GPIb-V-IX- and GPVI-specific intracellular signaling and their regulation by PKA/PKG-dependent inhibitory pathways in washed human platelets

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1 Introduction

1.1 Platelets- multifaceted physiological and pathological roles

Being the smallest circulating cells in the blood and having no nucleus, platelets were considered for a long time as passive and transient participants in hemostasis and coagulation[1]. They were also described as degenerated leukocyte fragments or fibrin clumps or some kind of microbe until 1882, when the pathologist Giulio Bizzozero discovered blood platelets and their outstanding role in hemostasis[2].

Platelets are derived from megakaryocytes, which are produced in the bone marrow. They have a size between 2 and 5 µm and a discoidal shape. Platelets have a relatively short life span of 5 to 9 days before they are destroyed by phagocytosis by the Kupffer cells in the spleen and liver[3]. Their discoid shape and their small size, in addition to their high count in blood (around 150,000– 400,000 platelets/µl), allow them to be pushed to the vessel wall upon injury and to be constantly present to ensure the integrity of the vessel[4].

However, any abnormal platelet function and/or platelet count known (thrombocytosis: >450,000/µl; thrombocytopenia: <150,000/100,000/µl), respectively, may cause thrombotic complications or bleeding disorders. Therefore, platelets are the central players in maintaining the hemostatic balance in the blood flow and wound healing. They minimize the blood loss by repairing the damaged vessel wall. However, platelet functions under quiescent conditions are tightly regulated[5] by endogenous inhibitors, mainly the nitric oxide (NO) and the prostaglandin I_2 (PGI_2) secreted by intact endothelial cells to prevent enhanced platelet activation.

Platelets are characterized by multifaceted roles in hemostasis and thrombosis, inflammation and infection and in tumor growth[6, 7]. Despite their beneficial role in hemostasis, platelets are involved in many pathological aspects in thrombosis such as the thrombus formation at sites of ruptured atherosclerotic plaques[8], which trigger heart attacks and strokes. Moreover, due to the high number of molecules secreted by platelets upon activation, such as cytokines, growth factors and serotonin, immune cells are recruited to the site of injury, increasing their ability to attack pathogens[9]. Furthermore, platelets can be stimulated by distinct mediators, which are activated on the surface of a tumor cell, such as thrombin and tissue factor that initiate the coagulation cascade on the surface of circulating platelets[10].

1.2 Platelets in thrombus formation

Upon a vascular damage or endothelial disruption, platelets are recruited to the vessel wall and form a platelet plug to close the wound, this stage is defined by the primary hemostasis. Activated platelets secrete the content of their granules, leading to catch and stimulate other platelets through the integrin outside-in activation mechanisms favoring a strong bridging

between platelets, leading to a full aggregation. Consequently, platelets acquire procoagulant characteristics leading to thrombin generation on their surface, initiating the coagulation cascade and fibrin formation, which stabilizes the thrombus and revealing the secondary hemostasis stage.

1.2.1 Primary phase of hemostasis

1.2.1.1 Platelet adhesion

Under physiological conditions, platelets are recruited to the subendothelial collagen matrix of the damaged vessel wall and to the collagen-immobilized plasma protein von Willebrand Factor (vWF), which has as ultra large multimeric protein a molecular weight of 20,000 kDa. Initially, vWF has a closed conformation in the plasma. Upon an injury, when it adhered to the vessel wall or under high shear conditions it opens its conformation and the different repetitive domains are exposed to multiple receptors on platelets.

vWF binds to collagen via its A3 domain and on the other side, it catches circulating platelets through its exposed A1 domain that binds selectively to its main platelet receptor, the glycoprotein GPIbα[11, 12], which is part of the receptor complex GPIb/V/IX. The crosslinking between vWF and GPIbα represents the first step during thrombus formation. Consequently, platelets bind to collagen via its specific receptors the GPVI and the integrin $\alpha_2\beta_1$ ensuring a firm adhesion of platelets to the subendothelium[13].

1.2.1.2 Platelet shape change and secretion

In response to GPIbα- and GPVI-mediated activation, a platelet shape change is induced due to cytoskeleton rearrangements and integrin $\alpha_{IIb}\beta_3$ inside-out signaling, which facilitate the binding of soluble fibrinogen from the blood. Additionally, platelets store molecules and soluble mediators in distinct granules such as α- and δ- granules, which are secreted upon platelet initial activation steps[8].

In the α-granules, large proteins are present such as vWF and thrombospondin-1, which play a role in platelet adhesion, in addition to some coagulation factors, protease inhibitors and the α-granule membrane protein CD62P (P-selectin), which is considered one of the platelet activation markers and it mediates platelet binding to neutrophils and monocytes[14]. Dense bodies (δ- granules) contain adenosine diphosphate (ADP), adenosine triphosphate (ATP), Ca^{2+}, serotonin; they alone act as weak platelet agonists, but are strong amplifiers of initiated platelet activation by other agonists (platelet feedback agonists)[15]. Ca^{2+} ions are also stored in the dense tubular system (DTS) and are released into the cytoplasm, which act as important second messenger for platelet activation[8]. Additionally, platelets contain few lysosomes containing glycohydrolases able to degrade glycoproteins, glycolipids and glycosaminoglycans[16, 17].

Proteomic qualitative and quantitative analysis of platelet releasates/granule protein content contributes significantly to get novel insights into hereditary platelet defects[18, 19].

In addition, platelets synthesize and release thromboxane A_2 (TxA_2) upon activation, a secondary mediator playing an important role as a feedback platelet agonist (more details in paragraph 1.5.2).

1.2.1.3 Platelet aggregation

Second messengers released by activated platelets play an essential role in emphasizing the primary phase of platelet aggregation and in recruiting more platelets to the hemostatic plug. Platelet aggregates are stabilized by fibrin bridges formed via the activated integrin $\alpha_{IIb}\beta_{3.}$

1.2.2 Secondary phase of hemostasis

1.2.2.1 Thrombin generation and fibrin formation

Upon strong activation platelets undergo many biochemical changes, such as cytoskeleton redistribution and rearrangement of the membrane phospholipids by flip-flop moves of the anionic phospholipids. For example, phosphatidylserines (PS), located in the inner leaflet of the membrane, flips towards the outer leaflet yielding in a negatively charged/procoagulant platelet surface for the binding of distinct coagulation (co)factors, the prerequisite for thrombin generation[12].

According to the cell-based model of coagulation platelets act as essential amplifiers of thrombin generation when activated by small amounts of thrombin, generated by tissue factor bearing cells[20]. Activated platelets enable the enhanced formation of the activated tenase-complex on the platelet surface leading to a burst of thrombin, which converts fibrinogen to fibrin. Fibrin is essential for stabilization of the platelet plug[8].

1.2.2.2 Fibrinolysis and clot retraction

A formed platelet-fibrin clot is resolved by a mechanism called fibrinolysis. This step is important to restore blood flow by lysing the fibrin network by plasmin, which is a protease that degrades the fibrin clot into soluble fibrin degradation products[21]. By cutting the fibrin, new binding sites are exposed, which enhance plasmin activity and therefore increasing the lysis rate[22].

Under physiological conditions, the coagulation cascade is tightly controlled by endogenous regulators and inhibitors interacting with platelets in order to keep hemostasis.

1.3 Platelet inhibition and negative regulation

Platelet activation is tightly regulated to maintain the balance of the blood flow. Physiological platelets inhibitors attenuate and inhibit platelet activation processes by controlling protein phosphorylation and dephosphorylation through protein kinases and phosphatases,

respectively[23]. Major platelet inhibitors are the endothelium-derived soluble mediators such as nitric oxide (NO) and prostacyclin (PGI_2), both inhibit the cytoskeleton rearrangement, integrin activation, granule secretion, TxA_2 synthesis and thereby platelet aggregation and the surface exposure of anionic phospholipids[24, 25].

Clinically used anti-platelet drugs are targeting important activation mechanisms of platelets, including major receptors (e.g. integrin $\alpha_{IIb}\beta_3$; $P2Y_{12}$, PAR-1) and TxA_2 synthesis[26]. However targeting distinct inhibitory signaling pathways is promising for selective targeting, which prevents overshooting platelet activity without provoking severe bleeding risks. Therefore, understanding the crosstalk mechanisms between platelet activation and inhibition may provide new therapeutic approaches in the field of thrombosis, inflammatory and immune diseases. One of the main goals of this study is to investigate the effects of the most potent platelet endogenous inhibitors, nitric oxide and prostacyclin on distinct platelet activation processes.

1.3.1 Nitric oxide

Nitric oxide (NO) is a free radical synthesized by different nitric oxide synthases (endothelial NO synthases, neuronal NO synthases and macrophagal NO synthases located in macrophages, neutrophils)[27]. NO diffuses to the vasculature, thereby inducing smooth muscle relaxation and vasodilation, and it diffused in the blood stream through the platelet membrane, activating directly the soluble guanylyl cyclase (sGC), which induces the production of the second messanger cyclic guanosine 3', 5'-monophosphate (cGMP)[28]. Increased levels of cGMP induced by endothelium-derived NO or by riociguat (Adempas®) (sGC stimulator, NO donor, indication for the treatment of chronic thromboembolic pulmonary hypertension), activate the protein kinase G (PKG) triggering the activation of its downstream effectors and the inhibition of major platelet activation aspects such as integrin activation, granule secretion, cytoskeletal rearrangements and the overall platelet aggregation as well as the surface exposure of anionic phospholipids such PS[29, 30] (Figure 1).

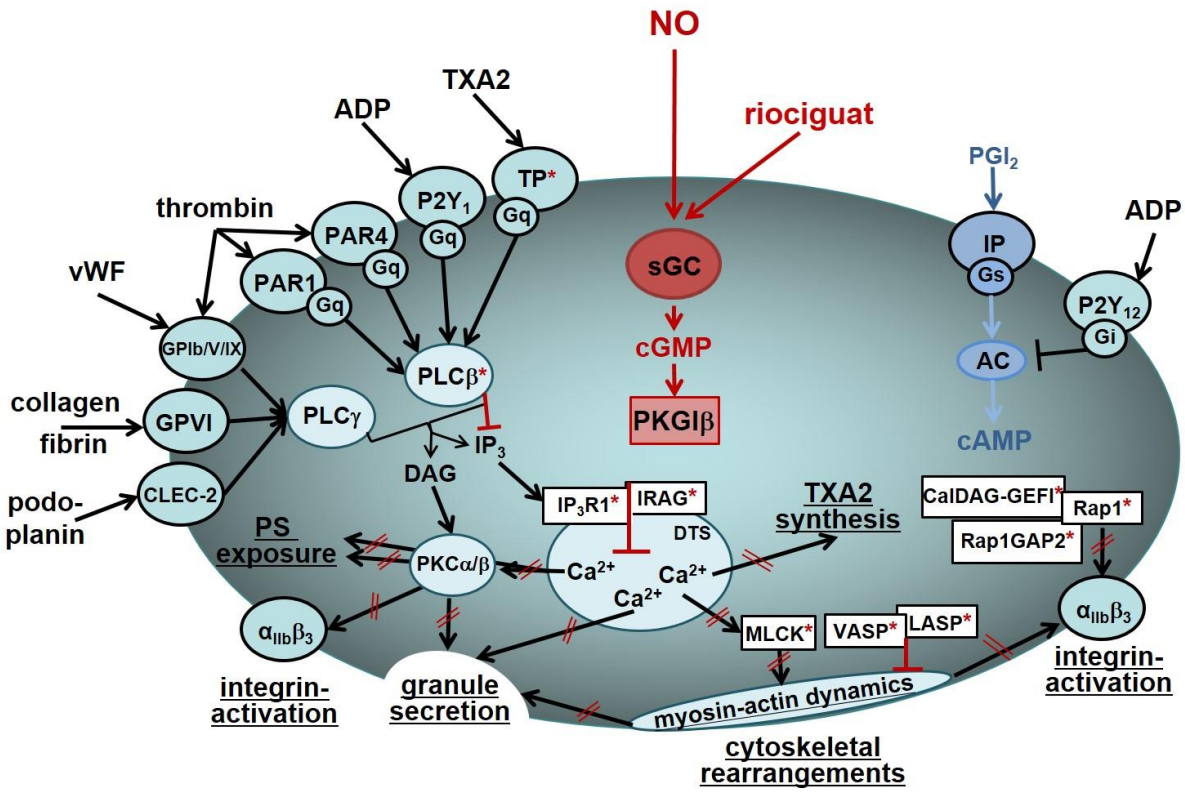

Figure 1. Regulation of protein phosphorylation and function of human platelets by the NO/sGC/cGMP and PGI_2/AC/cAMP pathways.
Soluble platelet agonists (thrombin, ADP, TXA2) and/or adhesion molecules (vWF, collagen, fibrin, podoplanin), via specific membrane receptors and subsequent signaling pathways, activate various platelet responses, e.g. myosin-actin dynamics with subsequent cytoskeletal rearrangements, integrin activation (e.g. integrin $\alpha_{IIb}\beta_3$), granule secretion (i.e. α-granules, δ-granules/dense bodies, lysosomes) and PS exposure. All these platelet activation responses are crucial for platelet adhesion, aggregation and thrombin generation. NO and sGC stimulators such as riociguat, via sGC/cGMP/PKGIß, inhibit many of these platelet activation responses at several sites of the activation pathways as shown by red blocked arrows. Similarly, PGI_2 increases the level of cAMP leading to the inhibition of platelet activation aspects. This includes inhibition of Ca^{2+} release from the intracellular Ca^{2+}-store DTS and inhibition of the activating PKCα and PKCβ. Platelet proteins shown to be phosphorylated by PKGIβ, as shown in previous studies and ongoing phosphoproteomic analysis, are marked by a red asterisk (*). Phosphorylation of these proteins, e.g. PLCβ, IP3R1, IRAG, MLCK, VASP, LASP, predominantly blocks distinct platelet activation steps (red blockades). In addition to cGMP, inhibitory cAMP is upregulated by the PGI_2/IP/AC pathway and downregulated by the ADP-mediated pathway. Scheme adapted and modified from Makhoul S. et al (2018)[30].

Abbreviations: PLC, phospholipase C; IP_3R1, inositol 1,4,5-trisphosphate (IP_3) receptor 1; IRAG (MRVI), IP_3 receptor-associated cGK I substrate protein; small GTPase system: (CalDAG-GEFI/RASGRP2; Rap1; Rap1GAP2); actin system: (MLCK, VASP, LASP); vWF, von Willebrand factor; TXA2, thromboxane A2; TP, thromboxane receptor; PGI2, prostacyclin; IP, prostacyclin receptor; PS, phosphatidylserine; PKC, protein kinase C; DTS, dense tubular system

1.3.2 Prostacyclin

Another potent physiological platelet inhibitor is the endothelial cell-derived prostacyclin (PGI_2). It binds to prostacyclin receptors (IP) present on the platelet surface as well to smooth muscle cells leading to platelet inhibition and vasodilation, respectively. Upon PGI_2 binding to IP receptors, the production of cyclic adenylyl monophosphate (cAMP) increases leading to activation of protein kinase A[31] (Figure 1). As anti-platelet drug, iloprost (Ilomedine ®) (a stable PGI_2 analog) is used for the treatment of pulmonary arterial hypertension[32] and for research

purposes. PKA activation inhibits procoagulant aspects of platelet by inhibiting PS exposure and thrombin generation induced by tissue factor during secondary hemostasis[33].PGI_2 works synergistically with NO to inhibit platelet aggregation and procoagulant activity and thereby balancing platelet reactivity[34, 35].

1.4 Platelet pattern-recognition receptors and their role in Syk-mediated signaling and platelet activation

Platelet receptors are at the forefront of research and interest of many scientists in the platelet field, since platelets have to response very fast to alterations in the blood and vascular compartment. Significant qualitative or quantitative defects of any platelet receptor can cause severe thrombotic or bleeding disorders due to the impairment of platelet responses initiated by these different receptors, the type of disorder occurs depending on the function of the altered receptor.

Platelet receptors important for adhesive functions, interaction with other blood and vascular cells and immune responses are so called pattern-recognition receptors, which signal via clustering in the platelet surface membrane and belong to different receptor classes[36-39]. Major platelet pattern-recognition receptors are described in detail in the following paragraphs and presented below (Figure 2).

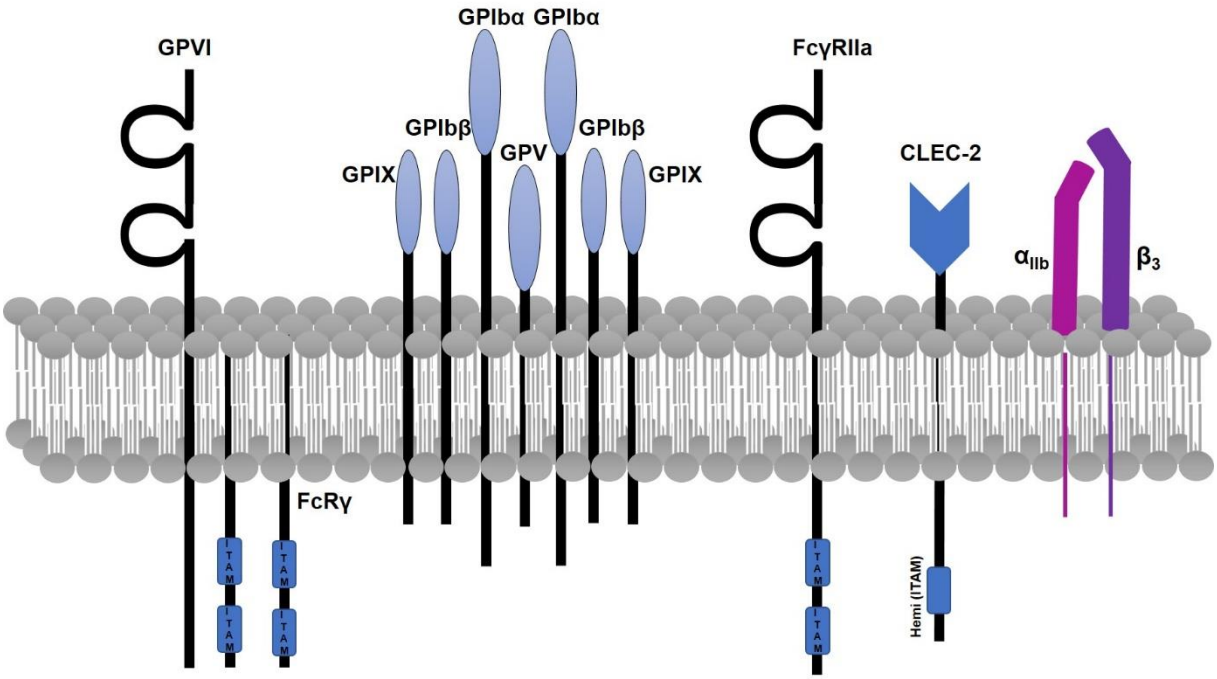

Figure 2. Major pattern-recognition platelet receptors.
From the left to right, the collagen receptor, GPVI, containing an extracellular domain, two IgG like domains. The ITAM-containing domain, FcRγ, the GPIb-IV-V complex, which is the main receptor of von Willebrand Factor (vWF), FcγRIIa, another ITAM-containing receptor, binds mainly immune complexes. The third hemi-ITAM containing receptor, CLEC-2, which binds mainly podoplanin and the integrin ($\alpha_{IIb}\beta_3$), which is the fibrinogen receptor. Scheme adapted from Senis Y. et al (2014)[40].

