activated by either  $Ca^{2+}$  or DAG.<sup>38</sup>

### **Rab GTPases**

SNAREs are not the only molecules that regulate membrane fusion of platelet granules with the plasma membrane. The Rab GTPases are also essential regulatory molecules in granule secretion. They are required upstream of the formation of the SNARE complexes. Rab GTPases belong to the Ras-related small GTPase superfamily and they function as molecular switches. Rabs cycle between the cytosol and the membrane of the trafficking organelle. This cycle is controlled by conformational changes, regulated by guanine-nucleotides. Like other GTPases, Rab proteins are active when they are bound to GTP and inactive when they are bound to GDP. The active GTP-bound Rabs exert their functions on the specific organelle membranes through binding to their effector molecules. Only the active GTP-bound form can associate with the effector molecules. Rab GTPases are also regulated by a unique negative regulator named Rab GDP dissociation inhibitor (GDI). Rab GDI is a cytosolic protein, which forms a complex with GDP-bound Rab proteins to inhibit the GDP-GTP exchange, and

extracts Rab proteins from the membrane into the cytosol. Moreover, Rab-GDI has recently been demonstrated to play also positive roles to accompany Rab proteins from cytosol to the proper membranes.<sup>42</sup>

There are several Rabs present in platelets, including Rab 1a, 1b, 3B, 4, 6c, 8, 11, 27a, 27b, and 31. Rabs 3b, 6c, and 8 are phosphorylated upon platelet activation.<sup>43</sup>

Rab4 is an essential regulator of the  $\alpha$ -granule secretion.<sup>42</sup> It is also shown that Rab proteins which are sensitive for RabGDI might not play important roles in the dense granule secretion, but only in  $\alpha$ -granule release.<sup>42</sup>

Rab27 regulates the Ca<sup>2+</sup>-induced dense granule secretion in platelets. There is also a GTP-Rab27-binding protein in platelets, Munc13-4, which mediates the function of GTP-Rab27 to promote the secretion. Rab27 in unstimulated platelets is predominantly in the GTP-bound form. The GTP-bound form of Rab27 drastically decreased upon granule secretion, due to enhanced GTP hydrolysis activity that was secretion-dependent. It is also shown that GTP hydrolysis of Rab27 is not necessary for dense granule secretion in platelets.<sup>44</sup> So it is likely that GTP-Rab27 would not mediate extracellular signals but rather keep the vesicles in a preparative state for the secretion of platelet granules. The increased calcium concentration is the trigger of the secretion. The Rab27 effector molecule, Munc13-4, has a calcium binding domain and mediates the calcium ion signal. PKC also mediates this signal.<sup>44</sup>

### **Calpain**

Platelet granule release is also influenced by intracellular proteases, such as calpain. Calcium-dependent protease (calpain) is an intracellular cysteine protease. There are two ubiquitous calpain isozymes,  $\mu$ -calpain and m-calpain, which are active at micromolar and millomolar calcium concentrations, respectively.<sup>45,46</sup> Their amounts differ from tissue to tissue. More than 90% of the calpain in platelets is  $\mu$ -calpain.<sup>47</sup> Both calpains have similar biochemical characteristics, except for the calcium concentration required for activation. Calpains are composed of a large catalytic subunit of 80 kDa and a small regulatory subunit of 30kDa. Each subunit contains a calmodulin-like domain at the C-terminus. After calcium binding to the calmodulin-like domains, calpains become active and begin to autolyze. The autolysis in the large subunit of  $\mu$ -calpain, which is accompanied by an increase in the sensitivity to calcium, is itself the activation step of calpain.  $\mu$ -calpain binds to the cytoplasmic surface of cell membranes as an 80-kDa proenzyme and is converted autolytically to the 76-kDa form via a 78-kDa intermediate on the membrane.<sup>47</sup>

SNARE proteins are susceptible to proteolysis. Calpain cleaves SNAP-23 on platelet activation.<sup>48,49</sup> VAMP-3 is also cleaved by calpain but seems to be susceptible to cleavage by other platelet proteases as well.<sup>49</sup> In contrast, neither syntaxin-2 nor -4 is cleaved by on platelet activation.<sup>48</sup>

Calpain cleaves also several other signaling molecules, such as PKC and PLC, that influence signaling of granule secretion. Protease-induced downregulation of PKC interferes with dense granule secretion.<sup>48,49</sup>

### **Actin**

Actin polymerization facilitates platelet  $\alpha$ -granule secretion. VAMP-8 and syntaxin- 2 associate with the actin cytoskeleton only following platelet activation while syntaxin-4 and SNAP-23 associate with

the actin cytoskeleton in resting and activated platelets. Actin in platelets binds directly to syntaxin 4.<sup>41</sup>

### **Granule contents**

Fusion of  $\alpha$ -granules with the plasma membrane results in the release of fibrinogen (Fg), P-selectin, vWf, thrombospondin, vitronectin (Vn), fibronectin (Fn), insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), VEGF, EGF, platelet-derived endothelial growth factor (PDEGF), TGF $\beta$ , TGF $\alpha$ , platelet factor 4 (PF-4) and interleukine-1 (IL-1). Fusion of dense granules with the plasma membrane results in the release of nucleotides such as ADP, ATP, calcium, serotonin, pyrophosphate and histamine.<sup>2</sup>

### **Short chapter summary**

Granule secretion is mediated by the SNARE 1 complex, formed by three membrane associated proteins: the syntaxins, the vesicle-associated membrane proteins (VAMPs) and the SNAP family proteins. SNARE proteins can be classified as vesicle-associated SNAREs (v-SNAREs), which are located on vesicles or granules, and target-associated SNAREs (t-SNAREs), which are located on target membranes. The v-SNAREs and the t-SNAREs form a heteromeric complex, which mediates membrane fusion and thus granule release. SNARE proteins are susceptible to proteolysis by calpain. Calpain cleaves also several other signaling molecules that influence signaling of granule secretion. Rab GTPases are involved in granule secretion also, they regulate alpha granule release.

# Chapter 6: GPIIb-IIIa (integrin $\alpha$ IIb $\beta$ 3) activation

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Integrins are transmembrane receptors that mediate connection between the cell and the extracellular matrix (ECM). Integrins play a role in the activation of intracellular signaling pathways after ligand binding, a process called outside-in signaling. Integrins can change between a high and a low affinity conformation for ligand binding, a process called inside-out signaling.<sup>50</sup> This change of affinity is strictly regulated by external signals that are transduced intracellularly and result in the direct binding of regulatory proteins to the short cytoplasmic domains of integrins.<sup>15,51</sup>

Integrins are heterodimers containing two distinct cytoplasmic chains, called the  $\alpha$  and  $\beta$  subunits. Next to the  $\alpha$  and  $\beta$  subunits, integrins have large extracellular domains (about 800 amino acids) that contribute to ligand binding and single transmembrane (TM) domains (about 20 amino acids). All three domains (the extracellular domain, the transmembrane domain and the cytoplasmic domain) are required to regulate the affinity of integrins. The  $\alpha$  and  $\beta$  cytoplasmic tails are very similar, in particular the membrane proximal region. Almost all  $\beta$  tails have two motifs that are part of a recognition sequence for phosphotyrosine-binding (PTB) domains. These are the membrane proximal NPxY (where x represents any amino acid) motif and the membrane distal NxxY motif. These motifs are binding sites for several integrin binding proteins, including talin and the kindlins.<sup>15,51,52</sup>

Integrins in a low affinity state for ligand binding have a bent, v-shape conformation with the head piece oriented toward the cell membrane. The ligand binding high affinity integrins have an extended upright conformation. In the bent conformation, the subunit transmembrane and cytoplasmic domains are in close contact and a salt bridge connects the  $\alpha$  subunit with the  $\beta$  subunit. In the high affinity conformation, the  $\alpha$  and  $\beta$  subunit are separated.<sup>15,53</sup> It is unclear whether ligand binding only occurs to the extended conformation or also to the bent conformation. There are two models of ligand binding to integrins. The first one is the switchblade model, which predicts that only extended integrins will bind ligand. The second one is the deadbolt model, which suggests that ligand binding occurs to the bent integrin and that extension occurs only following ligand binding.<sup>54</sup>

Regulation of the integrin affinity is important for several cellular functions. In response to injury, the fibrinogen receptor on platelets, GPIIb-IIIa, is rapidly activated to mediate platelet adhesion and aggregation in order to stop bleeding. Because  $\alpha$ IIb $\beta$ 3 integrins on platelets are constantly exposed to fibrinogen, it is essential to keep them inactive if there is no bleeding, to prevent pathological platelet aggregation and thrombus formation. Abnormal function of integrins or mutations in integrin-binding proteins required for integrin activation can result in bleeding disorders.<sup>50, 51</sup>