1.4.1 GPIb-V-IX receptor complex

The glycoprotein Ib-V-IX complex (GPIb-V-IX) is exclusively expressed by platelets and megakaryocytes. This complex consists of four different transmembrane proteins: 2 chains of GPIbα (135 kDa), 2 GPbIβ (26 kDa), 2 GPIX (20 kDa) and 1 GPV (82 kDa). It represents the second most abundant protein on the platelet surface (about 25,000 copies/ human platelet). GPIbα represents the main receptor for the adhesive ligand, the von Willebrand Factor (vWF). However, also other ligands exist, which show a high binding affinity to GPIbα such as thrombin, thrombospondin-1[41, 42] as well P-selectin, factor XII, factor XI[43, 44] , additionally to some snake toxins summarized in Table 1.

In the first step of platelet adhesion, the big multimeric plasma protein vWF immobilized on subendothelial matrix proteins or on activated endothelial cells binds with its repetitive domains to GPIbα, this binding induces the clustering of two GPIbα chains. This cross-linking can involve GPIbα chains from the same platelet and as well from different platelets representing the initial step upon ligand binding. Afterwards, distinct members of src family kinases (SFKs), which are associated or located near the tyrosine residues of the receptor cytoplasmic tails and are responsible to phosphorylate the tyrosine residues[45, 46], mediate the phosphorylation of downstream substrates leading to a full platelet activation mediated by the activation of the integrin $\alpha_{IIb}\beta_3$ responsible for a firm platelet adhesion, thereby trapping several platelets to the site of injury.

Qualitative or quantitative defects of the GPIbα-IX complex lead to the Bernard-Soulier Syndrome (BSS) having a prevalence of 1 person per million[47]. BSS is a rare autosomal, most cases recessive hereditary disorder, and rarely dominant[48]. Patients with BSS present a prolonged bleeding time, thrombocytopenia and larger platelets compared to healthy individuals[49].

1.4.2 GPVI receptor

The glycoprotein VI (GPVI) is expressed only in megakaryocytes and platelets, with a copy number of about 3,700 copies/ human platelet[50, 51]. GPVI is a 63 kDa transmembrane protein, which consists of two immunoglobulin-like domains in the extracellular region connected with a glycosylated linker, a transmembrane domain and a cytoplasmic tail. In addition, GPVI is associated with an adaptor transmembrane protein, the FcRγ chain containing one copy of an ITAM motif, facilitating the propagation of the signal[13].

GPVI is known as the major collagen signaling receptor, however collagen can also bind to the integrin $\alpha_2\beta_1$ on the platelet surface facilitating platelet adhesion to collagen[13, 52]. The binding of collagen fibers to GPVI induces engagement and dimerization of GPVI leading to activation of distinct SFKs and consequently the phosphorylation of the FcRγ ITAM chains[53]. This

mechanism represents the initial phase of the signal propagation downstream of GPVI that ends with a rapid platelet activation dependent on the spleen tyrosine kinase (Syk) followed by integrin activation and granule secretion.

Nevertheless, patients with altered GPVI receptor are very rare and they present only with a mild bleeding disorder. This impairment can be caused for example by an acquired deficiency mainly due to the presence of auto-antibodies against GPVI or by a genetically transmitted disorder where GPVI is not expressed[54].

1.4.3 Immunoreceptor tyrosine-based activation motif (ITAM) containing receptors

Many of the pattern-recognition platelet receptors belong to the ITAM- containing receptor family. An ITAM consists of a conserved sequence of 4 amino acids which is repeated twice within the cytoplasmic tail. ITAM sequence is formed by a tyrosine separated by two random aminoacids from a leucine (YxxL). Two ITAM motifs are then separated by 6 to 8 amino acids[55]. However, some receptors such as the C-type lectin-2 receptor (CLEC-2) contains only one sequence, therefore it is called a hemi-ITAM containing receptor.

ITAMs play an essential role in signaling in platelets and other immune cells. The binding of an agonist induces ITAM phosphorylation on its tyrosine residues by the SFKs forming a high affinity docking site for activation of further tyrosine kinases mainly the spleen tyrosine kinase Syk[40].

1.4.3.1 GPVI/FcRγ-chain

Fc receptor γ-chain (FcRγ) is an ITAM-containing adaptor protein. It is a transmembrane homodimer containing 2 chains covalently linked. In the intracellular tail of each chain one ITAM is found with dual YXXL aminoacid consenus sequences. FcRγ is a main receptor for the immunoglobulin G (IgGs) and it plays an important role in platelet activation and immune cells[56]. The presence of the FcRγ on the platelet surface is essential for the GPVI receptor[57]. The transmembrane domain of GPVI forms a salt bridge with D11 domain of the FcRγ chain, this interaction allow the expression of the complex at the platelet surface[58]. One dimer of FcRγ chain is associated with one GPVI receptor[13]. Moreover, FcRγ plays an important role in the GPIbα-mediated downstream tyrosine phosphorylation and platelet aggregation[59].

1.4.3.2 FcγRIIA

FcγRIIA is a transmembrane protein with a molecular weight of 40 kDa. It is a monomer that consists of 2 extracellular IgG-like domains, a single transmembrane domain and a cytoplasmic tail containing an ITAM peptide with dual YxxL amino acid consensus sequences. This receptor is expressed in classical immune cells such as monocytes and macrophages[60]. However, it is the solely expressed immune IgG-receptor of human platelets with a copy

number of 1,000 to 4,000 copies/platelet. Interestingly, murine platelets do not express FcγRIIA[61, 62].

The FcγRIIA binds IgGs of immune complexes but it has a low binding affinity to the monomeric IgG. However, FcγRIIA acts not only as IgG receptor but also plays an important role in platelet function via enhancing platelet integrin $\alpha_{IIb}\beta_3$-mediated spreading on fibrinogen in an IgG-independent manner[63, 64]. Furthermore, it ensures interactions between platelets and immune complexes and amplifies platelet responses to some bacteria[65].

The fact that FcγRIIA contains two extracellular IgG-like domains suggests that these are involved in the dimerization of this receptor upon ligand binding. Subsequently, ITAMs are phosphorylated by SFKs leading to signal transduction via Syk activation[66].

So far, no ITAM chains were reported to be directly associated to GPIbα, however, studies showed that the FcγRIIA was immunoprecipitated with GPIbα from human platelet lysates, suggesting the co-localization of those two receptors on the platelet membrane[66].

1.4.3.3 C-type lectin-2 (CLEC-2)

CLEC-2 is a membrane protein highly expressed in megakaryocytes and platelets with a copy number of 2,000- 4,000 copies/ human platelet[67]. The only endogenous ligand for CLEC-2 is podoplanin, which is expressed on the surface of kidney podocytes and lymphatic endothelial cells but not on platelets. However, the potent and specific activation of CLEC-2 by a snake toxin 'rhodocytin' helped to reveal the specific downstream signaling pathway mediated by CLEC-2[68].

In contrast to FcRγ and FcγRIIA, the CLEC-2 receptor contains only a single tyrosine residue in its hemi-ITAM. Agonist binding to CLEC-2 induces hemi-ITAM phosphorylation leading to a low binding affinity to one SH2 site of Syk. Despite this low binding affinity, the binding of the ligand to several CLEC-2 receptors induces a higher affinity of Syk to other CLEC-2 hemi-ITAM motifs, allowing the phosphorylation of Syk downstream signaling and full platelet activation[40].

Even if the endogenous agonist of CLEC-2 is absent in platelets and vascular endothelial cells, CLEC-2 has an important role in blood-lymphatic vessel separation[69]. Additionally, its ligand podoplanin is upregulated in cancer cells and upon an inflammation on macrophages, Th17 T cells and fibroblasts enhancing the role of platelets in cancer and inflammation[70].

1.4.4 Integrin $\alpha_{IIb}\beta_3$

GPIIb/IIIa or integrin $\alpha_{IIb}\beta_3$ is a surface-expressed integrin only of platelets and megakaryocytes. It represents the major platelet receptor due to its high copy number of 80,000 copies on the surface of unstimulated human platelet[71]. It is a heterodimer, contains

two distinct integrin subunits, the α_{IIb} (148 kDa) and β_3 (95 kDa) subunits assembled in the plasmic reticulum. The integrin $\alpha_{IIb}\beta_3$ is known to bind mainly fibrinogen but also vWF, fibronectin and thrombospondin[56]. The SFKs, Src and Fyn are associated to the β_3 subunit of the integrin, providing an essential role for a rapid signal transmission[40].

Upon an injury, platelets are activated by thrombin and ADP which cause the conversion of the integrin from a non-active into an active form. This conversion and extension is due to the binding of talin-1 and kindling-3 on different sites of the β_3 cytoplasmic domain, which attenuates the transmembrane and cytoplasmic domain interactions[72]. An active and extended integrin is then able to bind fibrinogen and vWF inducing platelet aggregation by bridging between adjacent platelets through these ligands[73].

The ligand-induced integrin activation known as 'outside-in' signaling is initiated by the clustering of the two subunits. However this binding can be reversible before it is progressively irreversibly activated by promoting granule secretion and secondary aggregation[74]. Following the integrin clustering, a trans-autophosphorylation of the tyrosine kinase Src which binds to the β_3 cytoplasmic domain of an activated integrin induces a signaling cascade leading to platelet spreading on fibrinogen-coated platelets and retraction of fibrin clots[74, 75].

Patients with a qualitative or quantitative defect of the integrin $\alpha_{IIb}\beta_3$ suffer from severe bleeding, such as patients with Glanzmann thrombasthenia (GT)[76]. GT is a rare autosomal predominantly recessive bleeding disorder (some cases with dominant variant were reported[77]) with frequency of 1:1,000,000. These patients lack platelet aggregation and suffer from excessive bleedings. Other manifestations of GT include a defective platelet spreading on collagen while clot retraction and integrin $\alpha_{IIb}\beta_3$-dependent fibrinogen storage in α-granules are variably defective depending on the type of the mutation[78].

1.5 Platelet activation by soluble feedback agonists via G-protein coupled receptors

A platelet monolayer is formed during the initial steps of thrombus formation, however this primary platelet aggregate is not firmly stable. Upon activation and aggregation, platelet secretes soluble mediators and molecules activating the same platelet and recruiting additional circulating platelets.

Some of the main soluble secondary mediators are the adenosine diphosphate (ADP), the adenosine triphosphate (ATP) and serotonin, which are secreted from the platelet dense granules, thromboxane A_2 (TxA_2) synthetized by the thromboxane synthase and cyclooxygenase 1 (COX-1) and thrombin generated on the platelet surface, which initiates the coagulation cascades[8]. These feedback agonists activate platelets by binding to the respective G-protein coupled receptors (GPCRs) ensuring a sustained platelet activation[79].

GPCRs are membrane proteins that consist of 7 transmembrane domains. They pass through the membrane 7 times and are coupled to heterotrimeric guanine nucleotide-binding proteins ($G_{\alpha\beta\gamma}$ proteins). These transmembrane proteins are able to propagate the signal from outside the cell and initiate several signaling pathways through the activation of the G-protein. This activation is caused by the conversion of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) on the G protein, allowing the interaction with specific downstream effectors such as the phospholipases Cβ (interacting with $G\alpha_q$), the guanine nucleotide exchange factors (GEF) (interacting with $G\alpha_{12/13}$) or the phosphoinositide 3-kinases (interacting with Gβγ)[80], which induce an intracellular increase of Ca^{2+}, granule secretion and platelet aggregation[12, 79].

1.5.1 Adenosine diphosphate (ADP)

ADP plays an important role in maintaining a normal hemostasis and this role is confirmed by the bleeding disorders in patients having a δ-storage pool disease. ADP stimulates platelets via two different GPCRs; $P2Y_1$ (coupled to G_q) and $P2Y_{12}$ (coupled to G_{i2}). The activation of the $P2Y_1$ initiates several platelet activation responses such as shape change and a weak platelet aggregation and $P2Y_{12}$ amplify ADP-induced platelet activation such as Ca^{2+} mobilization and the second wave of aggregation[81, 82]. Furthermore, ADP is an essential positive-feedback activator required for a sustained platelet activation, since platelet responses to low and intermediate concentrations of agonists such as collagen, thrombin and TxA_2 are reduced in the absence of ADP receptors[83]. The important role of ADP and ADP receptors (especially $P2Y_{12}$) in platelet physiology and pathophysiology is demonstrated by the recent medical progress that the irreversible binding and inactivation of the ADP receptor ($P2Y_{12}$) by thienopyridines such as clopidogrel, prasugrel, and others successful prevents secondary cardiovascular events[84].

1.5.2 Thromboxane A_2 (TxA_2)

Similar to ADP, TxA_2 is a positive-feedback agonist, produced by the conversion of the arachidonic acid by the cyclooxygenase-1 (COX-1). After release, TxA_2 activates platelets by binding to the GPCR, the prostanoid TP receptor (coupled to G_q and $G_{12/13}$)[85, 86] triggering platelet shape change and amplifying platelet aggregation by activating the inside-out signaling of integrin and granule secretion. The lack of the TP receptor in mice showed prolonged bleeding times occurred with unstable thrombus formation. Furthermore, deficiency in TP showed a protective role from atherosclerosis in mice where they showed a reduced progression of the disease[87]. A well-known potent anti-platelet drug, the acetylsalicylic acid (aspirin) is used to prevent the synthesis of TxA_2 by inhibiting irreversibly COX-1 by a selective acetylation on a serine residue (S530) which blocks COX-1 binding to arachidonic acid[88].

1.5.3 Thrombin

Thrombin is considered to be the important protease in the coagulation cascade and it is known as a strong platelet agonist. Prothrombin (precursor of thrombin) located in the plasma is cleaved into thrombin in presence of the tissue factor and the factor Xa. Briefly, platelets express many receptors that bind some coagulation factors (FIXa, FXa, FXI[89, 90]) and important for the assembly of the coagulation complexes, tenases and prothrombinases triggering the thrombin burst followed by fibrin polymerization[20]. The generated thrombin can bind to its respective platelet GPCRs coupled to G_q and $G_{12/13}$: the protease-activated receptors, PAR-1, PAR-4 (in human platelets) and PAR-3, PAR-4 (in mouse platelets). PAR-4 deficient mice showed a complete resistance to thrombin activation while PAR-3-deficient mice had a weak response to thrombin[91-93] highlighting the essential role of PAR-4 in mice platelets. In human platelets, PAR-1 (or PAR-3 in mice platelets) serves as a cofactor for PAR-4 activation triggering an enhanced PAR-4 cleavage[94].

Anticoagulants targeting thrombin generation or blocking the PARs are widely used in the clinic for the treatment of various cardiovascular disorders. For example; the direct thrombin inhibitor, dabigatran a non-vitamin K oral anticoagulant is used for prevention of stroke and systemic embolism in patients with non-valvular atrial fibrillation[95].

1.6 Role of of Src family kinases (SFKs) and spleen tyrosine kinase (Syk) in the initiation of platelet activation

1.6.1 Role of SFKs downstream different platelet receptors

The Src family kinases (abbreviation for ‘sarcoma’), SFKs, are expressed in high levels in platelets where 7 SFKs have been reported in human platelets (Src, Yes, Lyn, Hck, Fyn, Fgr and Lck). SFKs are involved in wide range of cellular processes such as proliferation, differentiation, survival and adhesion[40]. In platelets, SFKs play a major role in many platelet activation aspects. Knocking out the most highly expressed SFKs (Src, Lyn and Fyn) resulted in major platelet dysfunctions; Src-deficient mice represent a reduced integrin-mediated signaling and aggregation[96, 97], Fyn-deficient mice show a reduced GPVI-mediated aggregation and secretion[98] and Lyn-deficient mice show a reduced GPIb-IX-V-mediated spreading and GPVI delayed aggregation[98, 99]. Furthermore, different SFKs inhibitors were developed such as PP2 and contributed in elucidating the role of SFKs in human platelets.

SFKs are associated or located near the tyrosine residues of the receptor cytoplasmic tails[45, 46] and they are responsible to phosphorylate the tyrosine residues of the tyrosine kinase-linked receptors such as, GPIbα-V-IX complex, integrins α2β1 $\alpha_{IIb}\beta_3$, ITAM-containing GPVI/FcRγ, FcγRIIA and CLEC-2 receptors[40]. Most of the SFKs, except Src, are located in the lipid rafts within the plasma membrane which serve as organizing centers of signaling proteins and

receptors[40]. All SFKs contain two highly conserved tyrosine residues one in their C-terminal tail and the other in their activation loop. The regulation of these sites regulates SFKs activity; the phosphorylation of the C-terminal tyrosine site by Src kinase (Csk) or dephosphorylation of the activation loop by the protein tyrosine kinases (PTPs) inhibits SFKs activity, however, trans-autophosphorylation of the activation loop tyrosine residue or dephosphorylation of inhibitory C-terminal tyrosine residue triggers an increased SFKs activity[100-102].

The binding of the specific ligand to the tyrosine kinase-linked receptors induces receptor clustering and changes within the lipid rafts, these interaction induce the activation of the proximate SKFs by trans-phosphorylation of the tyrosine residue. The activated SFKs allow the interaction and binding of downstream tyrosine kinases and several adaptor proteins resulting in full platelet activation. Moreover, SFKs provide a supplemental role upon activation of the G_q-coupled receptors ($P2Y_1$, TP, PAR-1 and PAR-1, 4), which induce the activation of the phospholipase Cβ, leading to intracellular Ca^{2+} release. However, this involvement is not direct; the inhibition of the SFKs attenuates secondary aspects of platelets activation such as TxA_2 production and ATP release[103]. In contrast, SFKs play a central role in G_i-coupled receptors mediated signaling ($P2Y_{12}$, α_{2A}). Only $P2Y_{12}$ antagonism (and not $P2Y_1$) inhibited SFK phosphorylation induced by ADP[104].

The activation of the SFKs triggers the phosphorylation of several tyrosine kinases present in platelets such as the spleen tyrosine kinase (Syk), discussed in the next paragraph.

1.6.2 Spleen tyrosine kinase (Syk)

1.6.2.1 Biological and pathological role of Syk

The spleen tyrosine kinase, Syk, a 72 kDa kinase, was first detected in the thymus and the spleen[105]. Syk is highly expressed in hematopoietic cells such as B and T cells, mast cells, macrophages, neutrophils and platelets and is well expressed also in other cell types such as epithelial cells, hepatocytes and neuronal cells. Therefore, Syk represents a major player not only in immunological diseases but also in other biological pathologies, like cancer, vascular disorders, allergies and bone resorption disorders[106]. Moreover, mice embryos having a homozygous targeted mutation in Syk gene (by deletion of one exon on Syk gene encoding for 41 residues in Syk kinase domain in embryonic stem cells) die from severe hemorrhages before birth and a complete Syk deletion in lymphocytes induced an impairment in the differentiation of B-lineage cells and an incomplete maturation of pre-B cells[107]. Furthermore, mice platelets lacking Syk were protected from arterial thrombosis and ischemic stroke[108], highlighting the important role of Syk in platelets. As mentioned above, Syk is involved in the transduction of the signals downstream the major platelet pattern-recognition receptors.

1.6.2.2 Basic Syk structure

Syk contains two N-terminal Src homology 2 (SH2) domains separated by a linker interdomain A and a C-terminal kinase domain. N- and C- terminal domains are separated by the linker interdomain B (Figure 3). The inter-SH2 domain serves as a docking site for the immune receptor tyrosine-based activating motifs (ITAMs) found in the cytosolic part of the cell. The binding of Syk to those phosphorylated ITAMs induces Syk full activation and a fast signal transduction downstream of Syk[109].