In case of vessel injury or bleeding, it is important that the fibrinogen receptor, the  $\alpha$ IIb $\beta$ 3 integrin, becomes activated. This receptor triggers platelet aggregation and thrombus formation with the in order to stop bleeding. One of the first important observations in  $\alpha$ IIb $\beta$ 3 research was that mutation

of the highly conserved D<sub>723</sub> residue in the membrane proximal part of the  $\beta$ 3 subunit cytoplasmic tail or the comparable R<sub>995</sub> residue in the  $\alpha$  subunit membrane proximal part is enough to activate GPIIb-IIIa, both in vitro and in vivo. A deletion of these sequences also results in GPIIb-IIIa activation. These observations suggests that an ionic salt bridge between these two residues keeps the two cytoplasmic tails in close contact and stabilizes integrins in their low affinity state. The two residues are located in two highly conserved sequences in the membrane proximal part of the  $\alpha$  (GFFKR) and the  $\beta$  subunits (HDR(R/K)E) and form  $\alpha$ -helical coiled-coiled interactions.<sup>53,55</sup>

### **After GPIIb-IIIa activation**

The activation of GPIIb-IIIa involves the conformational change from a low to a high affinity state for ligand binding. Activated  $\alpha$ IIb $\beta$ 3 can bind to fibrinogen, but there are also other ligands that bind to the GPIIb-IIIa receptor. A common characteristic of the ligands that can bind to  $\alpha$ IIb $\beta$ 3 is the presence of the Arg-Gly-Asp (RGD) amino acid sequence.<sup>56</sup> RGD containing proteins are fibrinogen, vWF, fibronectin, vitronectin and thrombospondins. RGD-containing peptides inhibit the binding of adhesive proteins to  $\alpha$ IIb $\beta$ 3, which suggest that RGD mediates receptor binding. Other RGD-independent ligand binding sequences have also been identified.<sup>56</sup>

It is shown that after platelet activation, not only low affinity integrins change to high affinity conformations but that there is also mobilization of  $\alpha$ IIb $\beta$ 3 from internal stores.<sup>57</sup>

As said before, almost all  $\beta$  tails have two motifs that are recognized by phosphotyrosine-binding (PTB) domains of several integrin binding proteins, among which talin and kindlins. These proteins will now be explained in detail.

### **Talin**

Talin plays a role in the final activation step of integrins. It binds to the  $\beta$  integrin cytoplasmic domain of integrins and without this binding there is no integrin activation in platelets. There are two talin isoforms known in vertebrates, talin1 and talin2.<sup>58</sup> Talin1 is expressed widely and talin2 is found primarily in striated muscle and in the brain.<sup>59</sup> Lower eukaryotes encode only a single talin isoform corresponding to talin1.<sup>51,53</sup>

Talin is a 270kDa cytoplasmic protein bound to the cytoskeletal protein actin. Talin consists of a 50kDa globular head domain and a 220kDa rod like tail domain.<sup>59,60</sup>

#### *Talin structure: The head en tail domain*

The talin head domain contains a FERM (4-point-one/ezrin/radixin/moesin) domain, composed of 3 sub domains F1, F2 and F3.<sup>61</sup> The F3 domain contains a phosphotyrosine binding (PTB) domain.<sup>62</sup> The three subdomains assemble into a compact clovershaped module.<sup>61</sup> The FERM domain binds to the cytoplasmic domains (tails) of the  $\beta$  domain, including  $\beta$ 1A,  $\beta$ 1D,  $\beta$ 2,  $\beta$ 3,  $\beta$ 5, and  $\beta$ 7 integrin subunits.<sup>59</sup> The FERM domain can also bind to a region of phosphatidylinositol phosphate kinase type 1 $\gamma$ -90 (PIP1 $\gamma$ -90) and to layilin, a cell surface hyaluronan receptor.<sup>58,63</sup>

The talin rod like tail domain is composed of a series of helical bundles ( $\alpha$ -helices) that can bind to vinculin at multiple sites, especially if talin is subjected to mechanical stress. It also has an F-actin binding site near the C-terminus. With the binding to actin it provides a direct linkage between the  $\beta$ -integrin tail and the actin cytoskeleton.<sup>58,63</sup>

## *FERM*

As said before, the talin head domain contains a FERM composed of three sub domains F1, F2 and F3. The F3 and F1 sub domains will now be explained further, starting with the most important F3 domain.

### F3

The F3 domain contains the integrin binding site, the PTB domain. There is a second integrin binding site in the talin rod, but only FERMs containing the F3 domain are able to activate integrins by increasing the affinity.<sup>59</sup> The F3 domain binds with the PTB domain to the membrane proximal (MP) NPXY<sub>747</sub> motif and to the helical region of  $\beta$ -integrin tails. This binding disrupts the salt bridge between the  $\alpha$  and  $\beta$  integrin tails that normally keeps integrins in the low affinity state.<sup>60</sup> A mutation of the Y<sub>747</sub> residue or the upstream W<sub>739</sub> residue also disrupts the interaction between the  $\alpha$  and  $\beta$  integrin tails.<sup>60,53</sup> Mutations in the talin phosphotyrosine binding (PTB) domain prevent the binding of talin to the  $\beta$  integrin domain, which results in decreased integrin affinity.<sup>64,51,62</sup>

Only the non-phosphorylated NPXY<sub>747</sub> motif in the integrin  $\beta$  subunit can associate with the PTB-like F3 domain of the talin head, which suggests that phosphorylation of Y<sub>747</sub> in  $\beta$ 3 negatively regulates talin binding and integrin activation. It is shown that talin head binding to a single GPIIb-IIIa molecule inserted into a phospholipid layer is enough to induce the conformational change that converts  $\alpha$ IIb $\beta$ 3 from a bent to an extended receptor.<sup>65,53</sup>

It is noticeable that other PTB domain containing proteins known to interact with the NPXY motif of integrins, such as Dok1, tensin or Numb, are unable to induce integrin activation. This is caused by the existence of additional talin head contact sites located in the helix forming membrane proximal (MP) region of the  $\beta$ -integrin tail.<sup>66,62</sup>

Moreover, the F3 sub domain of talin FERM contains an extra loop of amino acids between strands S1 and S2, whereas this loop does not exist in the FERM domain of Dok1, tension or Numb. This loop forms a hydrophobic pocket that accepts the side chains of F727 and F730 of talin. The loop binds to membrane proximal (MP) sequences in the  $\beta$ 3 integrin tail. The talin mutation L325R in this pocket prevents binding to the MP region and thus prevents activation.<sup>64</sup> It is probable that talin first meets the  $\beta$  integrin tail by binding the NPXY motif through its PTB domain. After this binding the loop sequence interacts with the membrane proximal (MP) sequences within the  $\beta$  tail, to displace the  $\alpha$  integrin tail and separate the transmembrane TM domains.<sup>62</sup> Next to this, a K residue in the TALIN-FERM loop engages with the  $\beta$ 1D subunit D residue. This residue is known to form a salt bridge with the  $\alpha$  subunit. Thus, the binding of the K residue with the  $\beta$ 1D subunit D residue can break the salt bridge and activate integrins.<sup>62</sup>

Although the integrin binding site is localized in the F3 domain, other regions of the talin head that are not directly involved in integrin binding are also important in integrin activation.<sup>65</sup> Both the F2 and F3 domains contain groups of positively charged residues that contribute to integrin activation and integrin clustering through interaction with negatively charged phospholipids in the cell membrane. This causes reorientation of the talin head together with the integrin  $\beta$  subunit, promoting the separation of the transmembrane helices of the integrin  $\alpha$  and  $\beta$  subunits.<sup>60</sup>

## F1

The F1 domain has a 30 residue unstructured loop and is preceded by an additional F0 domain which, like F1, has a ubiquitin-like fold.<sup>53, 60</sup> Positively charged residues in the F1 loop that bind to negatively charged phospholipids in the cell membrane are also required for integrin activation. It is possible that folding of the loop brings the F1 domain closer to the membrane, and in this way contributes to integrin activation. The F0 domain is also essential for activation of  $\beta$ 1-integrins and enhances the activation of  $\beta$ 3-integrin, although the mechanism is currently unclear.<sup>60</sup>

### *Clustering of integrins into focal adhesions requires talin*

Another important subject is that full-length talin is required to cluster integrins into focal adhesions (FA). Although the talin head increases integrin affinity, the rod like tail is also required. Focal adhesions transmit signals from integrins to different cellular compartments. Cells that do not express talin are unable to undergo sustained spreading, which indicates an adhesion defect. Expressing the talin1 head in these cells partially restored the spreading defect, but FAs were still absent, demonstrating that the clustering of integrins into larger adhesion structures depends on both the head and rod of talin.<sup>60</sup>

Mutational analysis of talin indicates that a functional dimerization motif is both necessary and sufficient to localize talin to focal adhesions. Because talin contains two  $\beta$  integrin-binding sites, one within the FERM and the other within the rod domain, the talin homodimer has up to four integrin-binding sites, which may enable talin to act as an integrin crosslinker in order to promote clustering. Consistent with this hypothesis, cleavage of the talin head from the rod domain by the protease calpain induces focal adhesion disassembly.<sup>67</sup>

### **Regulation of activation**

Integrin activation has to be strictly controlled, so talin-integrin binding has to be strictly regulated also. Under normal conditions there is an autoinhibitory interaction between the C-terminus of talin and the PTB domain of talin. This interaction blocks the integrin binding pocket. So, when function of talin is not required it may be maintained in an autoinhibited state.<sup>68,69</sup> In case of injury, talin becomes activated at sites of focal adhesions. There are two processes in which talin might be activated. The first process involves the second messenger PIP2, the second process involves calpain, a calcium dependent protease.