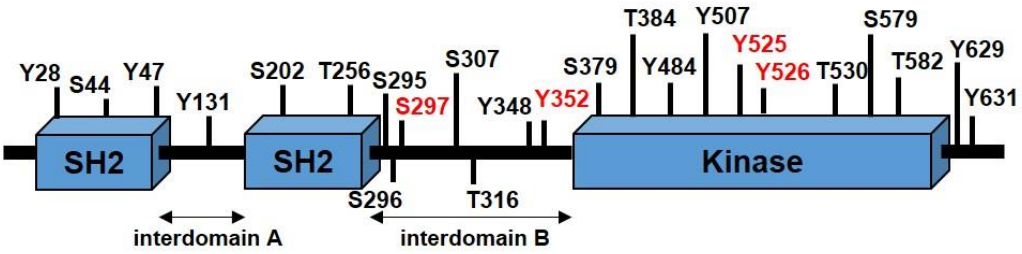

Figure 3. Structure of human Syk including major phospho-sites.
The Spleen Tyrosine Kinase (Syk) is formed by 629 amino acids. It contains 2 tandem Src homology domains (SH2) on the carboxy-terminal domain and a kinase domain or so called the activation loop at the C-terminal domain. SH2 domains are separated by the linker region (interdomain A) and separated from the kinase domain by the interdomain B. Serine 297 and tyrosine 352 are located in the interdomain B and tyrosines 525 and 526 located in the activation loop of Syk. Scheme adapted from Bohnenberger H. et al (2011)[110].

As shown in figure 3, Syk is a very complex protein in terms of regulation since it has 32 phosphorylation sites[110] (not all are shown in the scheme). The complex Syk regulation mechanisms are presented in the discussion part (paragraph 6.3).

1.6.2.3 Syk activation and regulation by multi-site phosphorylation

The regulation of Syk phosphorylation upon activation was identified in B cells using a high-resolution mass spectrometry study[110], 32 sites of Syk were identified: 15 tyrosine, 11 serine and 6 threonine (some are represented in Figure 3). The development of an antibody recognizing only the phosphorylation of the tyrosines in the activation loop (sequence 512-526) showed the tight correlation between Syk activity and the phosphorylation of the tyrosines in this sequence[111]. Therefore, the phosphorylation of Syk on tyrosine 525 and tyrosine 526 is considered as a marker of Syk kinase activity. Furthermore, tyrosine residues located in the SH2 kinase linker domain (interdomain B) showed an important role in the regulation of Syk activation mechanisms. For example, the phosphorylation of Syk on Y348 and 352 was the highest among the Syk phospho-acceptor sites[110]. Moreover, the substitution of Y352 with a phenylalanine in murine B cells inhibited Syk binding to the main Syk Y352 direct substrate, PLCγ1[112].

In addition, one of the serine sites of Syk was frequently detected, S297, located in the interdomain B. This site showed a 5-fold increased phosphorylation upon BCR stimulation in

human B cells[110]. Additionally, the phosphorylation of S297 decreased the binding of Syk to the membrane upon activation, therefore this phosphorylation may limit the activity of Syk and its capacity to bind to its downstream effectors[113]. These data show the potential role of S297 phosphorylation in Syk regulation, which is also a topic of this thesis and will be summarized in the discussion part.

1.6.2.4 Syk substrates and downstream signaling

Upon binding of an agonist to its (hemi)-ITAM receptor, ITAMs are phosphorylated mostly by the SFKs. This phosphorylation recruits Syk SH2 domains which lead to Syk activation and its recruitment to the cytoplasmic membrane. This activation triggers first the phosphorylation of several proteins, some are direct Syk substrates such as the phospholipase Cγ, the proto- oncogene vav1 and 2, the p85 α-subunit of the phosphoinositide 3-kinase (PI3K), the linker adaptor for T cells (LAT) and the SH2 domain-containing leukocyte protein 76 and 65 (SLP76 and 65)[68] and others can be indirectly activated such as the mitogen-activated protein kinase p38, the protein kinase B (or Akt)[114].Second, the hydrolysis of phospholipases into the diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) leads to Ca^{2+} release and PKC activation leading to granule secretion, shape change, spreading and platelet aggregation.

Among all Syk substrates and effectors, the focus in this study was mainly on LAT, PLCγ2 and Akt.

- The linker adaptor for T cells (LAT): is a 36 kDa tyrosine adaptor protein, located in lipid rafts, helps to bind proteins to form an activator complex (signalosomes) leading to a fast signal transduction downstream the receptor.
- The phospholipase Cγ2 (PLCγ2): is a 150 kDa 1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-2 tyrosine kinase, initially located in the cytosol. It is considered as crucial enzyme in the transmembrane signaling since it catalyzes the production of the production of the second messenger molecules diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) leading to granule and intracellular Ca^{2+} release, respectively. LAT activation recruits PLCγ2 to form a complex at the inner side of the membrane.
- The protein kinase B (Akt): is a 62 kDa serine/threonine protein kinase located in the cytoplasm. Its translocation to the membrane via its binding to the phosphatidylinositol 3-kinase (PI3K), which leads to Akt phosphorylation on threonine 308 and serine 473 and to phosphatidylinositol-3,4-bisphosphate (PIP2) and PIP3 formation. Akt is activated not only downstream of the tyrosine kinase-linked receptors but also downstream Gq-coupled receptors such as PARs and $P2Y_{12}$, which represents the direct way for Akt phosphorylation, since the blockage of any of these receptors greatly reduce this phosphorylation regardless the agonist used[115-118]..

1.7 Snake toxins as tools for selective stimulation of platelet GPIbα and GPVI

1.7.1 Overview

Snakes use venoms to defend against threats and to kill their prey. The venom is a highly modified saliva, secreted by the glandes situated on both sides of the snake's head below and under the eyes. It contains a mixture of toxic proteins that induce deadly damages in the biological system. Some toxins can affect the nervous system; they are called 'neurotoxins', which block neurotransmitters leading to paralyze the prey, such as dendrotoxins secreted by the snake mambas (*Dendroaspis polylepis*). And some other toxins, can act as cytotoxins such as hemotoxins that affect hemostasis[119].

Hemotoxins, mainly the C-type lectin- like proteins[120], affect hemostasis by acting on blood coagulation factors, vessel wall components or platelets[121] (Table 1). Global effect on hemostasis should be explained via activation or inhibition of hemostasis/coagulation.

Table 1: Overview of some snake toxins affecting hemostasis at different levels.

	Target	**Toxin**	**Toxin effect**	**Global effect on hemostasis**
Coagulation factors	FV	RVV-V (*Vipera russelli*)	FV activation	coagulation[122]
	thrombin	bothrojaracin (*Bothrops jararaca*)	thrombin inhibitor	bleeding[123]
Vessel wall	blood vessel extracellular matrix (ECM)	metalloproteinases (Crotalidae and Viperidae)	ECM degradation	bleeding[124]
	endothelial cell	aggregin (*Calloselasma rhodostoma*)	EC proliferation angiogenesis	coagulation[125]
Platelets	GPIbα	echicetin (*Echis carinatus sochureki*)	inhibiting vWF binding to GPIbα	bleeding[126]
		botrocetin (*Bothrops jararaca*)	increasing vWF binding (affinity) to GPIbα	coagulation[76]
		jararhagin (*Bothrops jararaca*)		agglutination in whole blood[127]
	GPVI	convulxin (*Crotalus durissus terrificus*)	GPVI activation and its mediated secretion	coagulation[128]
		jararhagin (*Bothrops jararaca*)	Blocking collagen binding	bleeding[127]
		jararcetin (*Bothrops jararaca*)	inhibiting GPVI-mediated	

	Target	Toxin	Toxin effect	Global effect on hemostasis
			platelet secretion	
	platelet cytoskeleton	phospholipase A2 (Pseudechis papuanus)	changing in platelet morphology	bleeding[129]

Echicetin and convulxin were used as specific platelet agonists. They belong to the C-type-like proteins or snaclecs (Snake venom C-type lectins)[130]. C refers for the requirement of Ca^{2+} ions for binding and lectins for sugar, typically galactose. Compared to the typical C-type lectin, this special group (snaclecs) lacks the Ca^{2+} binding site despite the high homology of the amino acid sequences.

1.7.2 Echicetin as selective GPIbα ligand

Initially reported in 1993 by Peng M. et al, echicetin was purified from the saw-scaled viper, *Echis carinatus*. Echicetin is a heterodimer protein consisting of two subunits, α and β with 131 and 123 amino acid residues, respectively. The amino acid sequences of α and β subunits show to have 35.5 % similarity[131]. They are tightly interconnected by a disulphide-bond between cysteine residue 78 in the α-subunit and Cys 75 in the β-subunit[132] (Figure 4).

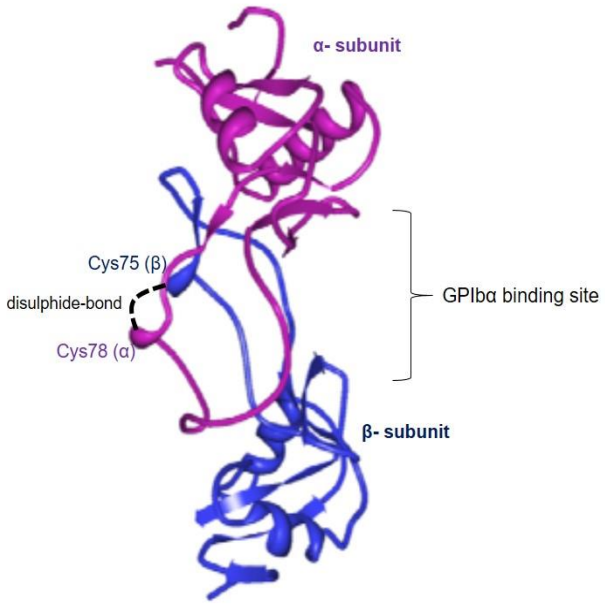

Figure 4. 3D structure of echicetin.
Echicetin α-subunit (in violet) is linked to the β-subunit (in blue) via a disulphide-bond between two cysteine residues, Cys78 (of the α-subunit) and Cys75 (of the β-subunit). This scheme shows the site where echicetin binds the GPIbα protein. Figure adapted from Jasti J. et al (2004)[132] and Matsui T.et al (2010)[133].

Echicetin does not require Ca^{2+} ions for binding due to the truncated Ca^{2+}-binding site, therefore, it belongs to the C-type lectin like protein family (snaclecs)[131].

1.7.2.1 Echicetin: the GPIbα antagonist

Echicetin was reported to bind through its concave domain to the GPIbα protein and to compete with the binding of vWF and thrombin[132-134], preventing the interaction of these ligands to GPIbα domain resulting in inhibition of platelet agglutination in washed platelets. In addition, echicetin injected in whole blood leads to an increase in the bleeding time in mice in a dose dependent manner. Bleeding times were increased up to 10 minutes with an ejection of 2 µg echicetin/ mouse[126].

On the other hand, data showed that blood infusion of echicetin induced thrombocytopenia in mice. Platelet counts significantly dropped down (20 to 40% compared to control values) after echicetin injections in whole blood and then recovered after several hours. Echicetin-induced thrombocytopenia in mice showed to be a result of echicetin's ability to bind to the IgMκ in plasma. IgMκ can bind up to 5 molecules of echicetin, indirectly cluster several GPIbα receptors of different platelets by echicetin leading to platelet agglutination[134]. However, the complex echicetin-IgMκ induces platelet activation and signal transduction through GPIbα and the phosphorylation of downstream kinases such as the spleen tyrosine kinase Syk[134, 135].

1.7.2.2 Echicetin beads: the GPIbα agonist

All data agree that echicetin is selective ligand of GPIbα. Navdaev A. et al developed a novel tool to investigate the specific signaling pathways mediated only by GPIbα[136]. Echicetin was coated on polystyrene latex beads of a diameter of 0.46 µm. Echicetin beads (EB) showed to induce a full platelet aggregation in washed platelets by clustering several GPIbα receptors together and induce a full platelet activation dependent on the integrin $\alpha_{IIb}\beta_3$.

However, some parameters have to be respected during coating the beads with echicetin. This group showed that the distance between each echicetin bound on the bead is essential for platelet activation and not the amount of echicetin used. A distance of less than 7 nm between each neighboring echicetin molecule is able not only to cluster GPIbα molecules but also to induce integrin $\alpha_{IIb}\beta_3$-dependent GPIbα-specific platelet activation, otherwise echicetin induces only platelet agglutination without intracellular signal transmission[134].

Some facts in numbers are provided according to the calculations provided by Navdaev A. et al (2014); for 0.3 mg/ml echicetin coated on polystyrene beads (concentration used in all experiments performed in this project), the distance between two neighboring echicetin is 4.7 nm, one bead is coated with 30,500 echicetin molecules and each protein occupies a surface on the bead of 21.8 nm^2 (total bead surface $1.247x10^{10}\mu m^2$)[136].

1.7.3 Convulxin, a selective GPVI agonist

Convulxin (72 kDa) is a protein derived from the South American rattlesnake, *Crotalus durissus terrificus*. Convulxin is very well-studied and its sequence was clearly identified as well as its

crystal structure[137, 138]. It is a tetramer formed by 4 α and 4 β chains interconnected by disulfide bridges between cysteine residues in the C-terminus of the α-chain and the N-terminus of the β-chain[133, 137, 138].

It was firstly isolated in 1981 by Franceschi J. et al[139] and characterized to induce convulsions and perturbation in both respiration and circulation systems. Convulxin was later identified as a potent platelet agonist[128] that bind to the collagen receptor GPVI with high affinity[140]. Convulxin-mediated platelet aggregation induces the activation of kinases that play a crucial role in platelet signaling such as the spleen tyrosine kinase Syk. Compared to collagen, convulxin binds only to GPVI and not to the integrin $\alpha_2\beta_1$[140]. Therefore, convulxin is widely used as GPVI-specific agonist for platelet function assessments and a useful tool to investigate the specific signaling pathways downstream of GPVI *in vitro*.

2 Aims of the study

The overall goal of this project was to enhance the understanding of the vWF-receptor GPIb-V-IX–mediated signaling in human platelets. However, GPIb-V-IX is not the only platelet receptor that binds vWF, which additionally binds to the integrin $\alpha_{IIb}\beta_3$ via its C4 domain inducing platelet activation. This interference obviously obscures the results and makes the study of GPIbα-specific signaling very difficult to establish. Therefore, I aimed in this project to establish the following goals:

1- To develop an established GPIbα ligand, the snake toxin echicetin, as selective agonist and/or antagonist of GPIbα.mediated intracellular signaling
2- To investigate the activation pathways induced by echicetin coated on polystyrene beads (EB) and the GPIbα-downstream intracellular signaling in human platelets.
3- To study the role of the src family kinases (SFKs) and the spleen tyrosine (Syk) for the initial GPIbα-mediated mechanisms of human platelet activation
4- To compare EB/GPIbα-mediated signaling pathways with the well-established collagen –receptor pathways, the convulxin/GPVI-mediated response.
5- To analyze the effects of platelet inhibitory pathways mediated by cAMP/PKA and cGMP/PKG on the GPIbα and GPVI-activation mechanisms and to study the crosstalk between these pathways.
6- To address the possible mechanism of regulation at the level of Syk tyrosine kinase.

3 Materials

3.1 Agonists

Product	Binding receptor	Company
ADP	$P2Y_1$ and $P2Y_{12}$	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
bovine thrombin	PAR receptor	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
collagen	GPVI, α2β1 integrin	Chrono-log corporation, Havertown, Pennsylvania
convulxin	GPVI	Enzo Life Sciences, Lörrach, Germany
ristocetin	increases the affinity of vWF to GPIbα	Loxo GmbH, Dossenheim, Germany
TRAP6	PAR receptor	Bachem, Bubendorf, Switzerland
U 46619 (TxA_2)	TP receptor	Tocris, Wiesbaden, Germany
venom from *Echis carinatus sockurechi*	GPIbα	Latoxan, Valence, France
Wilate® (human vWF)	GPIbα, $\alpha_{IIb}\beta_3$ integrin	Octapharma GmbH, Langenfeld, Germany
Wilfact® (human vWF)	GPIbα, $\alpha_{IIb}\beta_3$ integrin	LFB Biomedicaments Courtaboeuf Cedex, France

3.2 Inhibitors

Product	Function	Company
AR-C69931	$P2Y_{12}$ receptor inhibitor	The Medicines Company, Parsipany, NJ, USA
cOmplete, Mini	protease Inhibitor Cocktail	Roche, Mannheim, Germany
GF109203X	pan PKC inhibitor	Enzo Life Sciences, Switzerland
Ilomedine® or iloprost	ligand of the prostacyclin receptor	Bayer AG, Leverkusen, Germany
LY294002	PI3K inhibitor	USBiological, life sciences, USA
MRS2179	$P2Y_1$ receptor inhibitor	Viozol, Eching, Germany
OSXI-2	Syk inhibitor	Merck, Darmstadt, Germany
PhosStop	phosphatase inhibitor cocktail	Roche, Mannheim, Germany
piceatannol	Syk inhibitor	Sigma Aldrich, USA
PRT-060318	Syk inhibitor	Sellckem, USA
riociguat	soluble guanylyl cyclase activator (PKG activator)	Bayer AG, Leverkusen, Germany
SQ 29.548	TP- receptor inhibitor	Cayman chemical, USA
tirofiban (Aggrastat®)	integrin $\alpha_{IIb}\beta_3$ inhibitor	Iroko Cardio LLC, USA.

Product	Function	Company
wortmannin	PI3K inhibitor	Cayman chemical, USA

3.3 Chemical compounds and reagents

Chemicals	Company
1,4-Dithiothreitol (DTT)	Carl Roth GmbH, Karlsruhe, Germany
ammonium persulfate (APS)	Carl Roth GmbH, Karlsruhe, Germany
bovine serum albumin (BSA)	Capricorn Scientific GmbH, Ebersdorfergrund, Germany
bromophenol blue (BPB)	Merck KGaA, Darmstadt, Germany
calcium chloride ($CaCl_2$)	Carl Roth GmbH, Karlsruhe, Germany
Clarity™ Western ECL Substrate	BioRad Laboratories, Hercules, USA
complete mini protease inhibitor tablets	Roche GmbH, Mannheim, Germany
D(+) -Glucose	Carl Roth GmbH, Karlsruhe, Germany
dimethyl sulfoxide (DMSO)	Merck KGaA, Darmstadt, Germany
ethanol (EtOH) 99,5% (v/v), pure	Carl Roth GmbH, Karlsruhe, Germany
ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)	Carl Roth GmbH, Karlsruhe, Germany
ethylenedinitrilotetraacetic acid disodium salt dihydrate (Titriplex® III)	Merck, Darmstadt, Germany
fluo-3, AM (intracellular cytoplasmic Ca^{2+})	Life Technologies, USA
formaldehyde (FA) 37% (v/v)	Carl Roth GmbH, Karlsruhe, Germany
glycerol	Carl Roth GmbH, Karlsruhe, Germany
glycine	Carl Roth GmbH, Karlsruhe, Germany
hepes	Carl Roth GmbH, Karlsruhe, Germany
hydrochloric acid	Carl Roth GmbH, Karlsruhe, Germany
hydrochloric acid (2N) (HCl)	Carl Roth GmbH, Karlsruhe, Germany
iodacetamide	Merck KGaA, Darmstadt, Germany
isopropyl alcohol (isopropanol)	Carl Roth GmbH, Karlsruhe, Germany
magnesium chloride ($MgCl_2$)	Carl Roth GmbH, Karlsruhe, Germany
methanol	Carl Roth GmbH, Karlsruhe, Germany
N-ethylmaleimide	Sigma-Aldrich, Saint Louis, USA
polystyrene beads	Sigma-Aldrich GmbH, Seelze, Germany
potassium chloride (KCl)	Carl Roth GmbH, Karlsruhe, Germany
Precision Plus Protein Dual Color Standard	BioRad Laboratories, Hercules, USA

Chemicals	Company
protease inhibitor cocktail tablets	Roche, Basel, Switzerland
rotiphorese Gel (Acrylamide/ Bisacrylamide 30%/0.8%)	Carl Roth GmbH, Karlsruhe, Germany
silver nitrate ($AgNO_3$)	Merck KGaA, Darmstadt, Germany
sodium chloride (NaCl)	Carl Roth GmbH, Karlsruhe, Germany
sodium Dodecyl Sulfate (SDS)	Carl Roth GmbH, Karlsruhe, Germany
sodium hydroxide solution (2N) (NaOH)	Merck KGaA, Darmstadt, Germany
tetramethylethylenediamine (TEMED)	Sigma-Aldrich GmbH, Seelze, Germany
toyopeal DEAE-650S resin	Tosoh Bioscience GmbH, Griesheim, Germany
tris	Carl Roth GmbH, Karlsruhe, Germany
tris-natrium citrate dihydrate	Carl Roth GmbH, Karlsruhe, Germany
triton™X-100	Carl Roth GmbH, Karlsruhe, Germany
Tween®-20	Sigma-Aldrich GmbH, Seelze, Germany
β- mercaptoethanol	BioRad Laboratories, Hercules, USA

3.4 Antibodies

All antibodies (Abs) were diluted 1:1000 (v/v) in 5% (w/v) BSA in TBS-T unless stated otherwise:

Primary antibody	Recognized site	Source	Company
Syk	phosphorylated (p) pY525/526	rabbit monoclonal antibody (Ab)	Cell signaling technologies, USA
	pY352	rabbit polyclonal Ab	Cell signaling technologies, USA
	pS297	rabbit polyclonal Ab	Cell signaling technologies, USA
	total Syk (4D10)	mouse monoclonal Ab	Santa Cruz biotechnology, USA
$PLC\gamma_2$	pY759	rabbit polyclonal Ab	Cell signaling technologies, USA
	total PLCγ2 (B-10)	mouse monoclonal Ab	Santa Cruz biotechnology, USA
Akt	pS473	rabbit	Cell signaling technologies, USA

Primary antibody	Recognized site	Source	Company
		polyclonal Ab	
	total Akt1 (B-1)	Mouse monoclonal Ab	Santa Cruz biotechnology, USA
LAT	pY191	rabbit polyclonal Ab	Cell signaling technologies, USA
α-actinin	total α-actinin 1	rabbit polyclonal Ab	Cell signaling technologies, USA
β-actin	total β-actin	rabbit polyclonal Ab	Cell signaling technologies, USA
IgG_{2a}	normal IgG (used as negative control)	mouse monoclonal Ab	Santa Cruz biotechnology, USA

Secondary antibody	Source	Company
HRP-conjugated secondary mouse antibody 1:5000 (v/v)	goat polyclonal Ab	BioRad Laboratories, USA
HRP-conjugated secondary rabbit 1:5000 (v/v)		

3.5 Buffers

Buffers were prepared with ddH_2O. They are grouped following their purpose of use:

	Buffers and solutions	Composition
affinity chromatography	1x phosphate saline buffer (PBS) pH 7.4	137 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4*2 H_2O, 1.4 mM KH_2PO_4
	acetate buffer pH 2.7	0.2 M acetic acid + 1mM sodium acetate
anion exchange chromatography	start buffer A, pH 8.0	10 mM tris
	elution buffer B, pH 8.0	10 mM tris + 1 M NaCl
silver staining	gel fixation solution	40% (v/v) ethanol, 10% (v/v) acetic acid
	wash solution	30% (v/v) ethanol
	thiosulfate solution	0.02% (v/v) thiosulfate pentahydrate
	silver nitrate solution	0.2% (w/v) silver nitrate + 2 mM formaldehyde (37%)

	Buffers and solutions	Composition
	developing solution	6% (v/v) natrium carbonate, 16 µM thiosulfate pentahydrate, 5 mM formaldehyde (37%)
	stop solution	0.5% (v/v) Titriplex® (EDTA)
platelet isolation	CGS buffer pH 6.5	120 mM NaCl, 12.9 mM tri-Na-Citrate, 30 mM D-glucose
	hepes buffer pH 7.4	150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 10 mM hepes
sample preparation for Western Blot and silver staining	3x Laemmli buffer sample buffer	200mM tris/HCl, 15%(v/v) glycerol, 6%(w/v) SDS, 0.06% (w/v) bromophenol blue in ddH_2O
	DTT	0.2 M dithiothreitol in loading buffer
	IAA	778 mM iodacetamide in loading buffer, BPB solution
	BPB	10% bromophenol blue in loading buffer
	loading buffer	8 M urea, 0.1 M tris, 0.07 M SDS
	NEM	40 mM N-ethylmaleimide
electrophoresis	10x TGS running buffer (BioRad)	25mM tris, 192 mM glycine, 0.1%(w/v) SDS, pH 8.3
protein transfer	10x Transfer buffer	1.92 M glycine, 0.25 M tris
immunoprecipitation	2x lysis buffer pH 7.4	2% (v/v) NP40, 300 mM NaCl, 20 mM Tris-HCl, phosphatase and protease inhibitors (2 tablets/10 ml)
membrane stripping	SDS stripping buffer pH 2.2	1.5% (w/v) glycine, 0.1% (w/v) SDS, 1%(v/v) Tween®20

3.6 Consumables

Product	Manufacturer
14 ml tubes with two-position vent stopper, polypropylene	Greiner Bio-One GmbH, Frickenhausen, Germany
Amersham™ Hybond™ PVDF blotting membrane 0.45 µm and 0.2 µm	GE Healthcare Life Sciences, Amersham, UK
Cellstar Tubes® polypropylene, conical bottom (15 ml/50 ml) with and without foot (only 50 ml)	Greiner Bio-One GmbH, Frickenhausen, Germany
centrifugal filter units Amicon® Ultra 15, Ultracel® 10K	Merck Millipore, Darmstadt, Germany
mikro-cuvettes with stirring bar for APACT 4 dispo-system	DiaSys Greiner, Flacht, Germany

Product	Manufacturer
monovettes® 10 ml 9NC, with anticoagulant 0.106 M citrate	Sarstedt AG&Co KG, Nümbrecht, Germany
pipettes (2.5, 10, 20, 100, 200, 1000 µl) Eppendorf research plus	Eppendorf Vertrieb Germany GmbH, Wesseling,Germany
pipettes tips	StarLab, Hamburg, Germany
reaction tubes, safe seal 1.5 and 2 ml	Sarstedt AG&Co KG, Nümbrecht, Germany
safety-Multifly®-Set 21G with tube and adapter 0.8x19mm	Sarstedt AG&Co KG, Nümbrecht, Germany
serological pipettes 25/10/5 ml	Greiner bio-one GmbH, Frickenhausen, Germany
siliconized stirring bars	Chrono-Log Corp., Havertown, USA
transfer pipettes 3.5ml disposable pipettes with integral bulb	Sarstedt AG&Co KG, Nümbrecht, Germany
tricorn column, 5/50 column (1ml)	GE healthcare life sciences, Germany

3.7 Equipment

Device	Type	Company
anion exchange chromatography	Äkta pure	GE healthcare life sciences, Germany
automatic Haematology analyser	Sysmex® KX-21N	Sysmex Corporation Japan/Sysmex Germany GmbH
blot chamber	Trans-Blot® Cell	BioRad Laboratories, Hercules, USA
centrifuge 1	Allegra X-30R, rotors F2402, SX4400	Beckmann Coulter GmbH, Krefeld, Germany
centrifuge 2, tabletop for 1.5 and 2 ml reaction tubes	5418 R	Eppendorf AG, Hamburg, Germany
electrophoresis machine	mini-protean tetra cell	BioRad Laboratories, Hercules, USA
ELISA reader	Opsys MR™ microplate reader	Dynex technologies Inc,USA
flow cytometer/FACS	BD FACSCANTO II	BD biosciences, Heidelberg, Germany
gel casting module	mini-protean tetra cell casting module	BioRad Laboratories, Hercules, USA
light transmission aggregometer	Apact 4 S Plus	DiaSys, Flacht, Germany
pH meter	HI 2211	Hanna Instruments GmbH, Kehl am Rhein, Germany
shaker	DOS-10L	NeoLab® Migge GmbH, Heidelberg, Germany

Device	Type	Company
thermomixer	thermomixer compact 5350	Eppendorf Vertrieb Germany GmbH, Wesseling
vortexer	lab dancer S40	VWR International GmbH, Darmstadt, Germany
water bath	ED-AP (042) (5A max. 60°C)	Julabo GmbH, Seelbach, Germany
western Blot detection machine	fusion FX7	Vilber Loumat GmbH, Eberhardzell, Germany

3.8 Software

Software	Developer
FACSDiva software v6.1.3	BD biosciences, Heidelberg, Germany
GraphPad Prism 8 for Windows	GraphPad Software, San Diego, CA

4 Methods

4.1 Echicetin purification

Echicetin is a snake venom protein produced by the saw-scaled viper *Echis carinatus sochureki.* From the lyophilized venom, 50 mg were dissolved in 10 ml sterile PBS pH 7.4. The venom consists of different proteins, therefore, two purification steps were needed to obtain pure protein. Echicetin purification steps were adapted and modified according to Navdaev A. et al (2002)[135].

4.1.1 Affinity chromatography

To isolate selectively echicetin from the venom, a protein A-column coupled with rabbit polyclonal antibodies against echicetin was used. This column consists of protein A immobilized on sepharose beads type 4B. Protein A has a high affinity for binding IgGs (IgG anti-echicetin antibodies) through their Fc chain, keeping the Fab region free to bind to the antigen (echicetin).

One tablet of protease inhibitor cocktail was added to the dissolved mixture to inhibit all the proteases that might affect the antibodies coupled to the protein A. Before applying the mixture to the column, it was centrifuged at 16,000 xg for 10 minutes to get rid of the insoluble components.

The purification procedure was established mainly in 3 steps, which were performed all at room temperature.

a- **Sample application:** Dissolved venom containing protease inhibitors was applied to the affinity chromatography column and kept for one hour to make sure that echicetin binds to the antibodies.
b- **Washing:** The column was washed 5 times (5 column volumes, (CV)) with sterile PBS pH 7.4 in order to get rid of unbound proteins.
c- **Elution and regeneration:** Bound proteins to the column were eluted using a low pH buffer, acetate buffer 0.2 M, pH 2.7. The eluent was recuperated in 1.5 ml Eppendorf tubes containing 75% (v/v) 1M tris pH 9.0 to neutralize the acidic pH of the eluent. The column was washed again with 5 CV with sterile PBS and stored at 4°C in presence of 0.03% (w/v) sodium azide (NaN_3) to avoid any bacterial growth inside the column.

4.1.2 Anion exchange chromatography

Anion exchange chromatography was additionally performed to remove the remaining impurities of the sample. Proteins were separated using anion-exchange resin, which is coated with positively charged ions (diethylaminoethanol, DEAE).

Proteins with negative charge covalently bind to the cations on the resin matrix. A highly negative charged protein binds with higher affinity to the resin and vice versa. Proteins bound to the resin are retained and can be eluted in several ways. 1) Elution by increasing the salt concentration, negative ions concentration increases and competes with the bound proteins leading to their release. 2) Elution by decreasing gradually the pH, bound proteins become more positively charged, leading to the detachment of the bound proteins from the resin matrix.

Echicetin has an isoelectric point of 7.57, slightly negatively charged. Proteins eluted from the first purification step were transferred into a buffer A (tris 10 mM, pH 8.0). Negative charges provided by the buffer A help echicetin to bind more strongly to the cations on the resin matrix.

This purification was performed in 5 successive steps all at room temperature (Figure 5):

1. **Equilibration**: DEAE column was washed 5 column volums (CV) with the start buffer (buffer A), which has a low ionic strength.
2. **Sample application:** Negatively charged proteins bind stronger to the cations of the matrix. However, neutral and positive charged proteins are eluted directly during the sample application or during the column washing in the next step.
3. **Washing:** 20 CV of buffer A were applied to the column in order to wash away all unbound proteins.
4. **Elution:** Start buffer containing 1 M NaCl, pH 8.0 was applied gradually into the column from 0 to 1 M NaCl with a flow rate of 1ml/min. Cl^- ions competitively bound to resin cations, releasing the bound proteins from the column. As shown in Figure 5, during the elution step, two peaks appeared by increasing the salt concentration from 0 to 35%. By increasing the salt concentration up to 100%, therefore increasing the ion strength, all the bound proteins that might be still bound on the column should be eluted in this step.
5. **Column regeneration and equilibration:** A maximum salt concentration was applied to increase the ionic strength to the maximum. This high ionic strength helps to remove all the charged proteins that are tightly bound to the resin. This step is very important as a final step before equilibrating again the column with buffer A.

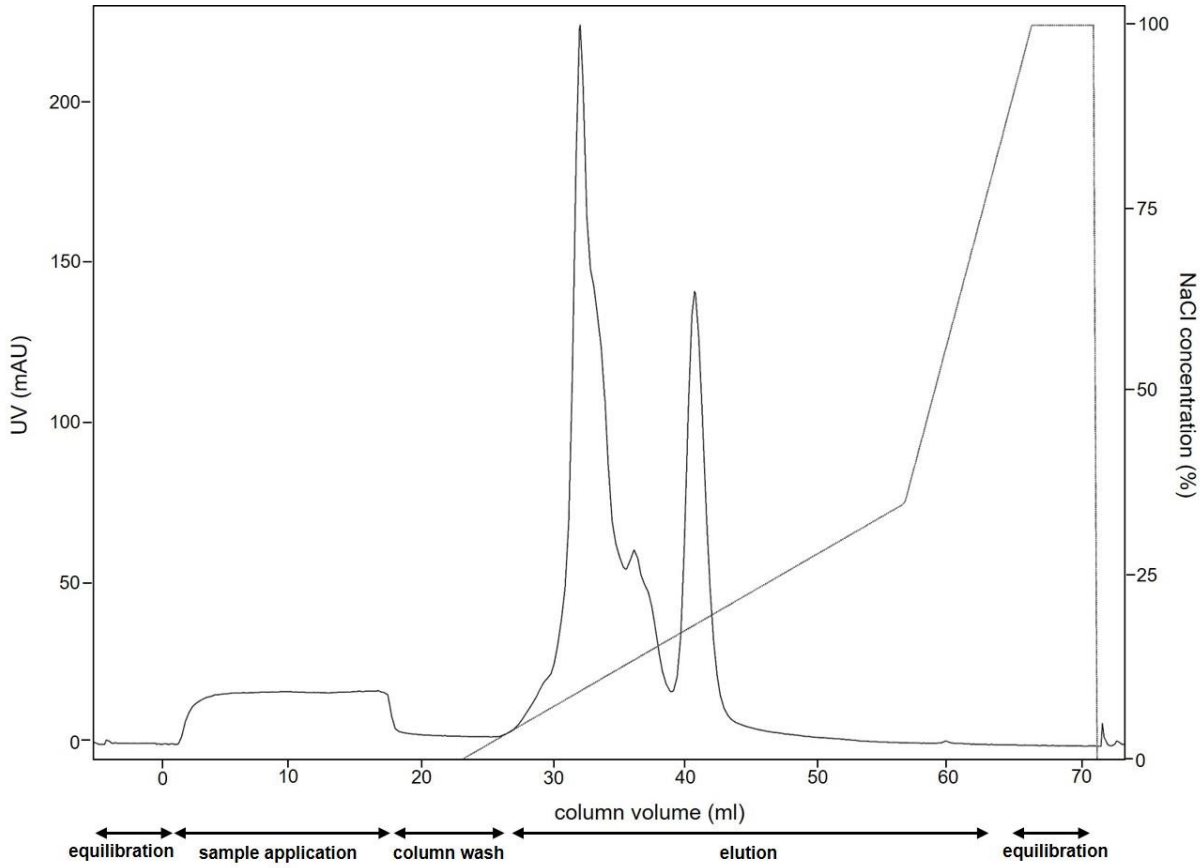

Figure 5. Anion exchange chromatography of echicetin.
An illustration graph showing echicetin purification using the Äkta™pure. The graph shows in the axis, the volume applied in each step in 'column volume' unit which is equal to 1 ml in function of the UV (mAU) on the left axis and the salt concentration (in %) on the right axis. After equilibrating the column with low ionic strength buffer, the sample containing echicetin is applied to the column, the UV line marks the presence of proteins during sample application. The column is washed again before elution. Two separated peaks are eluted while the concentration of salt is increasing, marking two different lots of proteins with different characteristics. A higher salt concentration is applied at the end to elute all proteins potentially stuck to the column before equilibrating it again.

The different fractions from the same peak were pooled together. The elution buffer was exchanged into PBS buffer pH 7.4 using Amicon® Ultra 15 ml centrifugal filter devices. Proteins from each peak were then concentrated using those devices as well.

4.2 SDS-PAGE

SDS-polyacrylamide gel electrophoresis is a method used to separate different proteins found in one sample according to their mass. Proteins negatively charged due to the SDS in the lysis buffer (see paragraph 4.11.2) migrate through the polyacrylamide layers of the gel. Different proteins are separated only depending on their size not of the charge; smaller proteins run faster towards the bottom of the gel. However, the bigger proteins migrate slower. Different acrylamide concentrations were used in order to better separate the different proteins inside the sample. A higher concentration of acrylamide is used to obtain a better resolution of smaller proteins, due to the smaller diameter of the gel pores. In the following experiments, 8, 10 and 15% (w/v) acrylamide gels were used with a gel thickness of 1.5 mm.

4.2.1 Gel preparation

Gels were prepared according to the appropriate acrylamide percentage (see tables below).

Table 2: Composition of the separating gel of different percentages of acrylamide gels.

Separating gel			
Components	**8% gel**	**10% gel**	**15% gel**
acrylamide/bisacrylamide	2 ml	2.5 ml	3.75 ml
1 M tris pH 8.8	3 ml	3 ml	3 ml
20% (w/v) SDS	38 µl	38 µl	38 µl
ddH_2O	2.43 ml	1.9 ml	680 µl
10% (w/v) APS	36 µl	36 µl	36 µl
TEMED	5 µl	5 µl	5 µl

Table 3: Composition of the stacking gel

Stacking gel (4% (w/v) acrylamide)	
acrylamide/bisacrylamide	660 µl
1 M tris pH 6.8	630 µl
20% (w/v) SDS	25 µl
ddH_2O	3.6 ml
10% (w/v) APS	25 µl
TEMED	5 µl

All components were mixed together in 50 ml falcon tube except for the APS and TEMED. Short plates and spacer plates (1.5 mm) were cleaned with ddH_2O followed by isopropyl and stacked together in the casting stand. APS and TEMED were added to the gel mixture to initiate gel polymerization and poured immediately between the two glass plates. The gel needs around 30 minutes to be completely polymerized. In order to linearize the gel surface and to avoid any air bubble formation, isopropyl was added on the surface directly after pouring the gel mixture.

After 30 minutes at room temperature, the isopropyl was removed and APS and TEMED were added to the stacking gel mixture and poured on top of the separating gel. Depending on the

sample number, combs of 10 or 15- wells were used. Gels were wrapped with wet tissues and stored at 4°C. They were used within 5 days.

4.2.2 Gel loading and gel run

20 µl of sample were loaded on the SDS-polyacrylamide gel, one lane is always reserved for the marker (5 µl). The marker consists of a mixture of molecules having a defined molecular weight and stained with 2 different colors. The marker helps to identify our protein of interest.

After loading the gel with the different samples, TGS buffer (running buffer) is poured inside the electrophoresis chamber. To start the electrophoresis, a current of 80 V is applied for 20 minutes and then increased until 120 V. The total running time is around 90 minutes. Proteins run from the cathode to the anode through the electrophoresis chamber.

4.3 Silver Staining (according to Blum H. et al (1987)[141])

Silver staining is known to be very sensitive method. It helps to detect proteins at very low concentrations as well as proteins with low molecular weight. This method is performed at room temperature and all buffers have to be freshly prepared before the experiment.