### *PIP2*

This process involves binding of talin to the lipid second messenger PIP2. PIP and PIP2 can bind to talin at early stages of adhesion. The head domain of talin contains mainly basic amino acids, and therefore talin is a good candidate for interacting with phosphoinositides.<sup>51</sup>

When phosphoinositides are associated with the phospholipid cell membrane, talin-phosphoinositide association is restricted to PIP2. This association leads to a conformational change of talin. Moreover, the interaction between integrin and talin is significantly enhanced by PIP2-induced talin activation. Sequestration of PIP2 by a specific pleckstrin homology domain confirms that PIP2 is required for accurate membrane localization of talin and that this localization is essential for the maintenance of focal adhesions.<sup>70</sup>

It is probable that PIP2 exposes the integrin-binding site on talin. PIP2 dependent signaling modulates assembly of focal adhesions by regulation integrin-talin complexes. This demonstrate that activation of the integrin binding activity of talin requires not only integrin engagement to the ECM but also the binding of PIP2 to talin, suggesting a possible role of lipid metabolism in organizing the sequential assembly of focal adhesion components.<sup>70</sup>

### *Calpain*

There is another mechanism of talin activation possible, that is the cleavage of talin by calpain. Calcium-dependent protease (calpain) is an intracellular cysteine protease. There are two ubiquitous calpain isozymes,  $\mu$ -calpain and m-calpain, which are active at micromolar and millomolar calcium concentrations, respectively.<sup>70,45</sup> Their amounts differ from tissue to tissue. More than 90% of the calpain in platelets is  $\mu$ -calpain.<sup>46</sup> The substrate of calpain is talin and calpain and talin colocalize at adhesion plaques. Calpain cleaves talin at an interdomain region, which results in production of the head en tail fragments.<sup>47,45</sup>

Both calpains have similar biochemical characteristics, except for the calcium concentration required for activation. Calpains are composed of a large catalytic subunit of 80 kDa and a small regulatory subunit of 30kDa. Each subunit contains a calmodulin-like domain at the C-terminus. After calcium binding to the calmodulin-like domains, calpains become active and begin to autolyze. The autolysis in the large subunit of  $\mu$ -calpain, which is accompanied by an increase in the sensitivity to calcium, is itself the activation step of calpain.  $\mu$ -calpain binds to the cytoplasmic surface of cell membranes as an 80-kDa proenzyme and is converted autolytically to the 76-kDa form via a 78-kDa intermediate on the membrane.<sup>46</sup>

### **Recruitment of talin to integrin tails**

In unstimulated platelets, talin resides in the cytoplasm, in an autoinhibited conformation, with the head and tail interacting in an intra- or intermolecular fashion to limit access to binding proteins. For binding to integrin tails and activation of integrins, talin must be recruited to the plasma membrane. This process involves Rap1a, a Ras subfamily member, and RIAM (Rap1-GTP-interacting adaptor molecule), a Rap1 effector. These proteins, when overexpressed, have the capacity to promote talin recruitment and subsequent  $\alpha$ IIb $\beta$ 3 activation.<sup>47</sup>

### *Rap1*

Ras proteins are small monomeric GTPases that cycle between the GTP-bound active form and the GDP-bound inactive form. Guanine nucleotide exchange factors (GEFs) promote Ras activity by exchanging bound GDP for GTP, whereas GTPase-activating proteins (GAPs) enhance the hydrolysis of Ras-bound GTP to GDP.<sup>71</sup>

In platelets, Rap1 is rapidly activated after binding of platelet agonists to platelet receptors by PI3K. A knock-out of Rap1B or of the Rap1GEF, RasGRP2 (CalDAG-GEFI), resulted in impaired  $\alpha$ IIb $\beta$ 3 dependent platelet aggregation. Thus, Rap1 is really essential in platelet aggregation.<sup>72</sup>

### *CalDAG-GEFI*

CalDAG-GEFI, a nucleotide exchange factor, activates Rap1 by promoting the release of GDP and loading of GTP in response to calcium and DAG. CalDAG-GEFI contains bindings sites for calcium and

DAG and a GEF domain catalyzing the activation of small GTPases of the RAS family, in particular Rap1 and Rap2. The nucleotide exchange activity of this protein can be stimulated by calcium and DAG.<sup>72,73</sup>

### *RIAM*

RIAM is a member of the MRL (Mig-10/RIAM/Lamellipodin) protein family and it is required for Rap1a to promote talin-dependent integrin activation. RIAM contains an Ras association (RA) domain, a pleckstrin homology (PH) domain, and several proline-rich sequences. RIAM functions as a scaffold that connect the membrane targeting sequences in Rap1a to talin, thereby recruiting talin to the plasma membrane, where it, after activation, binds to and activates integrins.<sup>74</sup>

RIAM binds directly to talin via a short, N-terminal fragment of 130 residues that contains an amphipathic  $\alpha$ -helix. RIAM binds to talin, even if it lacks the Rap1 binding RA domain, the PH domain, and most of the ENA/VASP binding motifs. However, for integrin activation, it is required that RIAM binds to both Rap1 and to talin. Without the talin binding domain or the Rap1 binding RA domain RIAM cannot promote integrin activation. Rap1 activity is not necessary for the association of talin and GPIIb-IIIa with RIAM.<sup>72</sup>

### **Kindlins**

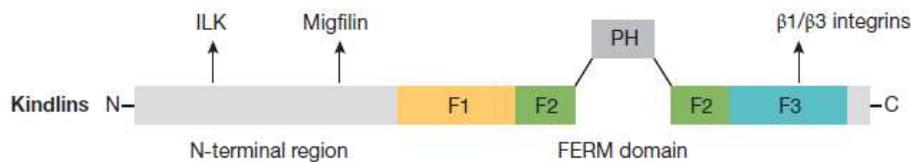
Although talin is the major activator of integrin ligand binding function, additional co-activators are required. There are numerous other proteins known to interact with integrin cytoplasmic tails including kindlins. The kindlin name refers to the Kindler syndrome first described by Dr. Kindler in 1954. Patients with this syndrome suffer from skin blistering, skin fragility and sun sensitivity, with often oral and colonic involvement.<sup>72</sup>

Three different kindlins (kindlin-1, -2, -3) are known, which are encoded by three separate genes and expressed in a tissue- or cell type-specific manner. Platelets lacking kindlin-3 cannot activate integrins even if talin expression is normal. This kindlin-3 deficiency results in severe bleeding and resistance to arterial thrombosis.<sup>53</sup>

Kindlin-1, also known as URP1 (Unc-112 related protein 1), kindlerlin and FERMT1, predominantly expressed in epithelial cells, is also found in a number of other tissues, including skin, heart, lung, liver, kidney, colon, prostate, ovary and pancreas. Kindlin-2, also known as MIG-2 (mitogen induced gene 2), is also expressed ubiquitously with the exception of hematopoietic cells and it is the only kindlin expressed in embryonic stem (ES) cells. The highest amounts of kindlin-2 are found in skeletal and smooth muscle cells. Kindlin-3, also known as URP2 (Unc-112 related protein 2), is only expressed in hematopoietic cells, mainly in megakaryocytes and platelets. Within the cells, all three kindlins co-localize to integrin-dependent adhesion sites. This suggest that they have very similar functions. Kindlins modulate signaling to and from integrins and loss of kindlins alters cell adhesion, shape and migration. Certain kindlins are also found to localize to unique structures. Kindlin-2 for example, is present in cell-cell contacts or in the nucleus. The three mammalian kindlins, kindlin-1, kindlin-2 and kindlin-3 exhibit high sequence similarities with identical domain architecture.<sup>75,53</sup>

Kindlins, like talin, contain FERM domains with the three subdomains F1, F2 and F3. However, talin and most other FERM-domain containing proteins have FERM domains in the N-terminal region, whereas kindlin FERM domains are located at the C-terminal region. moreover, the kindlins FERM

domains are split in the F2 subdomain by the insertion of a pleckstrin homology (PH) domain, suggesting that kindlins can be recruited to the cell membrane by binding to phospholipids. The structure of kindlins are shown in figure 3.<sup>75</sup>



**Figure 5. Kindlin domain structure and binding partners.**<sup>53</sup>

Among all FERM proteins, the kindlin FERM domains exhibit the highest sequence similarity with the talin FERM domains (>50%).<sup>76,76</sup> The kindlin FERM domain has, like the talin-FERM domain, a N-terminal FO domain and a large flexible F1 loop. The PTB-like subdomain within the kindlin FERM domain is also similar to that of talin. The only difference is that the FERM-PTB like subdomain binds to the membrane distal (MD) NPxY motif in  $\beta$ -integrin tails, whereas the talin FERM domain binds to the membrane proximal (MP) motif.<sup>53,53</sup>

The F3 subdomain of kindlins can interact directly with integrin  $\beta$  subunit cytoplasmic tails. All three kindlins interact with  $\beta 1$  and  $\beta 3$  integrin tails, while kindlin-3 additionally binds to  $\beta 2$  integrin tails. Kindlins might function as adaptor proteins promoting protein-protein interactions. Kindlins also interact with integrin-linked kinase (ILK) and the filamin-binding protein migfilin, two focal adhesion (FA) proteins that directly or indirectly regulate actin dynamics and integrin-dependent intracellular signaling pathways.<sup>75,53</sup>

Depletion of kindlin-1, -2 and -3 with siRNAs in mice and cells proofed that kindlins are essential regulators of integrin function because the conformational shift of integrins from the low to the high affinity does not occur in the absence of kindlins.<sup>76</sup>

### *Kindlin-3*

Kindlin-3 deletion causes severe bleeding. In kindlin-3 depleted platelets the integrins cannot bind ligands and platelet aggregation is defective even with normal amounts of talin. This prevents the formation of pathological thrombi. The same phenotype occurs in talin-deficient platelets, indicating that both proteins are required to regulate integrin affinity. Co-expressing kindlins with talin enhances talin-mediated activation of the platelet integrin  $\alpha IIb\beta 3$ .<sup>61</sup>

Thus, kindlin-3 is essential for platelet integrin activation and integrin outside-in signaling. It also regulates activation of  $\beta 3$  and  $\beta 1$  integrins. This suggests that kindlin-3, like talin, is a general regulator of integrin activation.<sup>61</sup>

### *Binding of kindlins to integrins*

Kindlin-1 and kindlin-2 bind directly to integrin  $\beta 1A$  cytoplasmic tails, with a partially conserved region in the F3 subdomain of the kindlin-1 and kindlin-2 FERM domain.<sup>75</sup>

Integrin binding activity correlates with kindlin-1 targeting to focal adhesions. Kindlin overexpression suppresses  $\beta 1$  integrin activation. This suggest that integrin activation depends on kindlin expression level and levels of active talin.<sup>61</sup>

### Short chapter summary

Activation of signaling pathways in platelets stimulated via GPVI, PAR-1 and P2Y12 result in integrin activation. This activation involves activation of PLC, PKC, talin and kindlins, also shown in figure 4.

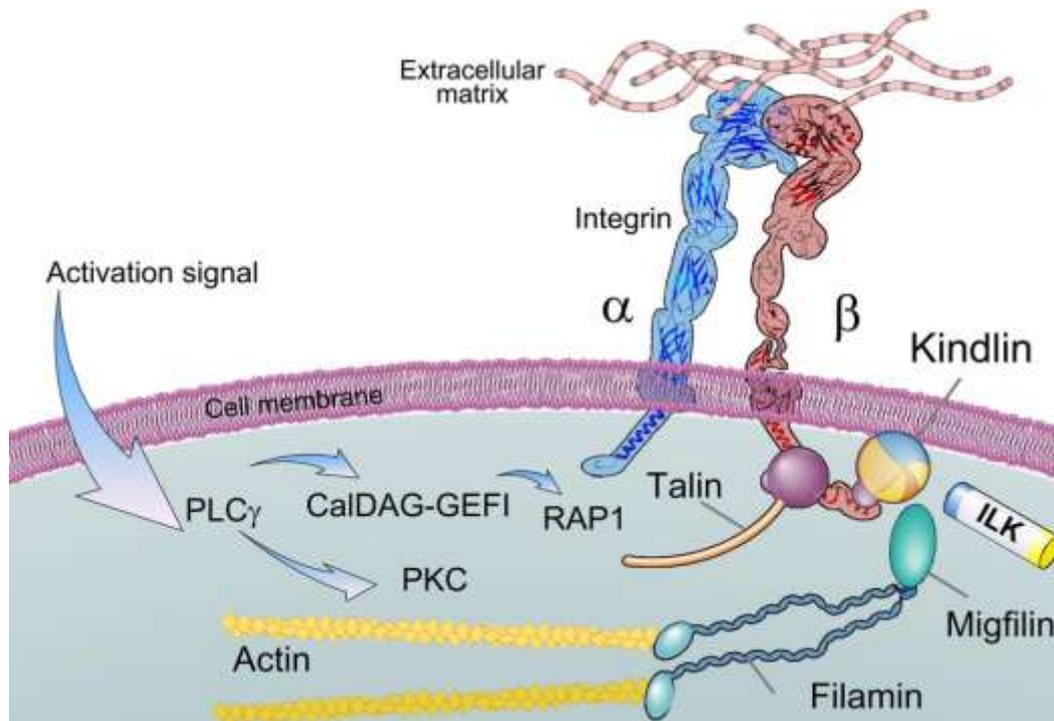


Figure 6. Schematic representation of the signaling pathway from an agonist to integrin activation in platelets.<sup>61</sup>

# Chapter 7: Experiments

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## Experiment 1: The effect of AR-C69931MX on platelet activation at different time points

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### Introduction

*The last years there is increasing evidence that granule release in platelets is moderated by a different signal transduction pathway than the activation of integrin  $\alpha\text{IIb}\beta\text{III}$ . It is known that inhibition of the P2Y12 receptor with AR-C69931MX (cangrelor, an analogue of ATP) blocks integrin activation. However, there is still some granule release, although in a decreased rate. This strongly suggest that  $\alpha$  granule release and integrin activation are different processes. However, it is still unclear how this regulation occurs. It is known that AR-C69931MX, a platelet antagonist, raises the platelet cAMP concentration to levels that inhibit platelet aggregation. This is mediated through activation of a separate G protein-coupled pathway, presumably an unidentified platelet  $G_s$ -protein that stimulates cAMP-mediated inhibition of platelet function.<sup>77</sup>*

*AR-C69931MX is a potent P2Y12 inhibitor that reaches a higher level of platelet inhibition than clopidogrel, which is also a platelet antagonist. It reversibly binds to the P2Y12 receptor and does not require conversion to an active metabolite for its antiplatelet action.*

**Aim:** to determine the activation response of platelets in response of the P2Y12 inhibitor AR-C69931MX at different time points, by determining p-selectin expression and GPIIb-IIIa activation after stimulation with ADP, and TRAP (thrombin receptor activating peptide) for 90 sec, 2 min, 5 min, 15 min.

### Materials and methods

#### Materials

PE- $\alpha$ -P-selectin was purchased from BD Pharmingen (San Diego, CA). FITC- $\alpha$ -Fibrinogen was purchased from DAKO (Glostrup, Denmark). AR-C69931MX was a gift from Astra Zeneca (R&D Mölndal, Sweden). ADP was purchased from Roche Diagnostics (Mannheim, Germany). TRAP was purchased from Bachem (Freiburg, Germany). The FACS machine (BD Canto 2) is purchased from Becton Dickinson (Heidelberg, Germany).

#### Methods

First two master mixes are prepared. The first one (control) contains 2750 $\mu\text{l}$  HBS, 110 $\mu\text{l}$  PE- $\alpha$ -P-selectin and 27,5 $\mu\text{l}$   $\alpha$ -Fibrinogen-FITC. The second one contains 2585 $\mu\text{l}$  HBS, 110 $\mu\text{l}$  PE- $\alpha$ -P-selectin, 27,5 $\mu\text{l}$   $\alpha$ -Fibrinogen-FITC and 27,5 $\mu\text{l}$  AR-C69931MX. Gradients of ADP and TRAP are made, with 8 different concentrations. For each sample and each time point strips with 200 $\mu\text{l}$  fixative (0,2% formaldehyde, 0,9% NaCL) are used. Then 15 $\mu\text{l}$  whole blood (obtained from healthy donors who signed an informed consent, UMC Utrecht ) is added to each ep and mixed. At the different time points, 20 $\mu\text{l}$  of each sample is taken and put in the strips with fixative. Then 100 $\mu\text{l}$  sample from each strip is taken and put in a well with 100 $\mu\text{l}$  fixative in a 96-wells plate. The samples are analyzed by FACS (BD Canto2).

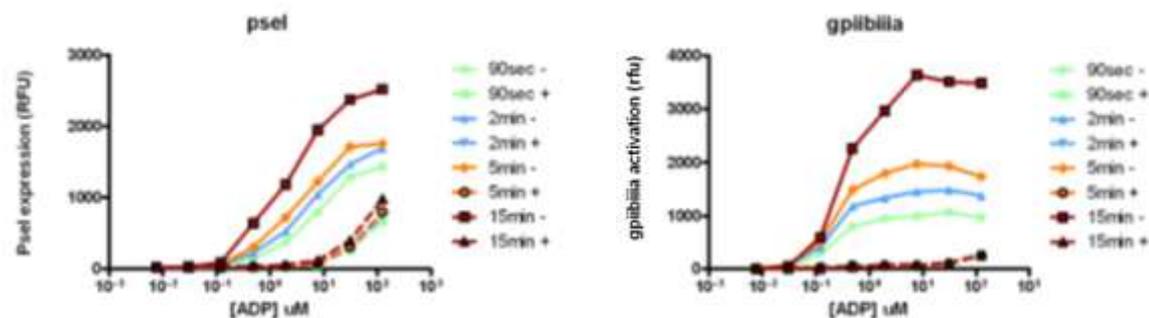
## Results and discussion

### Stimulation with ADP

In figure 1 the results of the experiment with ADP are shown. The first graph shows the P-selectin expression after platelet stimulation with ADP, with (illustrated with +) and without (illustrated with -) addition of AR-C69931MX. It is shown that after stimulation with ADP P-selectin is expressed on the platelet membrane. This is depending on the concentration of ADP that is used to stimulate the platelets. The higher the ADP concentration, the more P-selectin is expressed on the platelet membrane.