After electrophoresis, the gel was incubated overnight with a gel-fixing buffer containing 40% (v/v) methanol and 10% (v/v) acetic acid to tightly fixate proteins on the gel. The gel was washed 3 times with 30% (v/v) ethanol for 20 minutes to get rid of the rest of the fixing buffer. To increase the silver binding to the proteins, the gel was incubated for 1 minute with a sensitizer, 0.02% sodium thiosulfate solution. In addition, the thiosulfate solution helps to reduce unspecific silver binding in the background.

In the next step, the gel was incubated with silver nitrate (silver ions) for 20 minutes under gentle agitation at room temperature. Afterwards, it was washed 3 times with deionized water for 20 seconds. Silver is reduced to metallic silver by adding gradually the developing solution. Insoluble silver will deposit where proteins exist, then the color will turn into a dark brown color. Finally, the reaction was stopped by adding 0.5% (w/v) EDTA as stop solution. A picture of the gel was taken using BioRad ImageLab and a BioRad signal detection machine (Gel Doc™ EZ Reader).

4.4 Western blot

Western blot or protein immunoblot is an analytical technique widely used to detect proteins using specific antibodies[142]. After the electrophoresis proteins on the gel were transferred into a PDVF membrane then blocked with BSA before incubating with primary antibody, followed by washing step and incubation with HRP-conjugated antibodies to detect the signal.

4.4.1 Protein transfer

Proteins separated by electrophoresis were transferred into polyvinylidene difluoride membranes (PDVF) in the same order as they were on the gel. This membrane was placed on top of the gel covered from the down and upper sides with filter membranes, having the following order, from the positive to the negative side: filter membrane- membrane- gel- filter membrane). All together were placed in a 4°C room, inside a trans-blot machine filled with the transfer buffer. A current of 0.80 A was applied for 1 hour. During this time, proteins migrate from the negative to the positive side allowing them to be transferred onto the PDVF membranes.

4.4.2 Membrane blocking

In order to minimize unspecific antibody bindings and signals in the background, membranes were blocked with 5% (w/v) BSA or 5% (w/v) milk in tris-buffered saline (TBS) containing 0.1% Tween 20 (as detergent) at RT. This step is very important step to block the empty spaces of the membrane where proteins are not present.

4.4.3 Incubation with primary antibody

Detection of protein signals starts by incubating the membrane with a primary antibody against the protein of interest. For this reason, the corresponding primary antibody was diluted in a solution of 5% (w/v) BSA or 1% milk, which was used to incubate the membrane overnight under gentle agitation at 4°C.

4.4.4 Washing

The membrane was washed 3 times with TBS-T for 10 minutes each time at RT. The excess of non-bound primary antibodies is removed during this step.

4.4.5 Incubation with HRP-conjugated secondary antibody

Horseradish peroxidase-conjugated secondary antibodies are used to detect the primary antibody. The choice of the antibody has to be very specific. For example, a goat anti-rabbit antibody is used to detect rabbit primary antibody but cannot detect primary antibody derived from mouse. The amount of bound secondary antibody reflects the amount of existing protein. A dilution of 1:5000 (v/v) of the secondary antibodies is used to incubate the membrane for 2 hours at RT.

4.4.6 Washing

Similar procedure in step 5. The excess of secondary antibodies is removed.

4.4.7 Signal detection

Protein signals are detected by the enhanced chemiluminescence (ECL) method at RT. It consists of a commercially available kit, containing two different substrates. Shortly before signal detection, a mixed solution was freshly prepared with a 1:1 ratio ECL substrates 1 and

2. Depending on the size of the membrane, a volume of the mixed substrates was prepared in order to have 0.1 ml of solution per cm^2 of membrane. Chemiluminescence emitted by the HRP in presence of the substrates was captured by the camera of the Fusion-FX7 advanced machine. Images were captured at several exposure times in order to obtain the best signal of the studied proteins.

4.4.8 Mild membrane stripping

To obtain a loading control for some proteins such as LAT, or to make sure the regulation within the same sample, secondary antibodies and primary antibodies were removed following a mild stripping procedure. After detection of the initial signal, the membrane was covered with stripping buffer pH 2.2 at RT for 10 minutes. Before discarding the buffer and repeating this process once more. Then the membrane was washed twice with PBS buffer pH 7.4 for 10 minutes then twice with TBS-T pH 7.4 for 5 minutes each.

The membrane was again blocked with 5% (w/v) BSA for one hour at RT before incubating again overnight with the corresponding primary antibody.

4.4.9 Quantification and data analysis

Using the software of the Fusion X7 advanced machine, pixels of each band were given in an excel table format. Data are always normalized compared to a loading control such as α-actinin, β-actin or total protein compared to the phosphorylated form.

4.5 Protein quantification according to LOWRY

Total protein concentrations were measured using colorimetric techniques, which were firstly described by Lowry O. et al[143]. These techniques are based on redox reactions between an alkaline copper tartrate solution (reagent A) and folin reagent (reagent B). Reduced protein will manifest as a blue color with 750 nm as a maximal absorbance.

This method is able to detect proteins with concentrations from 0.3 mg/ml until 1.5 mg/ml. Standard solutions (0, 0.3, 0.6, 0.9, 1.2 and 1.5 mg/ml) were prepared from a stock solution of 3 mg/ml BSA in sterile PBS pH 7.4. 5 µl of each sample and each standard solution were added in 96-well flat bottom plate. 25 µl from reagent A were added to each well and then 200 µl of reagent B. The plate was incubated in the dark at RT for 15 minutes before measurement. Samples were measured using a spectrophotometer at 690 nm.

4.6 Echicetin beads preparation

After echicetin purification and determination of the concentration, proteins of peak 1 were used to coat the polystyrene beads.

A volume of 30 µl of the latex polystyrene beads (diameter 0.46 µm) were washed 3 times with sterile PBS pH 7.4 by centrifuging at 8,000 xg for 10 minutes to get rid of the surrounding

detergent that might affect the platelets during the experiment. Meanwhile 0.3 mg/ml of echicetin was prepared in a volume of 300 µl of PBS sterile pH 7.4. The washed beads were incubated with the echicetin solution for overnight at 4°C under gentle agitation. As control, the same volume of beads was added to a BSA solution (0.2 mg/ml BSA in sterile PBS).

After overnight coating, 900 µl of PBS were added to each EB preparation before centrifuging at 8,000 xg for 10 minutes at RT. The supernatant was discarded and the pellet was suspended with 1 ml of PBS and centrifuged again at the same speed. This step was repeated twice to get rid of the excess of protein. Then, echicetin beads (EB) were incubated with 1 mg/ml BSA in order to block empty spaces on beads that were not covered with the protein avoiding any unspecific binding. The blocking step was performed at RT for 2 hours under gentle agitation. EB were washed again 3 times with PBS. At the end, EB were suspended with 300 µl of PBS sterile to get rid of the excess of BSA and stored at 4°C.

4.7 Platelet isolation from whole blood

4.7.1 Blood withdrawal

Blood was collected from healthy donors who signed an informed consent. They were students or employees of the University Medical Center in Mainz. Using 10 ml monovettes containing 1:10 citrate, blood was collected by venipuncture from the superficial vein in the upper limb, precisely from the median cubital vein of the arm.

4.7.2 Platelet washing

After blood collection, a blood count was directly measured using an automated blood analyzer, Sysmex®, providing a general overview about the cell concentrations in the blood.

3 mM EGTA were added to each tube of anticoagulated whole blood in order to complex Ca^{2+} ions that might facilitate platelet preactivation during the washing procedure. The blood was then centrifuged at 200 xg for 10 minutes at RT.

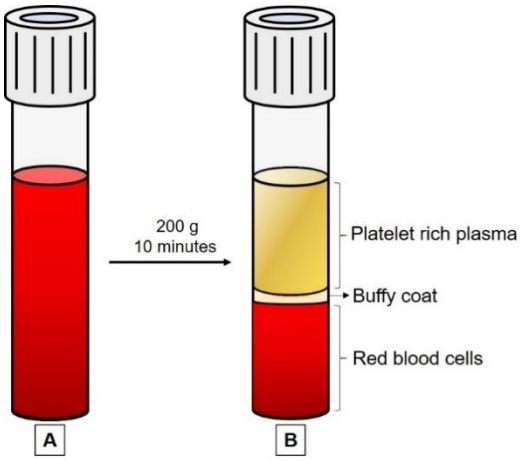

Figure 6. Representative scheme of separated platelets in plasma after the first centrifugation of citrated whole blood.
(A) Whole blood collected in a citrate 10 ml tubes, then centrifuged at 200 xg for 10 minutes with break of 2 (decel 2) at room temperature. (B) Blood is separated after centrifugation into 3 different layers (from down to up): According to different densities red blood cells are centrifuged down within the tube, the middle layer is the buffy coat where leukocytes (white blood cells) are predominantly present and the upper layer is the plasma rich in platelets.

The blood is separated into 3 different layers: the lower part consists of the accumulated red blood cells, the middle layer or the buffy coat consisting mainly of leukocytes/white blood cells and the upper layer (yellowish color) is the platelet-rich plasma (PRP) (Figure 6). The PRP was transferred into new 14 ml sterile polypropylene tubes and diluted 1:1 with a slight acidic CGS buffer pH 6.5. The low pH attenuates the platelet reactivity. Platelets were pelleted by centrifugation at 400 xg at RT for 10 minutes. 3 ml of CGS buffer were added to suspend the platelet pellet in each tube. After 10 minutes of resting time, platelet suspensions were centrifuged at 400 xg at RT for 10 minutes. In the last step, the platelet pellet was suspended using 2 ml of hepes buffer pH 7.4. This suspension was used to suspend the pellet in the next tube in a way to concentrate the platelets in a less volume.

Platelet concentration in the suspension was measured using the Sysmex®. A total volume of 100 µl of 1:10 diluted platelets is enough for the measurement. Platelets used in all experiments were adjusted to 3 x 10^8 platelet/ml. Platelets were kept in the water bath at 37°C for 15-20 minutes before starting the experiment

4.8 Light transmission aggregometry (LTA)

4.8.1 Principle

Light transmission aggregometry was first described in 1961 by Born and O'Brien[144]. This method remains the gold standard method for platelet function analysis. Platelets in suspension (in hepes buffer or in PRP) are placed in a cuvette containing a small stirring bar. This cuvette is placed between a light source and photocell. When platelets are in suspension, the light can hardly pass through the cuvette. However, once platelets are aggregated, the light

transmission through the cuvette increases. Results are giving by percentage of light transmission as a function of time. This percentage reflects the percentage of platelet aggregation (fibrinogen-mediated) or agglutination (vWF-mediated) as well.

4.8.2 Platelet lysis and sample preparation

Platelets are very sensitive and can be activated by many external stimuli. For this reason, normal stirring bars provided originally in the cuvettes were replaced with siliconized stirring bars to avoid additional stress through the sharp edges of the stirring bars.

After platelets were rest for 15- 20 minutes at 37°C, 200 µl of platelet suspension were placed in cuvettes at 37°C in the backside of the aggregometer. Depending on the aim of the experiment, platelets were treated, or not, with defined compounds and inhibitors before stimulation with the appropriate agonist (convulxin, EB, thrombin, ADP, etc). Platelet aggregation was stopped after specified time points (mainly after 1, 2 and 5 minutes unless stated different) by adding Laemmli buffer directly inside each cuvette.

4.9 Sample preparation for Western blot and silver staining

In order to detect protein regulation in platelets samples, it is recommended to have the protein reduced and unfolded or under its denatured form to obtain a better separation on the SDS-PAGE gel. However, in some cases, it is also important to keep the protein under its preserved conformation (non-reduced).

4.9.1 Non-reducing conditions

For echicetin, it was very important to show the preserved conformation (non-reducing conditions) in addition to the reduced form. For this reason, samples under non-reducing conditions were prepared and checked by Western blot and silver staining. 10 µl of sample from the pooled fractions after ion exchange chromatography of echicetin were boiled with 800 nM N-ethylmaleimide (NEM) for 10 minutes at 95°C 20 xg. Samples were cooled down at RT and then stored at - 20°C until the experiment has started.

4.9.2 Reducing conditions

4.9.2.1 Samples for echicetin validation

To check the reduced conformation of echicetin, a potent reducing agent was used dithiothreitol (DTT), which is able to disrupt the disulfide bonds between the cysteines residues in combination with a strong alkylating agent, iodoacetamide[145], which prevents the reformation of those disulfide bonds. 10 µl from IEX pooled samples were boiled with 4 mM DTT at 95°C for 10 minutes at 20 xg. Then a concentration 14 mM IAA was added. The whole mixture was kept at RT for 15 minutes under gentle agitation. Samples were stored at -20°C until performance of the experiment. They were used to be checked by silver staining and western blot.

4.9.2.2 Samples from platelets lysates

To check some specific proteins in platelets after being differently treated in comparison to basal conditions, platelet lysates were obtained by adding Laemmli buffer (dilution 1:2) directly inside the cuvette after specified time points (in our experiments 1, 2 and 5 minutes or 15, 60 and 120 seconds). After stopping the aggregation with the lysis buffer, platelet lysates were immediately transferred into 1.5 ml Eppendorf tubes then cooked at 95°C for 10 minutes at 20 xg. Samples were stored at - 20°C until the performance of the experiment.

4.10 Immunoprecipitation

The purpose of performing immunoprecipitation is to isolate a protein from a sample containing different proteins. This technique is based on precipitation the protein antigen using antibodies that bind specifically to this protein. Those antibodies are fixed on a solid matrix for example sepharose beads coupled to protein A or G. To confirm Syk phospho-regulation as observed on western blots, Syk-indirect immunoprecipitation was performed. Platelet lysates were first preincubated with the specific antibodies before adding the protein A sepharose beads, which act as a solid matrix to catch the antibodies through their Fab region.

4.10.1 Preparation of platelets lysates

Washed human platelets (200 µl, 3 x 10^8/ml) were treated differently and then lysed using an ice cold 2x NP 40 lysis buffer (1:1) containing protease and phosphatase inhibitors. Platelets lysates were kept on ice for 30 minutes, then centrifuged at 4°C at 13,000 xg in order to get rid of the insoluble debris. The supernatant was transferred into new 1.5 ml Eppendorf tubes.

4.10.2 Protein A-sepharose beads preparation

50% (v/v) of protein A sepharose were added to TBS-T pH 7.6. Beads were washed 3 times using TBS-T by centrifuging at 1,100 xg for 5 minutes.

4.10.3 Sample pre-clearing

To eliminate the proteins that may bind to the protein A sepharose, platelets lysates were kept overnight at 4°C under gentle rotation in the presence of 20 µl of protein A- coupled beads. Beads were pelleted down and the supernatant is transferred to a new 1.5 ml tube.

4.10.4 Immunoprecipitation

125 µl pre-cleared lysates were preincubated with 0.625 µg anti-Syk antibody (4D10) and 1.25 µg mouse IgG2a as a negative control. The tubes were placed on ice without any rotation for 90 minutes allowing the antibodies to bind to their specific ligand, the Syk protein.

As a second step, the protein A sepharose beads (20 µl) were added to each tube. At that time, the antibodies will be bound to their target, therefore the antibodies can only bind to

beads through their Fc region. Again, this mixture was kept at RT under gentle rotation for 90 minutes.

4.10.5 Washing

Beads were pelleted by centrifugation (1,100 xg, 5 min at RT). The supernatant was transferred into new 1.5 ml tubes and samples for western blot were prepared in order to check the unbound proteins.

The pellet containing the protein A sepharose beads was washed twice using 1x NP40 lysis buffer followed by a final washing step using TBS-T.

4.10.6 Sample preparation for western blot

As described in paragraph 4.9.2, Laemmli buffer was added respectively to the samples containing the beads and to the supernatant in a ratio of 1:2. Then samples were cooked at 95°C for 10 minutes at 20 xg and stored at -20°C until the performance of the western blot.

4.11 IP- One ELISA

4.11.1 Principle of the assay

The IP-One ELISA is a competitive immunoassay used to measure the phospholipase C activity by quantitative determination of D-*myo*-inositol 1 phosphate (IP1). The assay is based on the competition between free IP1 and IP1-HRP conjugate for a limited number of binding sites on an anti-IP1 monoclonal antibody.

The ELISA plate was coated with goat anti-mouse IgG. Anti-IP1 monoclonal antibodies were added to each well, including the standards and they would bind to the anti-mouse IgGs. The supernatant containing the free IP1 was then added followed by the IP1-HRP conjugate. Both IP1 would bind competitively to the IP1 monoclonal Abs, which has a determined binding capacity (Figure 7).

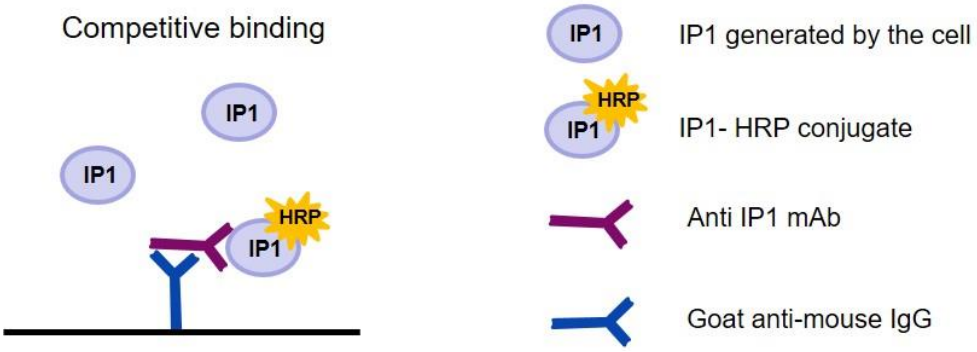

Figure 7. IPOne competitive ELISA.
Free IP1 and HRP-coupled IP1 competitively bind to the IP1 monoclonal antibody. A higher level of IP1-HRP bound reflects a lower concentration of free IP1 (less IP1 generated by the cell). Scheme adapted from cisbio IP-One ELISA data sheet.

After 3 hours of incubation at RT with gentle shaking, the plate was washed 6 times with the wash buffer containing 0.05% Tween 20®. Color development was performed by the addition of 3, 3', 5, 5'- Tetramethylbenzidine (TMB) to each well. The incubation with TMB was performed at RT for 20-30 minutes until the TMB blue color is developed. At the final step, the reaction was stopped by adding a stop solution (sulfuric acid solution) (provided by the company). The blue color was turned into yellow color. A high color intensity reveals a lower concentration of accumulated IP1 in the cell. The optical density was measured at 450 nm with a correction of 630 nm.

4.11.2 Sample preparation

Washed human platelets were prepared as mentioned in paragraph 4.7. At the end, they were suspended with hepes buffer containing 1 mM LiCl in order to inhibit the inositol monophosphatase (IMPase) leading to the accumulation of the inositol monophosphate (IP1) in the cytoplasm of the platelet (Figure 8).

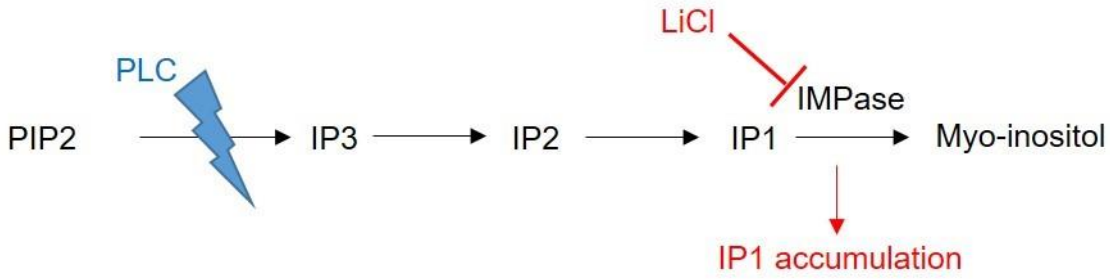

Figure 8. Mechanism of IP3 production and IP1 accumulation.
Upon platelet activation, the phospholipase C (PLC) hydrolyzes the phosphatidylinositol 4,5-biphosphate (PIP2) located in the plasma membrane inducing the inositol triphosphate (IP3) formation. IP3 is quickly degraded into IP2, IP1 and then myo-inositol. The use of LiCl in the platelet buffer inhibits the degradation of the IP1 therefore it is accumulated in the cell.