In the presence of AR-C69931MX, P-selectin expression is decreased. The higher the ADP concentration, the less AR-C69931MX can inhibit P-selectin expression. At the lowest ADP concentrations the expression is completely blocked.

The right graph shows the activation of GPIIb-IIIa after stimulation with ADP, with (illustrated with +) and without (illustrated with -) AR-C69931MX. The higher the ADP concentrations, the more GPIIb-IIIa is activated, although there is some decreased activation at the two highest ADP concentrations. In addition of AR-C69931MX, the activation of GPIIb-IIIa is almost completely blocked. Only after stimulation with the highest ADP concentration in addition of AR-C69931MX, there is some GPIIb-IIIa activation after 15 minutes.

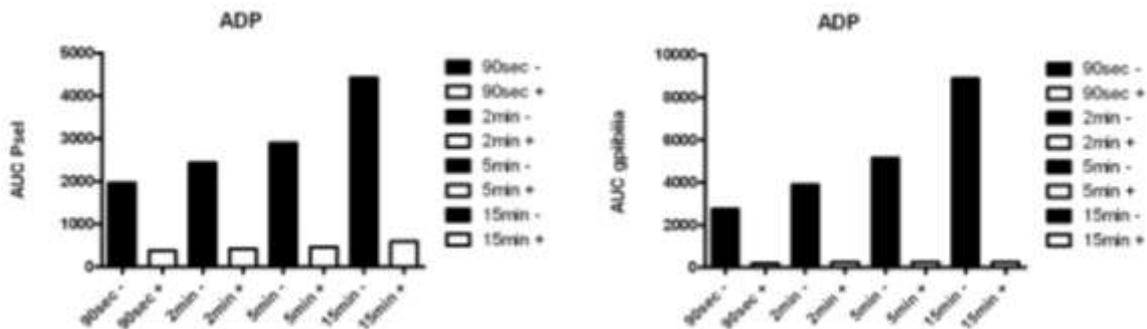


**Figure 1.** P-selectin expression and GPIIb-IIIa activation after stimulation with ADP, in the absence (-) and presence (+) of AR-C69931MX. The continuous line is after stimulation without AR-C69931MX compound. The broken line shows the expression with AR-C69931MX compound.

### Area under the curves

In figure 2 the area under the curves (from figure 1) are shown. The left graph shows the area under the P-selectin expression curves. There can be seen that in the absence of AR-C69931MX the higher is the incubation time, the higher is the AUC. When AR-C69931MX is added, the AUC is significantly decreased. But there is still some AUC left, which means that AR-C69931MX was not able to completely inhibit P-selectin expression.

The right graph shows the area under the GPIIb-IIIa activation curves. The AUC is increasing with the incubation time. In addition of AR-C69931MX, the AUC is almost zero. This means that AR-C79931MX almost completely inhibited GPIIb-IIIa activation after ADP stimulation.



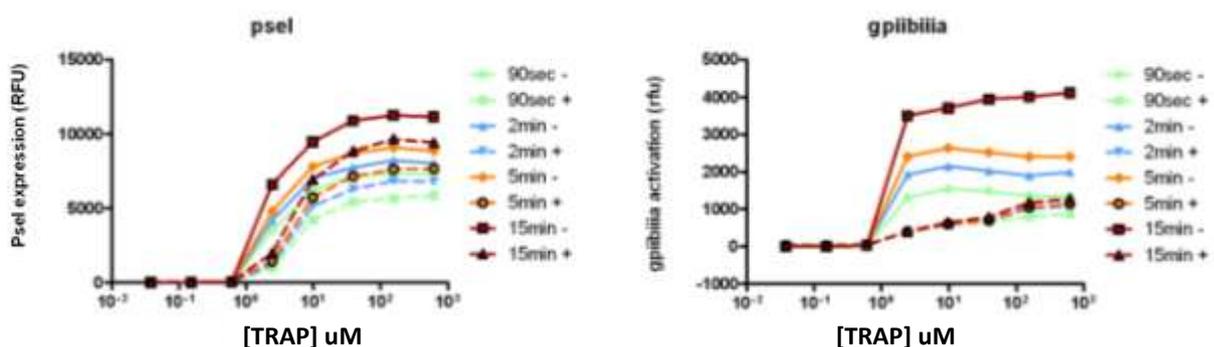
**Figure 2.** Area under the curve (AUC) after stimulation with ADP at different time points, in the absence (illustrated with -) and presence (illustrated with +) of AR-C69931MX.

### Stimulation with TRAP

In figure 3 the results of the experiment after stimulation with TRAP are shown.

The left graph shows the P-selectin expression after PAR-1 stimulation with (illustrated with +) and without (illustrated with -) addition of AR-C69931MX. If there is no AR-C69931MX added, P-selectin expression is increasing with the time and with TRAP concentrations. When AR-C69931MX is added, this expression is decreased, but there is still expression of P-selectin. The expression, in the presence of AR-C69931MX, is also increasing with the time and with TRAP concentrations. This suggests that AR-C69931MX cannot completely inhibit P-selectin expression after stimulation with TRAP.

The right graph shows GPIIb-IIIa activation after TRAP stimulation. GPIIb-IIIa activation is increasing with the time of incubation. When incubation takes 15 minutes, the GPIIb-IIIa activation is increasing with the PAR-1 concentration. Incubation for 90 seconds, 2 minutes and 5 minutes shows first an increasing GPIIb-IIIa activation with increasing TRAP concentration, but this activation is a little decreased at higher TRAP concentrations. In the presence of AR-C69931MX, GPIIb-IIIa activation is significantly decreased. This suggests that AR-C69931MX can block GPIIb-IIIa activation after TRAP stimulation and that GPIIb-IIIa activation is ADP-dependent.

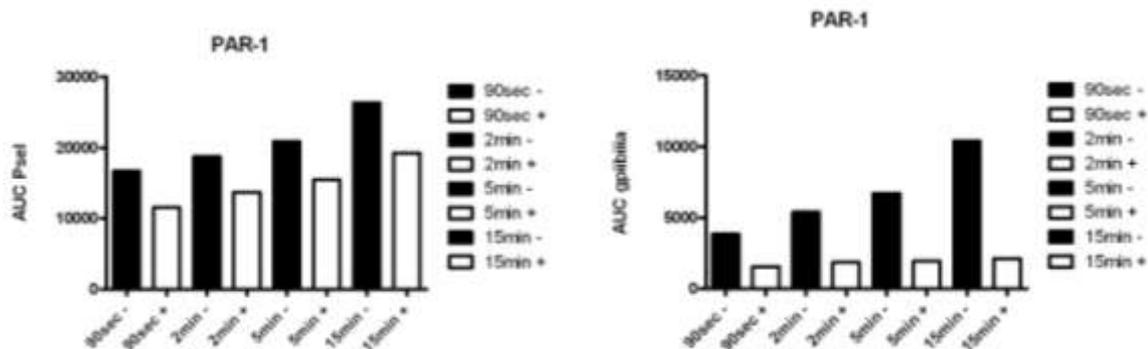


**Figure 3.** P-selectin expression and GPIIb-IIIa activation after stimulation with TRAP, in the absence (-) and presence (+) of AR-C69931MX. The continuous line is after stimulation without AR-C69931MX compound. The broken line shows the expression with AR-C69931MX compound.

### Area under the curves

In figure 4 the area under the curves (from figure 2) are shown. The left graph shows the area under the P-selectin expression curves. There can be seen that in the absence of AR-C69931MX the higher

the incubation time, the higher is the AUC. When AR-C69931MX is added, there is a little decrease in the AUC. This means that AR-C69931MX was not able to completely inhibit P-selectin expression. The right graph shows the area under the GPIIb-IIIa activation curves. The AUC is increasing with the incubation time. In the presence of AR-C69931MX, the AUC is significantly decreased, but there is still some GPIIb-IIIa activation. This means that AR-C79931MX is not able to completely inhibited GPIIb-IIIa activation after ADP stimulation. This suggest that GPIIb-IIIa activation is ADP dependent.



**Figure 4.** Area under the curve (AUC) after stimulation with TRAP at different time points, in the absence (illustrated with -) and presence (illustrated with +) of AR-C69931MX.

## Experiment 2: The effect of AR-C69931MX on platelet activation at different time points.

### Introduction

The first experiment showed that AR-C69931MX is able to decrease P-selectin expression and GPIIb-IIIa activation after stimulation with ADP or TRAP. The levels of P-selectin expression and GPIIb-IIIa activation were decreased after stimulation with ADP in particular.

To find out what the role of ADP is and if the decrease of granule release and GPIIb-IIIa activation is only a cause of competition for the receptor or if it is also a cause of a raise in cAMP levels the experiment is repeated with apyrase instead of AR-C69931MX. Apyrase is an enzyme, which catalyzes the hydrolysis of ATP and ADP to AMP and inorganic phosphate. Thus, when P-selectin expression levels and GPIIb-IIIa activation levels are also decreased in the presence of apyrase, AR-C69931MX may also act via a raise in cAMP. If these levels are not decreased in the presence of apyrase, AR-C69931MX may act as a competitive antagonist for ADP only. This could only be determined by measuring cAMP levels.