Platelets were preincubated with the suitable inhibitor prior to activation by EB or convulxin. The aggregation was stopped after 5 minutes using the lysis buffer provided by the company.

Platelet lysates were kept on ice for 30 minutes then centrifuged at 16,000 xg for 10 minutes to get rid of the cellular debris. The supernatant was transferred to new 1.5 ml Eppendorf tubes. Samples were freshly prepared before performing the IP-One ELISA test.

4.12 Flow cytometric measurement of intracellular Ca^{2+} release

Flow cytometry is a cell biology laser-based technology used to count, sort and profile cells and their functions in a heterogeneous fluid mixture. Cells are characterized individually, rapidly and quantitatively. In this study, the fluorescence-activated cell sorting technique (FACS) was used to investigate the Ca^{2+} release upon platelet stimulation with EB or convulxin.

Washed human platelets were washed as previously described and preincubated with a Ca^{2+} indicator dye, fluo-3 acetoxymethyl (AM) esters (5 µM) for 30 minutes at 37°C. Fluo-3 AM (excitation/emission of Ca^{2+} bound form of 506/526) is able to enter the cell and it exhibits an increased fluorescence intensity after Ca^{2+} binding which is detected by the cytometer.

Platelets were preincubated with different treatments prior to activation by EB or convulxin. Ca^{2+} signaling was monitored directly after adding the agonist into the platelet samples for 2 minutes. Data represent the mean of fluorescence measured during 2 minutes.

5 Results

5.1 Echicetin purification: Affinity chromatography, anion exchange chromatography and mass spectrometry

5.1.1 Silver staining

To analyze the purity of echicetin, samples from each purification step were analyzed by silver staining. This method is very sensitive; proteins at low nanogram ranges can be detected, which helps to track the degree of impurities in the sample. During the affinity chromatography, echicetin was retained by the rabbit polyclonal antibodies directed against echicetin in contrast to the other proteins not bound to the antibodies. Those impurities were washed away during the column-washing step with sterile PBS pH 7.4 (Figure 9).

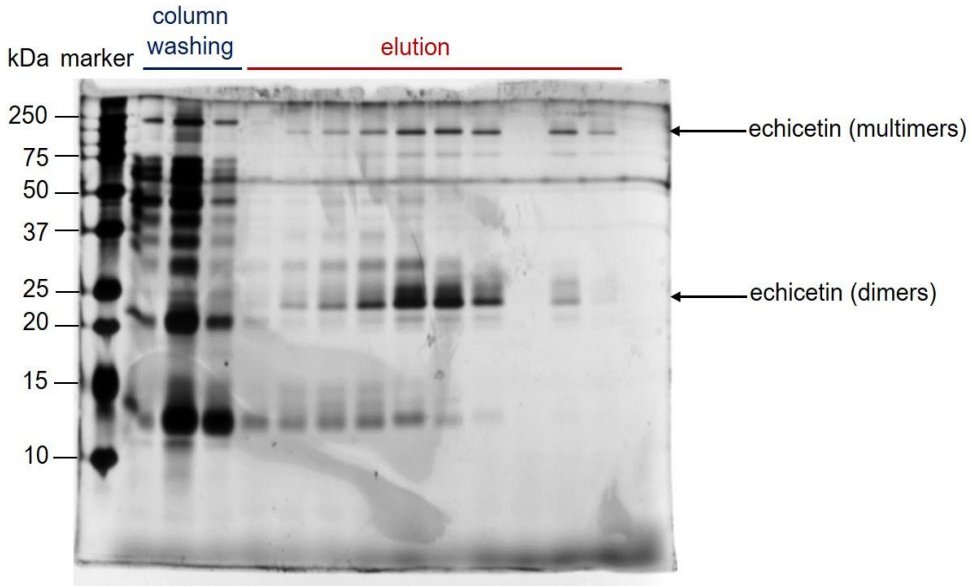

Figure 9. Qualitative validation of echicetin isolation by SDS-PAGE and silver staining.
Samples from the different steps (during column washing and elution) of the affinity chromatography were taken and analyzed by silver staining using 15% acrylamide gel under non-reducing conditions.

The second part of the silver stained gel (Figure 9) shows the presence of an isolated protein in the eluent, which was not washed away. The approximate size of this band is around 25 kDa, fitting to echicetin size. In addition, another band was eluted at higher molecular weight (150 kDa) suggesting the presence of echicetin as multimers. However, some proteins were still present at 13, 30 and 75 kDa, therefore an additional purification step was required in order to eliminate those proteins.

In the next step, the eluent of the affinity chromatography was collected and applied on a weak anion exchange DEAE column. A gradient of salt was applied, two major peaks appeared, the peak 1 starting at 62.5 mM NaCl and the peak 2 at 167 mM NaCl (corresponding to 6.25 and

16.7% of NaCl) (Figure 10). Fractions of each peak were collected together and buffer was exchanged by sterile PBS pH 7.4.

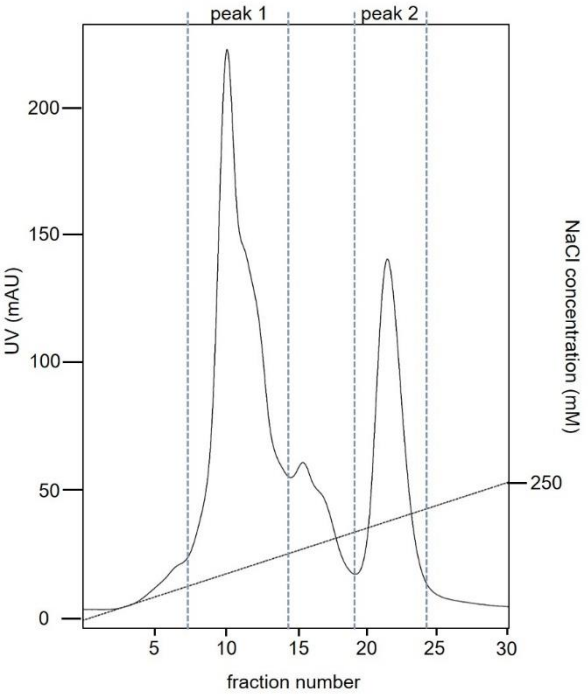

Figure 10. Elution profile of echicetin using anion exchange chromatography.
Echicetin isolated by affinity chromatography was again purified by anion exchange chromatography using DEAE column. Elution was performed with a gradient of NaCl, from 0 to 1 M at a flow rate of 1 ml/min. Two main peaks were separately eluted (P1 and P2) at around 62.5 mM and 167 mM NaCl, respectively.

Samples were checked again under non-reducing and reducing conditions using the silver staining method (Figure 11).

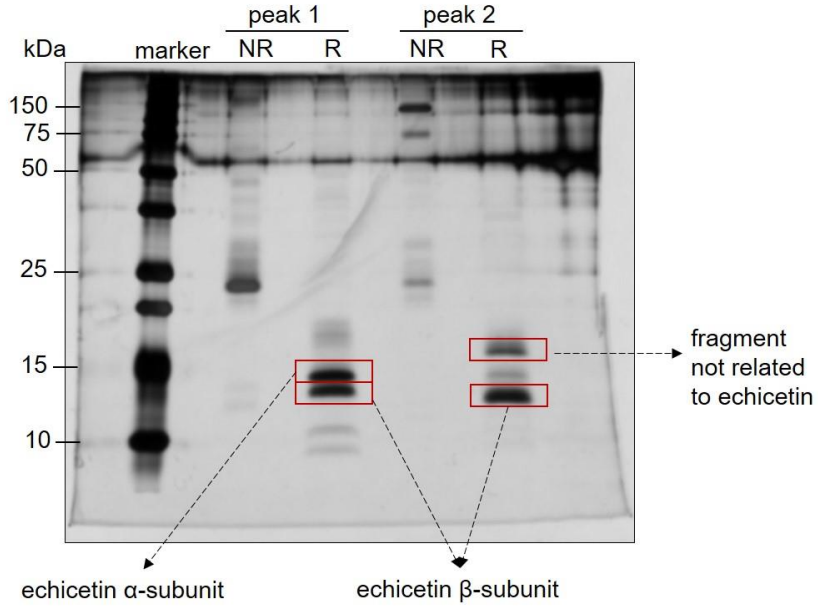

Figure 11: Qualitative validation of echicetin after the anion exchange chromatography and MS-analysis.

Samples from P1 and P2 were analyzed by silver staining under non-reducing (NR) and reducing conditions (R) in a 15% SDS-gel. Bands detected under reducing conditions were cut from the gel, digested using trypsin, and analyzed by MS-analysis. The upper and lower bands of peak 1 were identified under the uniprot IDs: P81017 (echicetin α-subunit) and P81996 (echicetin β-subunit), respectively. However, the upper band of peak 2 was not related to echicetin and the lower band was identified under the uniprot ID: P81996 (echicetin β-subunit).

Peak 1 shows under native conditions one main band at 25 kDa and two other bands at 50 and 150 kDa. The reduced form of those proteins shows two bands at 14 and 16 kDa, which fits exactly with the echicetin properties. Peak 2 shows under non-reduced conditions a main band at around 150 kDa and 2 minor bands at 75 and 100 kDa. The reduced form of this sample shows two major bands at 14 and 17 kDa.

5.1.2 Western blot

To identify bands detected in the silver stained gel, which represent echicetin, western blot analysis was performed using a polyclonal rabbit antibody against echicetin. The reduced form of the peak 1 confirmed the two subunits of echicetin, which means that under non-reducing conditions echicetin is present as monomer (25 kDa), dimer (50 kDa) and multimer (150 kDa).

In peak 2, polyclonal antibodies against echicetin recognized 4 bands under non-reducing conditions (25, 50, 100 and 150 kDa). However, under reducing conditions, echicetin antibodies identified only the lower band (Figure 12). This result shows that the peak 2 contains echicetin but also different proteins.

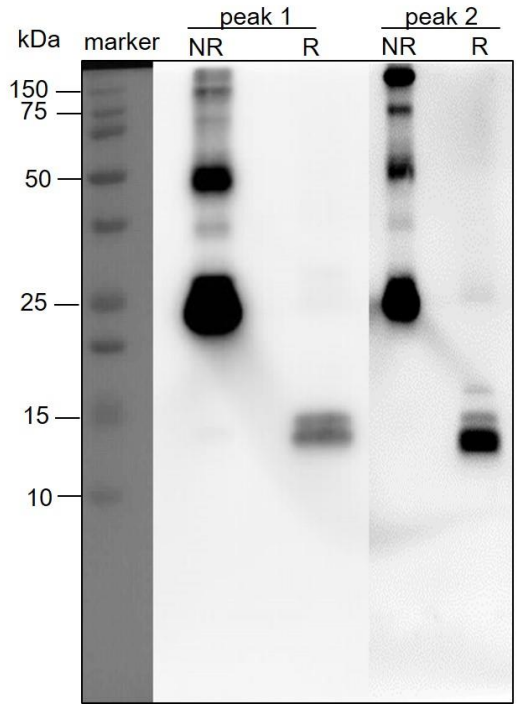

Figure 12: Western blot analysis of peak 1 and peak 2.
The same samples analyzed with silver staining (Figure 11) were checked by Western blotting (15% SDS gel) using polyclonal rabbit antibodies against echicetin. Both sample peaks were studied under non-reducing (NR) and reducing conditions (R).

5.1.3 Mass spectrometry analysis

Polyclonal antibodies may be unspecific and may cross react with different proteins. Therefore, reduced proteins in the peak 1 and 2 were analyzed by mass spectrometry to identify the sequence of the found peptides (Table 4). Proteins of both peaks were separated by electrophoresis using 15% SDS-PAGE gels. Gels were stained using InstantBlue™. Bands in the reduced sample lanes (Figure 11) were cut from the gel and digested using trypsin. Protein sequences were analyzed by mass spectrometry in the mass spectrometry core facility of the group of Prof. Stefan Tenzer at the University Medical Center of the Johannes Gutenberg University, Mainz. The analysis shows that the upper band of peak 1 corresponds to the α-subunit of echicetin and the lower band to the β-subunit (Figure 13). Furthermore, the lower band of peak 2 was identified as the β-subunit by MS whereas the upper band (not echicetin) was matched into two different sequences different from echicetin sequence (Figure 14) and from the *Echis carinatus sochureki* species, the small band in between refers to the α-subunit of echicetin similar to peak 1. According to these data, the highly enriched proteins from peak 1, which have an equal proportion of α and β subunits of echicetin were used in this study.

Table 4. Mass spectrometry data of the protein sequences in peak 1 (P1) and peak 2 (P2)
*percent identity calculated using protein BLAST program.

		Protein accession numbers	**Name**	**Species**	**Identity %***
P1	upper band	sp|P81017|SLA_ECHCS	Snaclec echicetin subunit alpha OS=Echis carinatus sochureki PE=1 SV=1	ECHCS	99%
	lower band	sp|P81996|SLB_ECHCS	Snaclec echicetin subunit beta OS=Echis carinatus sochureki PE=1 SV=1	ECHCS	96%
P2	upper band	tr|A0A0A1WDW9|A0A0A1WDW9_ECHCO (sequence A in Figure 14)	C-type lectin J OS=Echis coloratus PE=2 SV=1	ECHCO	45%
		tr|V8N8G6|V8N8G6_OPHHA (sequence B in Figure 14)	uncharacterized protein (fragment) OS=Ophiophagus hannah GN=L345_16333 PE=3 SV=1	OPHHA	33.33%
	lower band	sp|P81996|SLB_ECHCS	Snaclec echicetin subunit beta OS=Echis carinatus sochureki PE=1 SV=1	ECHCS	98.37%

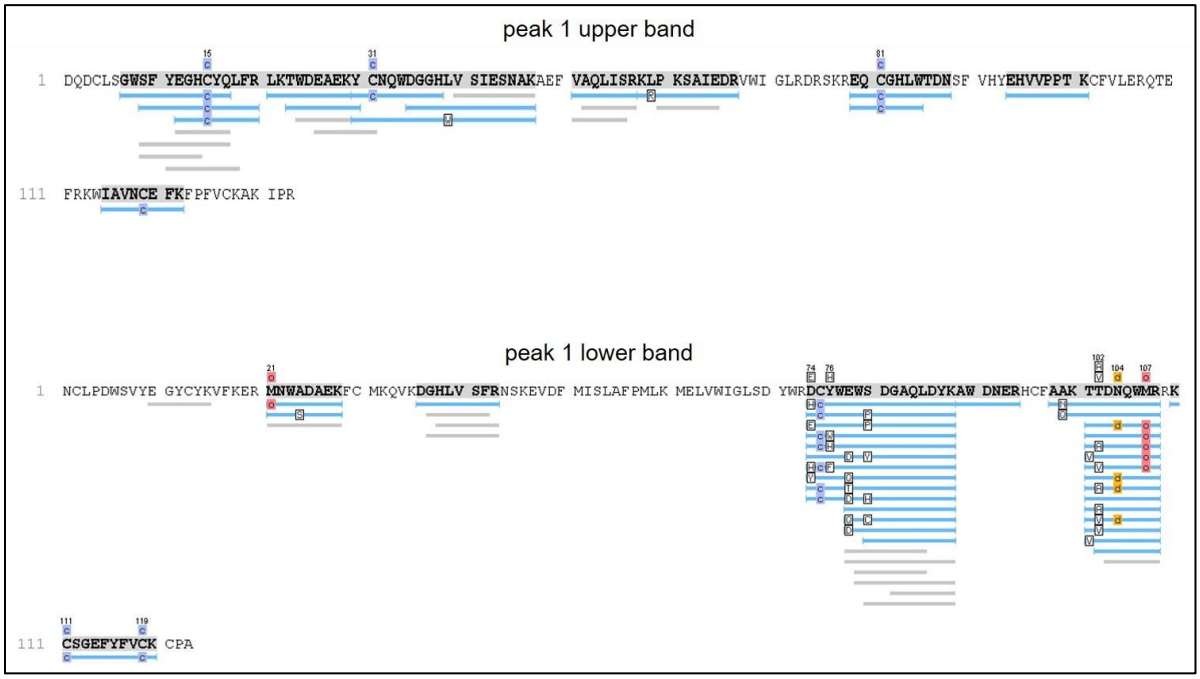

Figure 13. Sequence alignment results of peak 1.
Sequences of peak 1, upper and lower bands shown the silver stained gel in Figure 11, are compared to the sequence of echicetin.

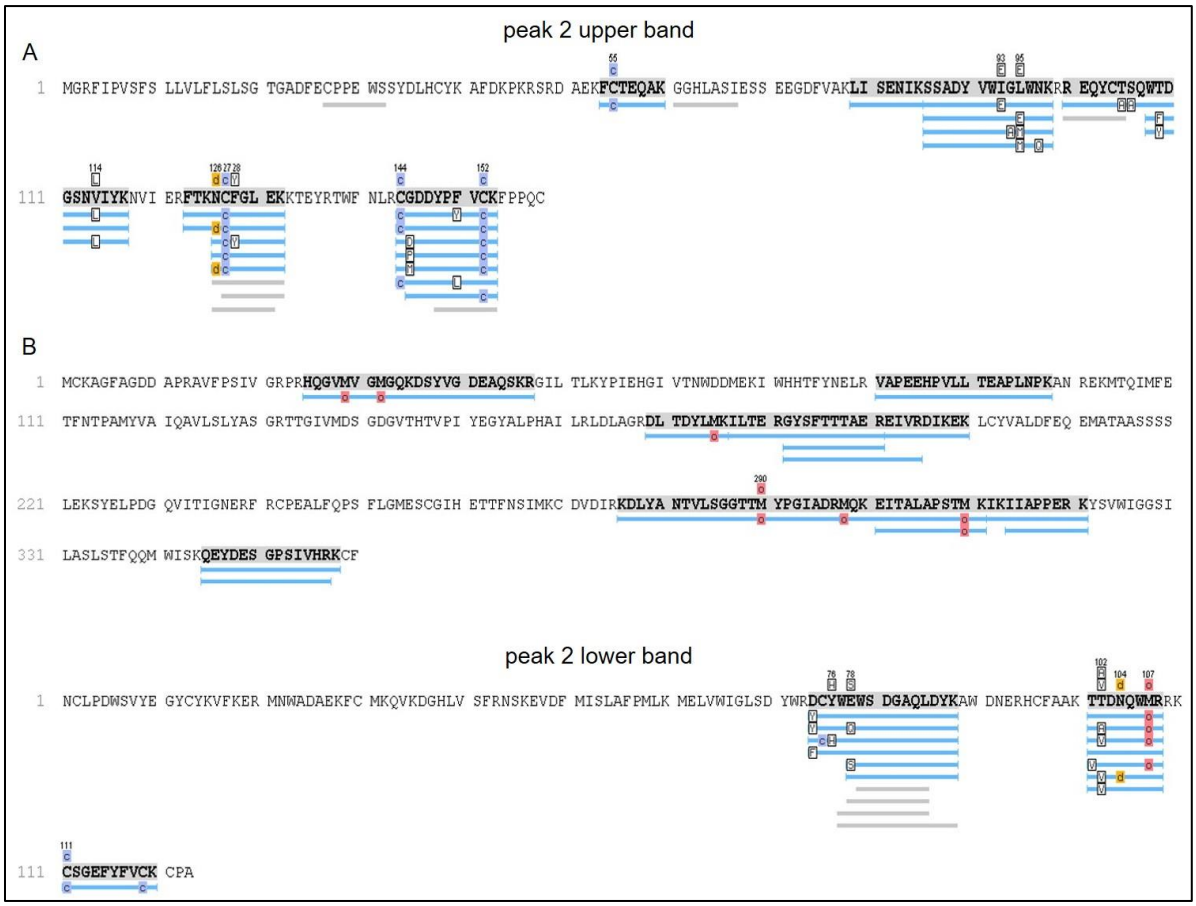

Figure 14. Sequence alignment results of peak 2.