However, in this experiment, the effect of ADP on P-selectin expression and GPIIb-IIIa activation are measured only. The levels of cAMP are not determined. So, with this experiment the importance of ADP can be shown, but not the role of ADP in raising the cAMP concentration.

**Aim:** to determine the activation response of platelets in response of apyrase at different time points, by determining p-selectin expression and GPIIb-IIIa activation after stimulation with ADP, and TRAP for 90 sec, 2 min, 5 min, 15 min.

## Materials and Methods

### Materials

PE- $\alpha$ -P-selectin was purchased from BD Pharmingen (San Diego, CA). FITC- $\alpha$ -Fibrinogen was purchased from DAKO (Glostrup, Denmark). Apyrase was purchased from Sigma Chemical Co (Poole, UK). ADP was purchased from Roche Diagnostics (Mannheim, Germany). TRAP was purchased from Bachem (Freiburg, Germany). The FACS machine (BD Canto 2) is purchased from Becton Dickinson (Heidelberg, Germany).

### Methods

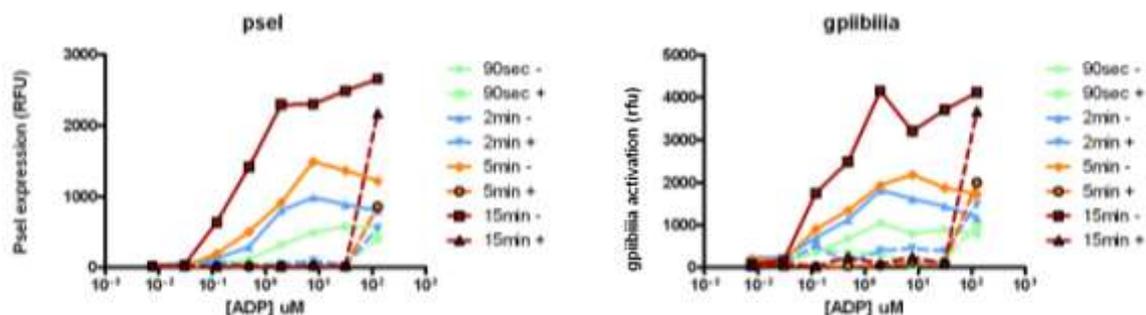
First two master mixes are prepared. The first one (control) contains 2612,5 $\mu$ l HBS, 110 $\mu$ l  $\alpha$ -P-selectin and 27,5 $\mu$ l  $\alpha$ -Fibrinogen-FITC. The second one contains 2590,5 $\mu$ l HBS, 110 $\mu$ l  $\alpha$ -P-selectin, 27,5 $\mu$ l  $\alpha$ -Fibrinogen-FITC and 22 $\mu$ l apyrase. Gradients of ADP and TRAP are made, with 8 different concentrations. For each sample and each time point strips with 200 $\mu$ l fixative (0,2% formaldehyde, 0,9% NaCl) are used. Whole blood is centrifuged for 15 minutes (160crf, RT), where after the upper layer with platelets is used for this experiment. Then 15 $\mu$ l platelet rich plasma (obtained from healthy donors who signed an informed consent, UMC Utrecht) is added to each ep and mixed. At the different time points, 20 $\mu$ l of each sample is taken and put in the strips with fixative. Then 100 $\mu$ l sample from each strip is taken and put in a well with 100 $\mu$ l fixative in a 96-wells plate. The samples are analyzed by FACS.

## Results and discussion

### Stimulation with ADP

As shown in figure 5, there is P-selectin expression after ADP stimulation. This expression is increasing with the increasing ADP concentration, but at higher ADP concentrations this level is a little decreasing for 90 seconds, 2 minutes and 5 minutes of incubation. When apyrase is added, the P-selectin expression is completely inhibited for all time points and all ADP concentrations, except for the highest ADP concentration, where it is decreased but not completely inhibited. This means that apyrase inhibits P-selectin expression, but it is not capable to do this for the highest ADP concentration.

In the right graph the GPIIb-IIIa activation is shown. This activation is increasing when the ADP concentration becomes higher, but it decreases at the highest ADP concentrations. In the presence of apyrase, the GPIIb-IIIa activation is lowered in all conditions, except for the highest ADP concentration for 2 and 5 minutes of incubation. This graph shows that apyrase is able to inhibit GPIIb-IIIa activation after ADP stimulation, but not for the highest ADP concentrations.

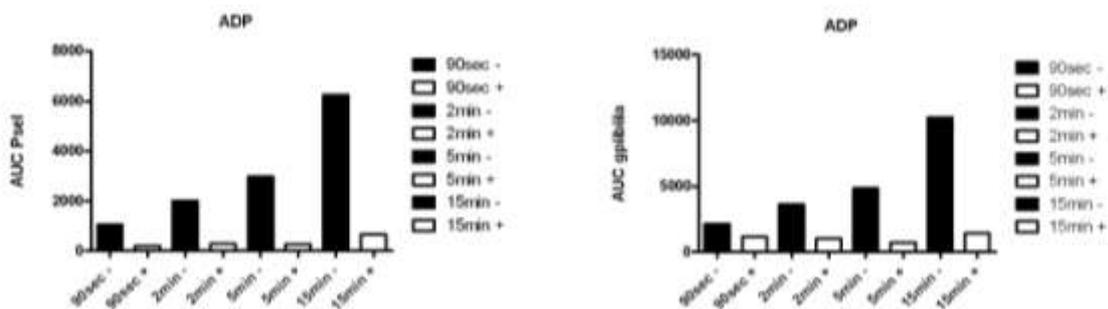


**Figure 5.** P-selectin expression and GPIIb-IIIa activation after stimulation with ADP, in the absence (-) and presence (+) of apyrase. The continuous line is after stimulation without apyrase compound. The broken line shows the expression with apyrase compound.

Area under the curves

In figure 6 the area under the curves from figure 5 are shown. The left graph shows the area under the P-selectin expression curves. It is shown that the AUC is increasing with the time of incubation. This AUC is decreased in the presence of apyrase. This means that apyrase inhibits P-selectin expression. But this inhibition is not complete, because stimulation with the highest ADP concentration gives still a little P-selectin expression. This suggests that there was not enough apyrase to inhibit P-selectin expression completely.

In the right graph the area under the GPIIb-IIIa activation curves is shown. The AUC is increasing with the time of incubation. The AUC is decreased in the presence of apyrase, which means that apyrase decreases GPIIb-IIIa activation.

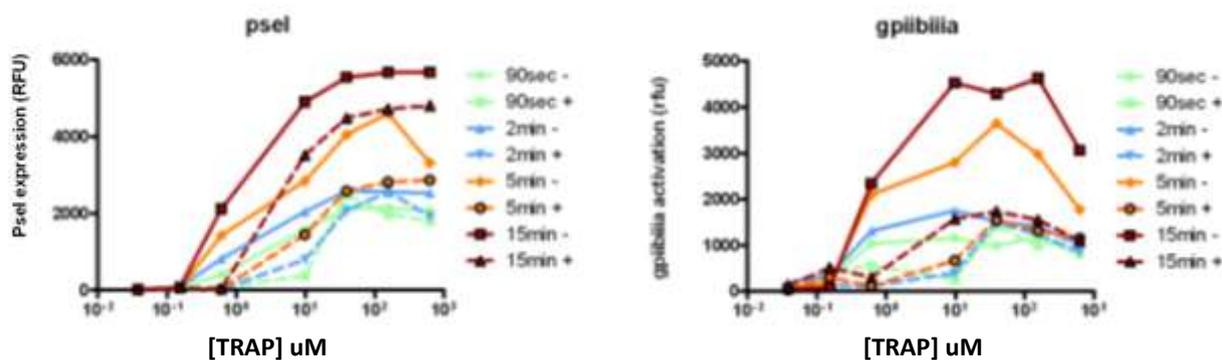


**Figure 6.** Area under the curve (AUC) after stimulation with ADP at different time points, in the absence (illustrated with -) and presence (illustrated with +) of apyrase.

Stimulation with TRAP

As shown in figure 7, there is GPIIb-IIIa expression after TRAP stimulation. This expression is increasing with the TRAP concentration, but at higher TRAP concentrations this level is decreasing for 5 minutes of incubation. When apyrase is added, this expression is decreased for all time points and all TRAP concentrations. This means that apyrase decreases P-selectin expression, but only for a small part. It cannot completely block P-selectin expression.

In the right graph the GPIIb-IIIa activation is shown. This activation is increasing when the TRAP concentration becomes higher, but it decreases with the highest TRAP concentrations. In the presence of apyrase, the GPIIb-IIIa activation is lowered in all conditions. This suggests that apyrase is able to decrease GPIIb-IIIa activation after PAR-1 stimulation.

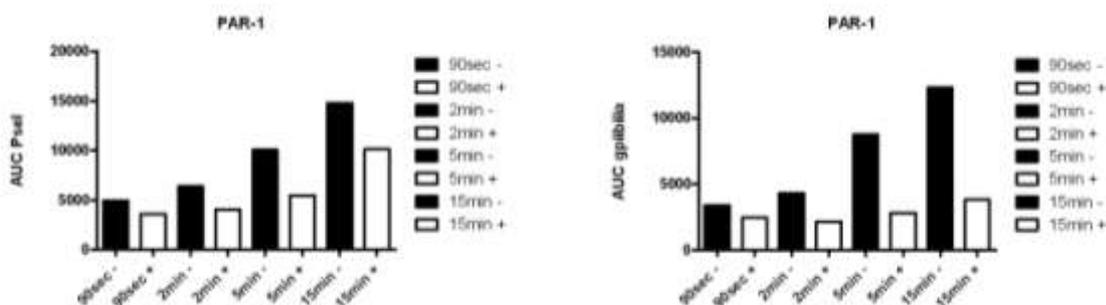


**Figure 7.** P-selectin expression and GPIIb-IIIa activation after stimulation with PAR-1, in the absence (-) and presence (+) of apyrase. The continuous line is after stimulation without apyrase compound. The broken line shows the expression with apyrase compound.

#### Area under the curves

In figure 8 the area under the curves from figure 7 are shown. The left graph shows the area under the P-selectin expression curves. It is shown that the AUC is increasing with the time of incubation. This AUC is decreased in the presence of apyrase. This means that apyrase decreases, but not completely inhibits, P-selectin expression.

In the right graph the area under the GPIIb-IIIa activation curves is shown. The AUC is increasing with the time of incubation. The AUC is decreased in the presence of apyrase, which means that apyrase can decrease GPIIb-IIIa activation.



**Figure 8.** Area under the curve (AUC) after stimulation with PAR-1 at different time points, in the absence (illustrated with -) and presence (illustrated with +) of apyrase.

#### Conclusion

The aim of this experiment was to determine the importance of ADP for P-selectin expression and GPIIb-IIIa activation. In particular the GPIIb-IIIa activation is ADP-dependent. P-selectin expression is a little ADP-dependent also, but not in such way as GPIIb-IIIa activation. The next question was if AR-C69931MX, besides being a competitive antagonist, causes a raise in cAMP levels also. P-selectin expression levels and GPIIb-IIIa activation levels are decreased in the presence of apyrase, which means that AR-C69931MX may cause a raise in cAMP levels (higher cAMP levels can inhibit platelet activation). To confirm this hypothesis, other experiments should be done in which cAMP levels will be measured.

### **Experiment 3:**

#### **The effect of AR-C69931MX and apyrase on GPVI platelet activation at different time points**

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In chapter one, the activation of platelets via GPVI can be read. In the following experiment the effect of AR-C69931MX and apyrase on platelet activation via stimulation of GPVI with CRP (collagen related peptide) is determined.

**Aim:** to determine the activation response of platelets in presence of the P2Y12 inhibitor AR-C69931MX and apyrase at different time points, by determining p-selectin expression and GPIIb-IIIa activation after stimulation of GPVI with CRP for 90 sec, 2 min, 5min and 15min.

#### **Materials and methods**

##### *Materials*

PE- $\alpha$ -P-selectin was purchased from BD Pharmingen (San Diego, CA). FITC- $\alpha$ -Fibrinogen was purchased from DAKO (Glostrup, Denmark). AR-C69931MX was a gift from Astra Zeneca (R&D Mölndal, Sweden). Apyrase was purchased from Sigma Chemical Co (Poole, UK). CRP was obtained from Richard Farndale (Cambridge, UK) The FACS machine (BD Canto 2) is purchased from Becton Dickinson (Heidelberg, Germany).

##### *Methods*

First three mastermixes are prepared. The first one (control) contains 2612,5 $\mu$ l HBS, 110 $\mu$ l  $\alpha$ -P-selectin and 27,5 $\mu$ l  $\alpha$ -Fibrinogen-FITC. The second one contains 2585 $\mu$ l HBS, 110 $\mu$ l  $\alpha$ -P-selectin, 27,5 $\mu$ l  $\alpha$ -Fibrinogen-FITC and 27,5 $\mu$ l AR-C69931MX. The third one contains 2590,5 $\mu$ l HBS, 110 $\mu$ l  $\alpha$ -P-selectin, 27,5 $\mu$ l  $\alpha$ -Fibrinogen-FITC and 22 $\mu$ l apyrase. A gradient of CRP is made, with 8 different concentrations. For each sample and each time point strips with 200 $\mu$ l fixative (0,2% formaldehyde, 0,9% NaCl) are used. Whole blood is centrifuged for 15 minutes (160crf, RT), where after the upper layer with platelets is used for this experiment. Then 15 $\mu$ l platelet rich plasma (obtained from healthy donors who signed an informed consent, UMC Utrecht) is added to each ep and mixed. At the different time points, 20 $\mu$ l of each sample is taken and put in the strips with fixative. Then 100 $\mu$ l sample from each strip is taken and put in a well with 100 $\mu$ l fixative in a 96-wells plate. The samples are analyzed by FACS.

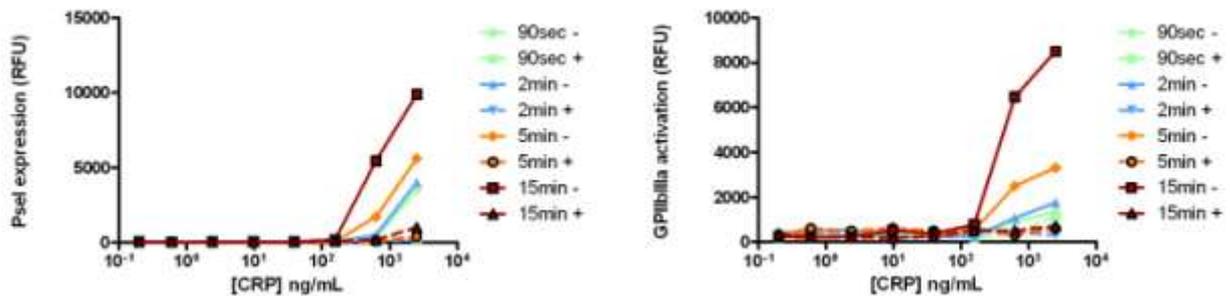
#### **Results and discussion**

##### *Stimulation with CRP, with and without AR-C69931MX*

In figure 9 the results of the experiment with CRP and AR-C69931MX are shown. The first graph shows the P-selectin expression after platelet stimulation with CRP, with (illustrated with +) and without (illustrated with -) addition of AR-C69931MX. It is shown that after stimulation with the highest CRP concentrations P-selectin is expressed on the platelet membrane. In the presence of AR-C69931MX this P-selectin expression is almost decreased to zero. Stimulation with the lowest CRP concentrations give no results, which means that there was too less CRP to activate platelets.

The right graph shows the GPIIb-IIIa activation after stimulation with CRP. Only stimulation with the highest CRP concentrations results in the activation of GPIIb-IIIa. In the presence of AR-C69931MX

this activation is almost zero, except for the incubation time of 90 seconds. There it is almost the same with and without AR-C69931MX compound. Stimulation with the lowest CRP concentrations give no results, which means that there was too less CRP to activate platelets.

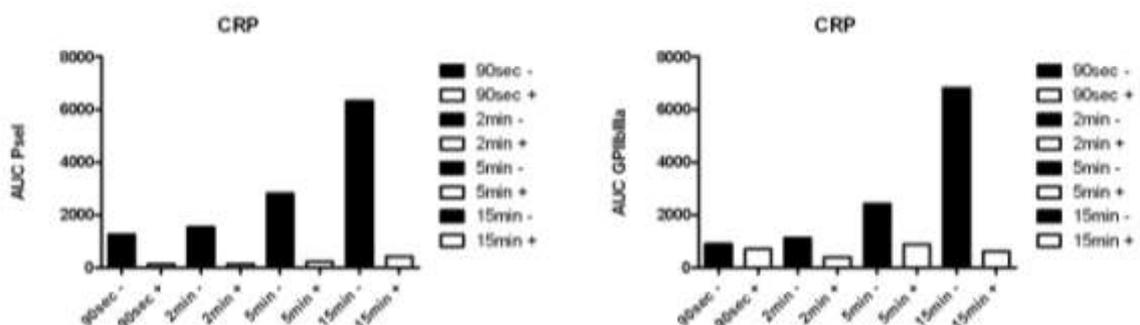


**Figure 9.** P-selectin expression and GPIIb-IIIa activation after stimulation with CRP, in the absence (-) and presence (+) of AR-C69931MX. The continuous line is after stimulation without AR-C69931MX compound. The broken line shows the expression with AR-C69931MX compound.

#### Area under the curves

In figure 10 the area under the curves (from figure 9) are shown. The left graph shows the area under the P-selectin expression curves. There can be seen that in the absence of AR-C69931MX the higher is the incubation time, the higher is the AUC. When AR-C69931MX is added, the AUC is significantly decreased and it is almost zero in all incubation times. This means that AR-C69931MX was able to inhibit P-selectin expression after CRP stimulation, probably by the activation of an inhibiting signal transduction pathway (a raise in cAMP).