Sequences of peak 2, upper and lower bands shown the silver stained gel in figure 11 are compared to the sequence of echicetin. Peak 2 has two different matches which are both different from echicetin sequence.

5.2 Functional validation of purified echicetin

5.2.1 Effect of echicetin on vWF-mediated agglutination of washed platelets

After the biochemical characterization of echicetin, the aim was to study the functional aspect of the protein. Therefore, washed human platelets were preincubated for 3 minutes with 10 µg/ml of echicetin from peak 1 or peak 2 prior to stimulation with vWF/ristocetin. Echicetin binds selectively to GPIbα, competing the binding of the vWF to this receptor. In Figure 15, echicetin from peak 1 inhibited completely the platelet agglutination induced by GPIbα/vWF binding. Peak 2 containing mainly the β-subunit of echicetin inhibited also the binding of vWF to the GPIbα receptor.

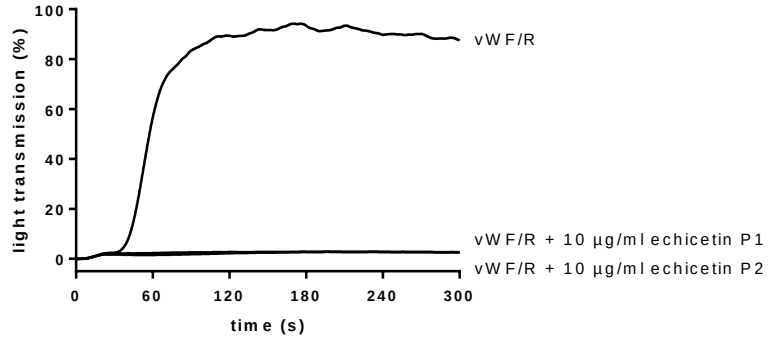

Figure 15. Functional validation of purified echicetin.
Washed human platelets were preincubated with purified echicetin, derived from peak 1 or peak 2 (10 µg/ml, 3 min) at 37°C prior to stimulation with vWF/ristocetin.

These data demonstrate that the purified echicetin competitively binds to GPIbα as also reported in earlier studies[126, 136].

5.2.2 Effect of echicetin on GPVI- and Gq-coupled receptor-mediated platelet aggregation

Furthermore, the possible effect of echicetin on other platelet receptors was investigated. Echicetin as purified protein, called echicetin monomer (EM), showed no effect on collagen-induced platelet aggregation (GPVI-mediated activation) (Figure 16A, D). Additionally, echicetin did not affect ADP-($P2Y_1$, $P2Y_{12}$ receptors) or TxA_2-(TP receptors) mediated platelet aggregation (Figure 16B, C, E, F).)

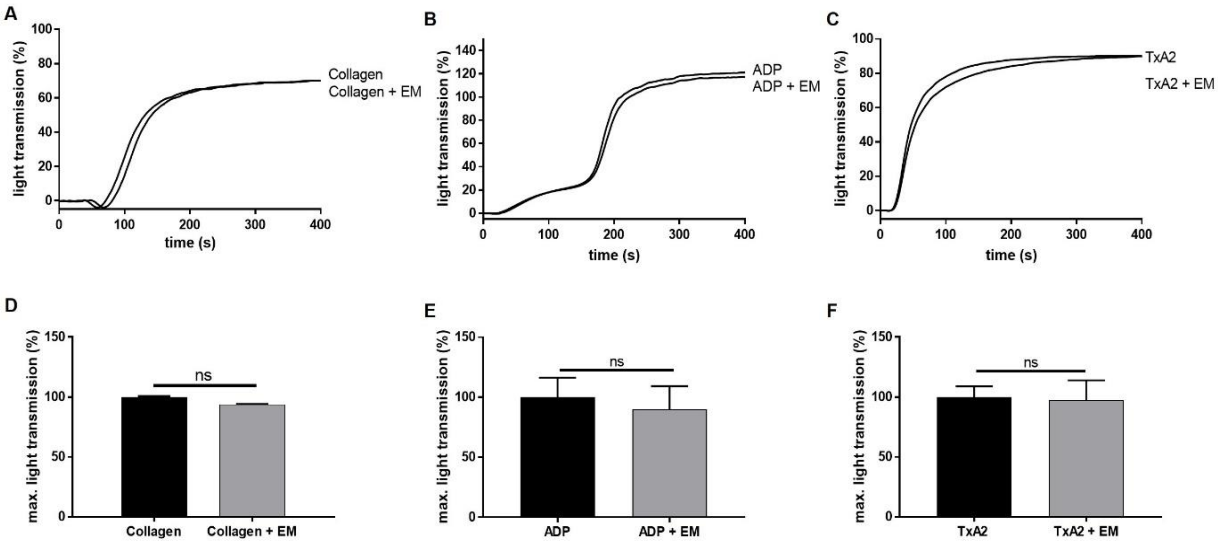

Figure 16. Echicetin monomers do not affect aggregation induced by GPVI- or by ADP/TxA_2- receptors. WP were preincubated with 25 µg/ml echicetin monomer for 3 minutes before stimulating with (A) collagen (3 µg/ml), (B) ADP (1.75 µM), (C) TxA_2 (60 nM). Quantitative data are means ± SD for collagen (D), ADP (E), TxA_2 (F). Data are presented from 3 different experiments with platelets from 3 different healthy donors. Statistical analysis was performed using student t-test (p> 0.05, n.s).

Thrombin activates human platelets via the G-coupled receptors PAR-1 and PAR-4 as well asat low concentrations via GPIbα[11, 146]. Therefore, the effect of echicetin on thrombin-induced platelet activation was tested. Echicetin did not show any effect on the thrombin receptor activator peptide 6 (TRAP-6)-induced platelet aggregation, which is mediated via PAR-1 (Figure 17A, D). As well, echicetin did not affect platelet aggregation mediated by a high thrombin concentration (0.1 U/ml) (Figure 17B, E). However, only at low dose of thrombin (0.025 U/ml), a small inhibition of aggregation was seen (Figure 17C, F).

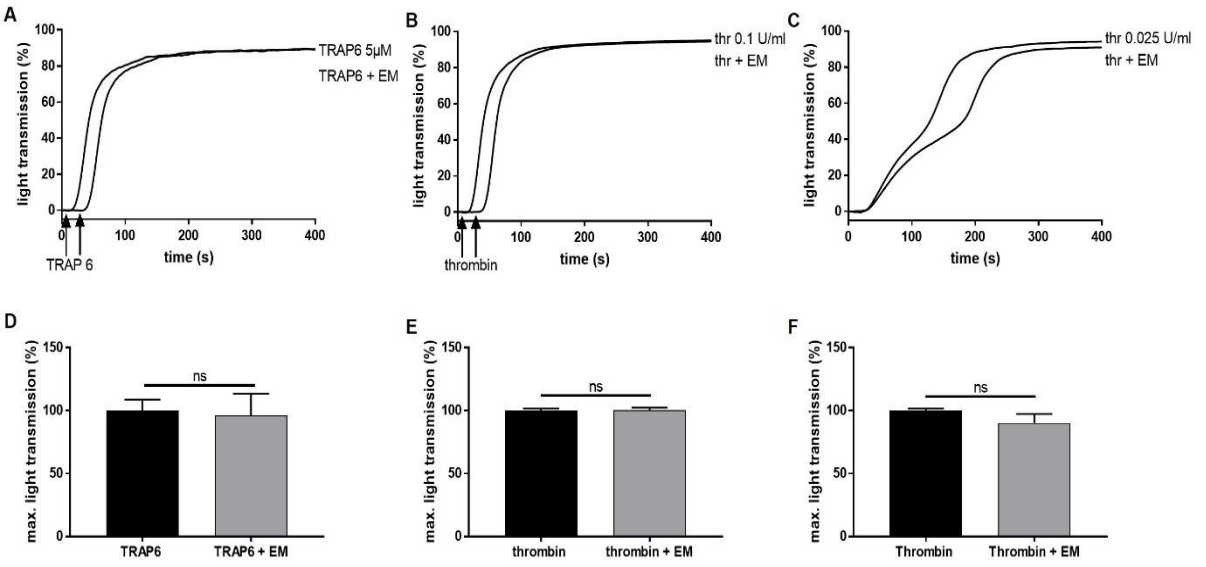

Figure 17. Echicetin monomers (EM) do not affect aggregation induced by the thrombin receptor PAR-1.

WP were preincubated with 25 µg/ml echicetin monomer for 3 minutes before stimulating with (A) TRAP-6 (5 µM/ml), or high (B) or low (C) concentrations of thrombin. Quantitative data are means ± SD for TRAP-6 (D), thrombin (0.1U/ml) (E), thrombin (0.025U/ml) (F). Data are presented from 3 different experiments with platelets from 3 different healthy donors. Statistical analysis was performed using student t-test (p> 0.05, n.s).

5.3 Investigation of GPIbα-mediated platelet signaling using echicetin beads

5.3.1 EB induce GPIbα-specific platelet aggregation dependent on the $\alpha_{IIb}\beta_3$ integrin activation

For an additional proof of echicetin specificity, we had the opportunity to use a murine model in which the extracellular domain of platelet GPIbα was replaced by the human interleukin-4 receptor (IL4R). These mice were provided by the group of Prof. Wolfram Ruf (CTH, Mainz), and these experiments were performed by Dr. med. vet. Katharina Trabold during her dissertation. This murine model was used to validate the specificity of EB for GPIbα (Figure 18). EB induced full platelet aggregation with platelets from WT mice, however, no effect was shown in the IL4R/GPIbα-chimera[95]. Furthermore, EM showed a similar effect as in human platelets, they bound competitively to the GPIbα preventing any activation by EB.

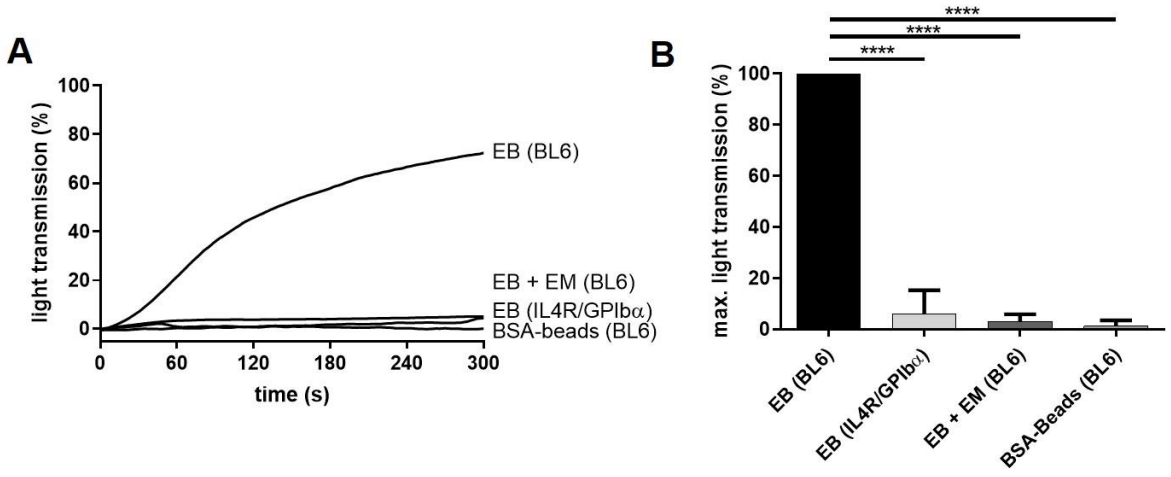

Figure 18. Echicetin beads (EB) do not induce platelet aggregation in washed IL4R/GPIbα murine platelets. Washed murine platelets (WP) from wildtype mice (C57BL6/J, BL6) or GPIbα KO mice (IL4R/GPIbα) were stimulated with EB or BSA-beads. WP from WT mice were preincubated with 25µg/ml EM prior to stimulation with EB. Platelet aggregation was monitored until 300s. (B) Results are normalized to 100% compared to EB (BL6). They are shown are represented as mean ± S.D of 3 different mice from each type (****p<0.0001)

To study the effect of EB on washed human platelets, light transmission was monitored using the LTA. EB induced a fast aggregation with a maximum of 70% aggregation (Figure 19). In contrast, beads coated with BSA did not induce platelet aggregation. Furthermore, platelets preincubated with tirofiban ($\alpha_{IIb}\beta_3$ inhibitor) did not show any aggregation in response to EB. These results indicate that EB induced full platelet activation, which is highly dependent on the integrin $\alpha_{IIb}\beta_3$ activation. In agreement with previous work[136], platelets incubated with echicetin

monomers (EM) did not show any activation after stimulation with EB. EM blocked GPIbα receptors preventing the binding of echicetin beads (EB) and subsequent aggregation.

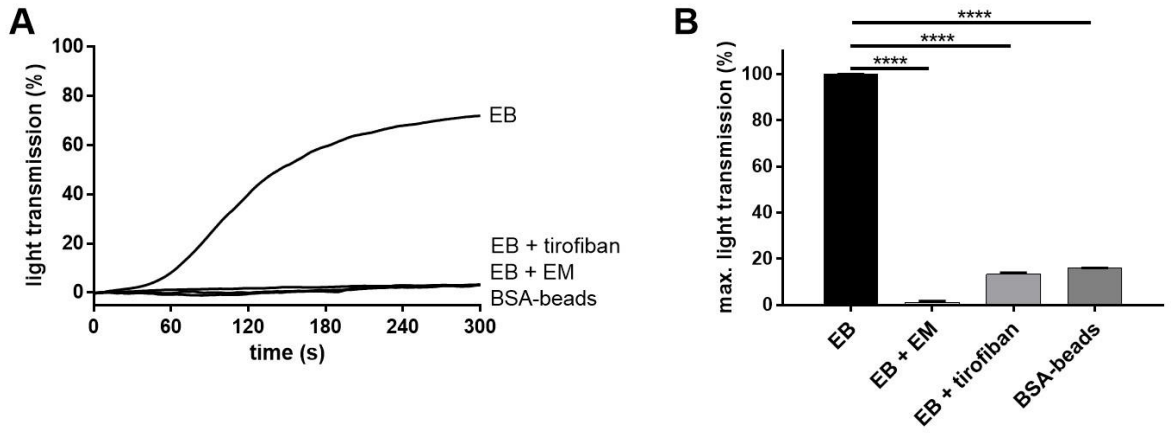

Figure 19. EB induce platelet aggregation in a $\alpha_{IIb}\beta_3$ dependent manner.
(A) Washed human platelets (WP) were stimulated under stirring conditions with EB or BSA-coated beads (as negative control). WP were preincubated with echicetin monomers (EM) (25µg/ml, 3min) or with tirofiban (1.25µg/ml, 1min) prior to stimulation with EB. Platelet aggregation was monitored until 300s. (B) Results are normalized to 100% compared to EB. They are shown are represented as mean ± S.D of 3 independent experiments with platelets from 3 healthy donors (****p<0.0001)

5.3.2 EB initiate Syk-mediated signaling downstream of GPIbα

Furthermore, the signaling pathway downstream of GPIbα that leads to $\alpha_{IIb}\beta_3$ activation has been investigated. Phosphorylation of the spleen tyrosine kinase Syk was studied in a time dependent manner by western blot, and data are representative of 3 independent experiments. Additionally, Syk downstream substrates/ effectors were studied to confirm Syk activity. Figure 20A, B shows the kinetics of Syk phosphorylation on Y352, Y525/526 and S297. A maximum of phosphorylation was reached within 1 minute and then the phosphorylation declined.

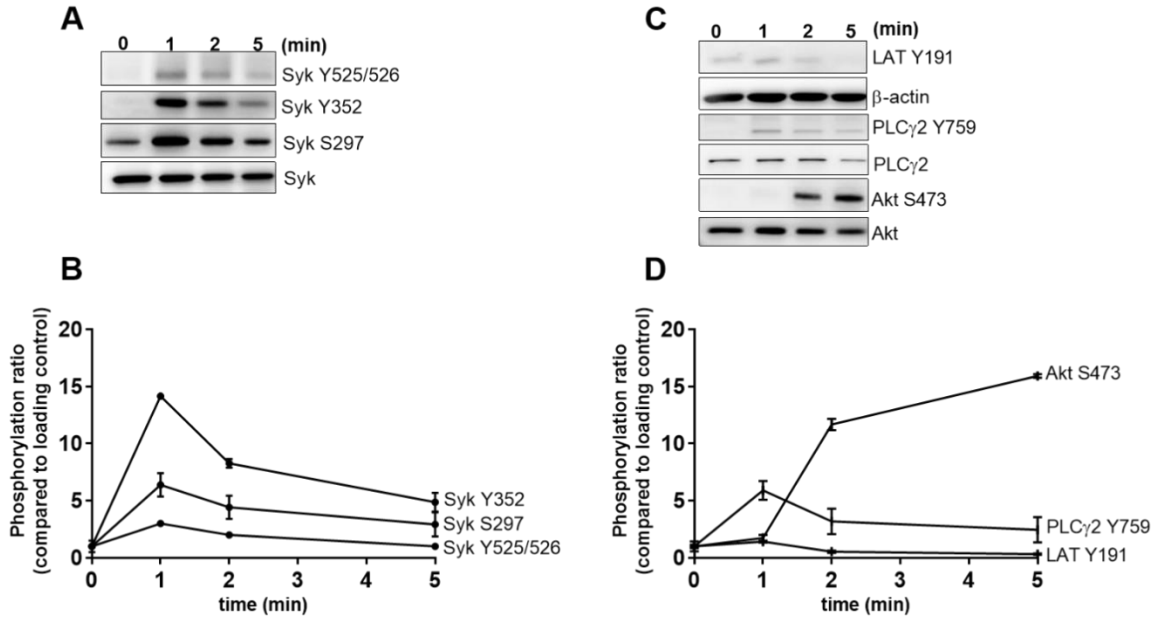

Figure 20. EB mediate the activation of the spleen tyrosine kinase and its downstream effectors.
EB-mediated platelet aggregation was stopped after 1, 2 or 5 minutes by adding Laemmli buffer. Platelets lysates were boiled for 10 minutes. Tyrosine and serine phosphorylation patterns were detected by western blot. (A and C) Phosphorylation profiles of Syk tyrosine sites (525/526 and 352), serine site (S297) and PLCγ2 Y759, LAT Y191 and Akt S473, respectively. (B and D) Data from at least 3 independent experiments with at least 3 different donors were analyzed compared the corresponding loading controls. They are represented as mean ±S.D.

The phosphorylation of the linker adaptor protein LAT on its tyrosine 191 was detectable but weak whereas phospholipase Cγ2 (PLCγ2) Y759 phosphorylation showed a similar time-dependent phosphorylation pattern as Syk. In contrast, one of Syk downstream effectors and important PI3K effectors, Akt, showed a more delayed phosphorylation pattern on S473 (Figure 20C, D) and confirms that Akt is an indirect Syk effector.

Phosphoproteomic data (Beck F. et al, 2017) showed that ADP stimulates Syk phosphorylation on S297 site in human platelets[147]. Therefore, I aimed to validate this phosphorylation in washed human platelets using immunoblotting (Figure 21). ADP induced Syk S297 phosphorylation in a time dependent manner, which declined after 4 to 5 minutes of stimulation.