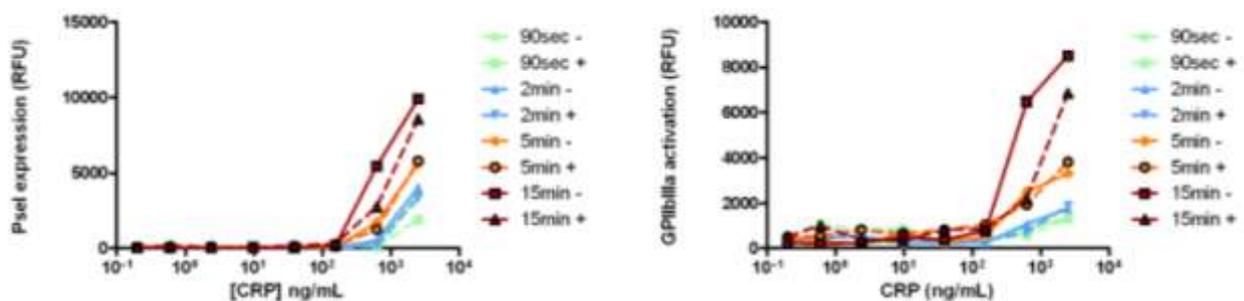
The right graph shows the area under the GPIIb-IIIa activation curves. The AUC is increasing with the incubation time. In addition of AR-C69931MX, the AUC is significantly decreased. This means that AR-C69931MX can decrease GPIIb-IIIa activation after CRP stimulation. These results suggest that GPIIb-IIIa activation is ADP dependent. For the incubation time of 90 seconds the AUC is almost the same with and without AR-C69931MX. It could be that these platelets were a little pre-activated before the addition of AR-C69931MX.



**Figure 10.** Area under the curve (AUC) after stimulation with ADP at different time points, in the absence (illustrated with -) and presence (illustrated with +) of AR-C69931MX.

Stimulation with CRP, with and without apyrase

In figure 11 the results of the experiment with CRP and apyrase are shown. The first graph shows the P-selectin expression after platelet stimulation with CRP, with (illustrated with +) and without (illustrated with -) addition of apyrase (to prevent platelet activation by secreted ADP). It is shown that after stimulation with the highest CRP concentrations P-selectin is expressed on the platelet membrane. In the presence of apyrase this P-selectin expression is almost the same, which means that apyrase was not able to block P-selectin expression. Stimulation with the lowest CRP concentrations give no results, which means that there was too less CRP to activate platelets. The right graph shows the GPIIb-IIIa activation after stimulation with CRP. Only stimulation with the highest CRP concentrations results in the activation of GPIIb-IIIa. In the presence of apyrase this activation is almost the same, which means that apyrase was not able to block P-selectin expression.

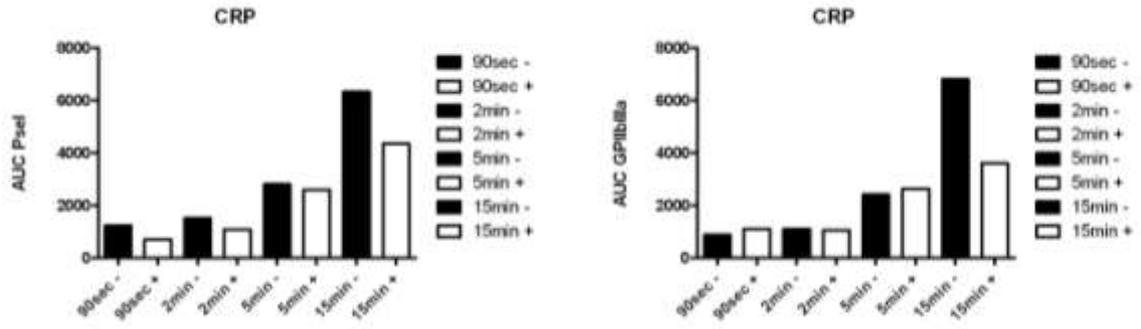


**Figure 11.** P-selectin expression and GPIIb-IIIa activation after stimulation with CRP, in the absence (-) and presence (+) of apyrase. The continuous line is after stimulation without apyrase compound. The broken line shows the expression with apyrase compound.

#### Area under the curves

In figure 12 the area under the curves (from figure 9) are shown. The left graph shows the area under the P-selectin expression curves. There can be seen that in the absence of apyrase the higher is the incubation time, the higher is the AUC. When apyrase is added, the AUC is a little decreased. This means that apyrase was able to partially inhibit P-selectin expression after CRP stimulation. These results suggest that CRP can activate platelets to express P-selectin, without the help of secreted ADP. However, the activation is a little higher in the presence of ADP, so it is partially ADP-dependent. Probably there was not enough apyrase to convert all the ADP into AMP and phosphate to completely inhibit P-selectin expression.

The right graph shows the area under the GPIIb-IIIa activation curves. The AUC is increasing with the incubation time. In addition of apyrase, the AUC is only significantly decreased for the incubation time of 15 minutes. For the other incubation times, the AUC is almost the same. This means that apyrase has almost no effect on GPIIb-IIIa activation after stimulation with CRP. These results suggest that CRP can activate GPIIb-IIIa, without the help of secreted ADP. Only for the highest incubation time, the activation is partially ADP-dependent. Probably there was not enough apyrase to convert all the ADP into AMP and phosphate to completely inhibit GPIIb-IIIa activation.



**Figure 12.** Area under the curve (AUC) after stimulation with ADP at different time points, in the absence (illustrated with -) and presence (illustrated with +) of apyrase.

# Chapter 8: Conclusion

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The main question of this thesis was: ***What are the most important intracellular signaling pathways in the activation of platelets?*** The sub questions were: What are the most important platelet receptors? How are these receptors activated? How are the signals from these receptors transduced intracellularly? How will lead stimulation of platelet receptors to platelet granule release and GPIIb-IIIa activation? How are platelet granule release and GPIIb-IIIa activation regulated?

The most important platelet receptors are GPVI, PAR-1 and P2Y<sub>12</sub>, which are activated with the agonists collagen, thrombin and ADP, respectively.

Upon vessel wall injury, collagen in the sub endothelium becomes exposed to the bloodstream, which leads to the binding of GPVI to collagen and thereby platelet activation. This activation involves activation of PLC $\gamma$ , PKC and PI3K. Activated platelets can spread and form pseudopods on their surface, which facilitate binding and aggregation of platelets.

Most of the platelet agonists transduce signals via seven transmembrane domain receptors that are coupled to heterotrimeric G-proteins. Upon stimulation of the heterotrimeric receptor, the  $\alpha$ -subunit is released from the  $\beta\gamma$ -complex. The liberated subunits are second messengers and may exert either a stimulatory or inhibitory role, depending on the receptor they were bound to. A major effector that is activated via a heterotrimeric receptor is PLC. PLC cleaves PIP<sub>2</sub> in the membrane to form DAG and IP<sub>3</sub>. DAG remains in the membrane, but IP<sub>3</sub> enters the cytosol and binds to the IP<sub>3</sub>-receptor on the ER. This results in the release of Ca<sup>2+</sup> from the ER, and subsequently an increase in the cytosolic calcium concentration. The presence of both DAG and Ca<sup>2+</sup> leads to the activation of calcium-dependent isoforms of PKC. PKC phosphorylates its downstream targets, which finally results in granule secretion, activation of ion-exchangers and regulation of the integrin  $\alpha$ IIb $\beta$ 3 affinity.

Vessel injury also initiates the coagulation cascade, resulting in the conversion of prothrombin into thrombin. Thrombin converts fibrinogen into fibrin, which in turn stabilizes the thrombus. Thrombin is in addition to collagen a second platelet agonist. One of its receptors is PAR-1. This receptor is cleaved by thrombin and the new amino acid terminus acts as a tethered ligand, which auto-activates the PAR-1 receptor. This platelet activation results in activation of PLC $\beta$ .

Platelet activation via GPVI and PAR-1 results in platelet granule release. Platelets contain dense granules,  $\alpha$ -granules and lysosomes. The dense granules contain nucleotides (e.g. ADP). ADP can activate platelets via two receptors, P2Y<sub>1</sub> and P2Y<sub>12</sub>. Stimulation of P2Y<sub>12</sub> with ADP leads to activation of PLC $\beta$  and PKC.

The final result of all platelet stimulations is the release of granule contents and the activation of GPIIb-IIIa. Granule secretion is mediated by the SNARE 1 complex, formed by three membrane associated proteins: the syntaxins, the vesicle-associated membrane proteins (VAMPs) and the SNAP family proteins. SNARE proteins can be classified as vesicle-associated SNAREs (v-SNAREs), which are located on vesicles or granules, and target-associated SNAREs (t-SNAREs), which are located on target membranes. The v-SNAREs and the t-SNAREs form a heteromeric complex, which mediates membrane fusion and thus granule release. SNARE proteins are susceptible to proteolysis by calpain. Calpain cleaves also several other signaling molecules that influence signaling of granule secretion.

Rab GTPases are involved in granule secretion also, they regulate alpha granule release.

GPIIb-IIIa activation involves the binding of talin and kindlins to the integrin tails and the subsequent conformational change from a low to a high affinity state for ligand binding. The binding of talin and kindlins to integrin tails is strictly regulated, for example via the second messenger PIP2 and calpain.

The experiments, performed in chapter 7, show a few things. The main observation was that ADP is very important for GPIIb-IIIa activation. AR-C69931MX and apyrase are platelet inhibitors which act via inhibition of ADP. They can completely or partially inhibit P-selectin expression and GPIIb-IIIa activation, dependant on which platelet stimulation is used.

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