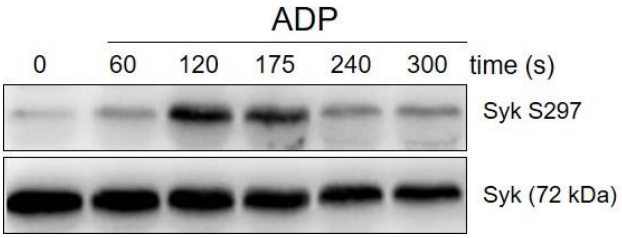

Figure 21. Platelet Syk S297 phosphorylation was detected in ADP-induced activation.
WP were stimulated with 25 µM ADP. Aggregation was stopped as indicated above by adding directly Laemmli buffer in the cuvettes after each indicated time points. Western blot analysis was performed using antibodies against Syk S297 or total Syk antibody, which is used as loading control. Blots are representative of 2 independent experiments performed using washed platelets from 2 healthy donors.

Therefore, it was of considerable interest to study not only the EB regulation of Syk tyrosine sites (Y525/526, Y352) but also the S297 site, which was not studied so far in platelets.

5.3.3 Effect of Src family kinases on EB-mediated platelet activation

In order to study the involvement of the SFKs in GPIbα-mediated platelet aggregation, two distinct SFK inhibitors PP2 and dasatinib were used. PP2, a non-ATP-competitive inhibitor of Src family kinase, potently inhibits Lck/Fyn at nanomolar ranges (IC50 of 4 nM/5nM in cell free assays) but it is 100-fold less potent with EGFR and inactive for ZAP-70, JAK2 and PKA[148, 149]. Dasatinib (Sprycel®) is a potent oral ATP-competitive inhibitor of tyrosine kinases. This drug is a second-generation BCR-ABL tyrosine kinase inhibitor, but also inhibits the Src family kinases (SFKs)[150].

5.3.3.1 Effect of PP2 and dasatinib on EB-induced platelet aggregation

First, the effect of SFKs inhibitors on EB-mediated platelet aggregation was checked. Washed human platelets were preincubated with 10 µM PP2 or 100 nM dasatinib for 5 minutes prior to treatment with EB. PP2 and dasatinib strongly inhibited platelet aggregation in response to EB (Figure 22).

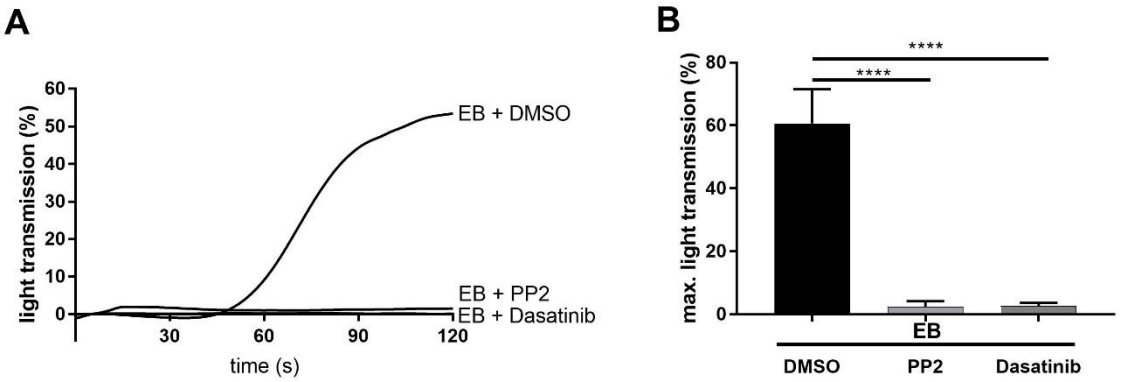

Figure 22. EB-induced platelet aggregation depends on src family kinases.
WP were preincubated for 5 minutes at 37°C with vehicle control (DMSO) or 10µM PP2 or 100 nM dasatinib prior to stimulation with EB. (A) representative aggregation curves of the effect of PP2 and dasatinib on platelet aggregation. Platelet aggregation was monitored until 120s.(B) Results are represented as mean ± S.D of 3 independent experiments with platelets from 3 healthy donors (****p<0.0001).

5.3.3.2 Effect of PP2 and dasatinib on tyrosine phosphorylation

First, I obtained a quick and general overview about the effect of SFKs in tyrosine phosphorylation induced by EB. Using a pan-tyrosine antibody, the general tyrosine phosphorylation pattern was analyzed (Figure 23). PP2 as well dasatinib strongly inhibited tyrosine phosphorylation mediated by EB.

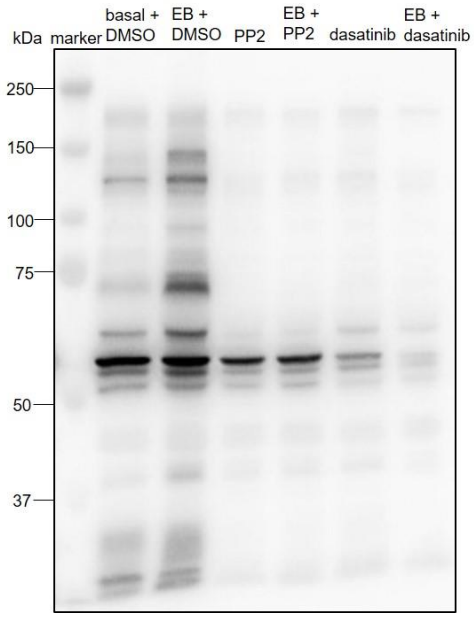

Figure 23. EB-mediated tyrosine phosphorylation strongly depends on SFKs.
WP were treated as mentioned previously with DMSO, PP2 or dasatinib prior to stimulation with EB. Platelet aggregation was stopped after 1 minute by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C for 10 minutes. General tyrosine phosphorylation was analyzed by western blot using a pan-tyrosine antibody.

5.3.3.3 Effect of tyrosine kinase inhibitors (TKIs) on platelet aggregation induced by different platelet agonists and their effect on EB-mediated Syk phosphorylation

To validate the role of tyrosine kinases downstream of the different platelet receptors, the effect of their inhibition was studied on the GPIbα, GPVI, $P2Y_1$ and $P2Y_{12}$ –mediated platelet aggregation in platelet-rich plasma (PRP). Therefore, two different tyrosine kinase inhibitors (TKIs), imatinib and dasatinib, were used. These commercially available drugs were initially used to treat patients with chronic myeloid leukemia (CML). Imatinib (Gleevec®, Glivec®) is a selective but relatively weak inhibitor of the bcr-abl (the Abelson proto-oncogene) tyrosine kinase, c-kit (proto-oncogene c-kit or tyrosine kinase Kit) and PDGF-R (platelet-derived growth factor)[151]. Dasatinib (Sprycel®), compared to imatinib, inhibits additionally the SFKs in platelets[150].

As first part, GPIbα-mediated platelet aggregation in PRP was checked by adding 1 mg/ml ristocetin in the presence or absence of the TKIs (Figure 24). Ristocetin induced full platelet aggregation in PRP represented in a small agglutination (first wave) and followed by the aggregation (second wave). These two waves are clearer in the presence of the vehicle control and imatinib. There was no significant effect of imatinib on the overall ristocetin-mediated platelet aggregation (Figure 24A, C). However, dasatinib inhibited significantly both waves of platelet aggregation (Figure 24A, B). As positive control, tirofiban andintegrin GPIIb/IIIa inhibitor, was used to distinguish the vWF/GPIbα-mediated platelet agglutination phase, which cannot be inhibited by tirofiban.

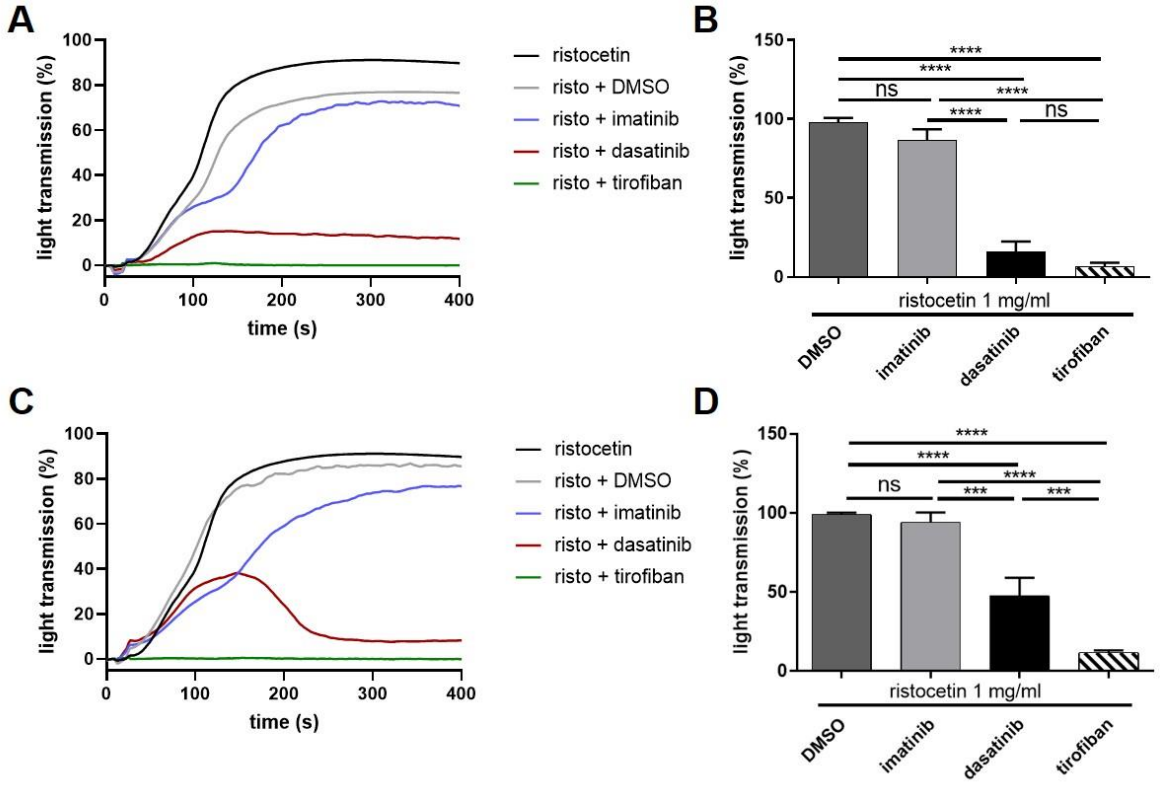

Figure 24. The tyrosine kinase inhibitor, dasatinib, inhibits GPIbα-mediated platelet aggregation in PRP. Platelets preincubated with DMSO (0.1%), imatinib (1.5 µM) or dasatinib (400 nM) for 5 minutes at 37°C prior to stimulation with 1 mg/ml ristocetin (representative curves A and C). Platelet aggregation was monitored until 400s. (B and D) Results are normalized to 100% compared to ristocetin plus DMSO. They are represented as mean ± S.D of 3 independent experiments with platelets from 3 healthy donors (***p<0.001, ****p<0.0001, p>0.5 ns. not significant)

Interestingly, a donor-dependent variability of the effect of dasatinib was observed (Figure 24A/B vs. C/D). For some healthy donors, dasatinib inhibited only the second wave of ristocetin-mediated platelet aggregation (Figure 24C, D).

Furthermore, the effect of TKIs on GPVI-mediated platelet aggregation was investigated by stimulating platelets with 5 µg/ml collagen. Imatinib did not show any significant effect on platelet aggregation compared to control. However, dasatinib inhibited completely platelet aggregation but not the shape change (Figure 25A, B).

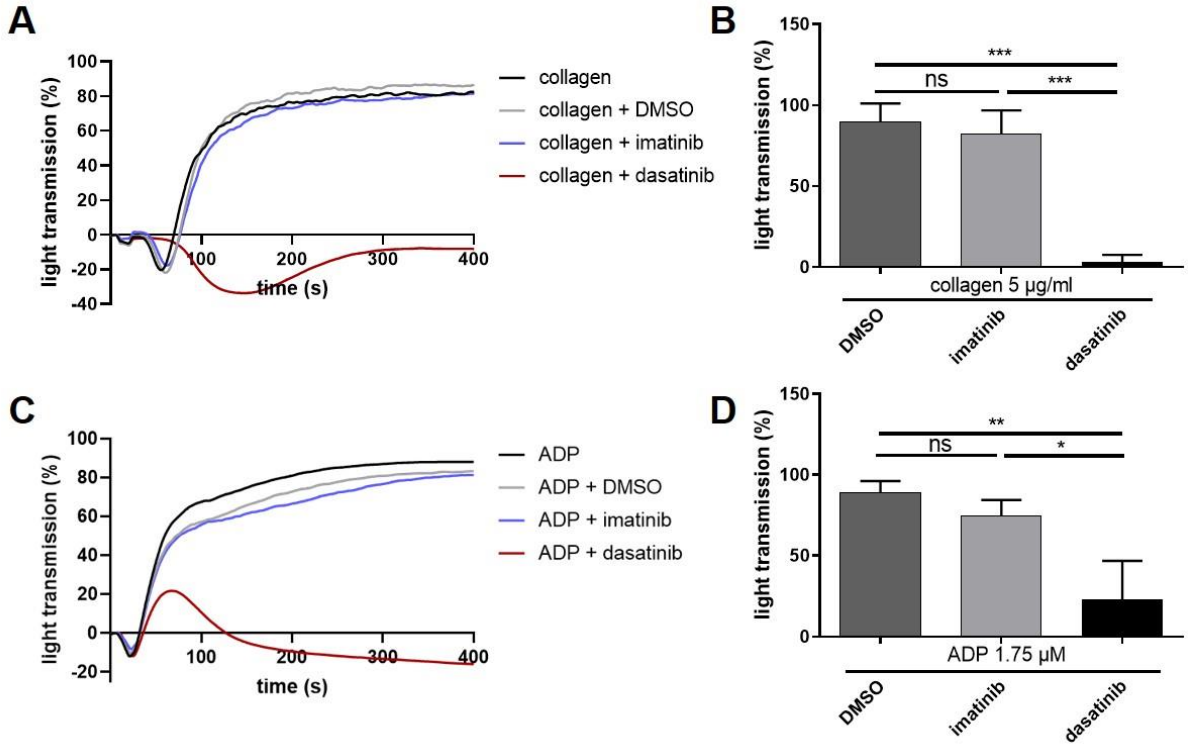

Figure 25. The tyrosine kinase inhibitor, dasatinib, inhibits GPVI and $P2Y_1$/$P2Y_{12}$-mediated platelet aggregation in PRP.
Platelets preincubated with DMSO (0.1%), imatinib (1.5 µM) or dasatinib (400 nM) for 5 minutes at 37°C prior to stimulation with 5 µg/ml collagen (A) or 1.75 µM ADP (C). Platelet aggregation was monitored until 400s. (B and D) Results are normalized to 100% compared to ristocetin plus DMSO. They are represented as mean ± S.D of 3 independent experiments with platelets from 3 healthy donors (*p<0.05, **p<0.01, ***p<0.001, ns. not significant)

Finally, the effect of the TKIs on $P2Y_1$ and $P2Y_{12}$ receptors was studied by stimulating platelets with 1.75 µM ADP. Similarly to the data with collagen, only dasatinib showed a significant inhibition (disaggregation, only first wave of aggregation) of platelet aggregation compared to control (Figure 25C, D).

Compared to other tyrosine kinases, these data demonstrate the essential role of the SFKs downstream of the main platelet receptors.

In order to confirm the role of SFKs in the observed EB-mediated Syk activation (see paragraph 5.3.2), the phosphorylation of Syk on its tyrosine sites (525/526 and 352) was analyzed. Figure 26 shows that PP2 and dasatinib abolished Syk tyrosine phosphorylation time-dependently.

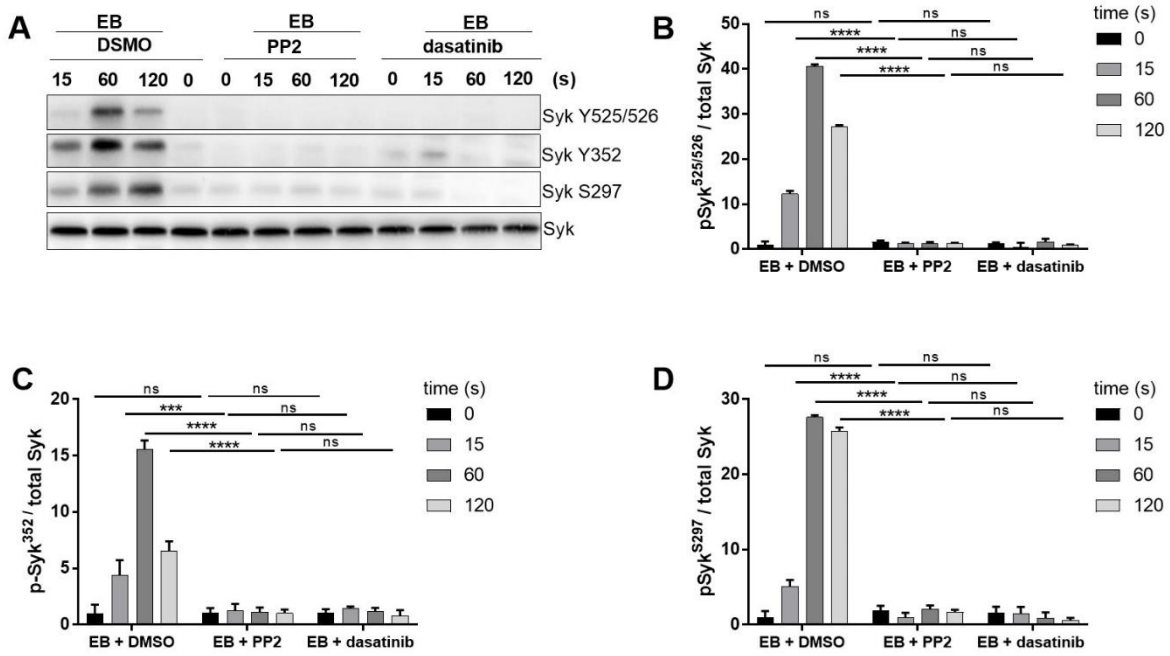

Figure 26. Syk tyrosine phosphorylation is dependent on SFKs.
WP were treated as mentioned previously with DMSO, PP2 or dasatinib prior to stimulation with EB. Platelet aggregation was stopped after 15, 60 or 120 seconds by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C for 10 minutes. (A) Western blot analysis was performed using antibodies against Syk Y525/526, Y352 and S297. Quantitative analysis of Syk Y525/526 (B), Y352 (C) and S297 (D) are from at least 3 independent experiments with platelets from at least 3 different donors. They were analyzed compared to the total Syk and represented as mean ±S.D. ns: not significant, ***p<0.001, ****p<0.0001.

5.3.4 Effect of Syk inhibition on EB-mediated platelet activation

5.3.4.1 Effect of OXSI-2 and PRT318 on EB-induced platelet aggregation

To investigate the role of Syk in EB-mediated platelet activation, platelets were preincubated with two structurally different Syk inhibitors, inhibiting the Syk kinase activity in an ATP-competitive manner[152-154]. First, OXSI-2, which has a dose-dependent effect on EB-induced platelet aggregation (Figure 27A). OXSI-2 only at concentration of 1 µM or higher inhibited platelet aggregation and granule secretion[153]. Second, PRT318, another Syk inhibitor that shows higher specificity for Syk inhibition[153, 154].PRT inhibited in dose dependent manner platelet aggregation and shape change (Figure 27B). For further experiments, washed platelets were preincubated with 2 µM OXSI-2 and 1 µM PRT prior to stimulation with EB. Platelet aggregation was completely inhibited by both inhibitors (Figure 28).

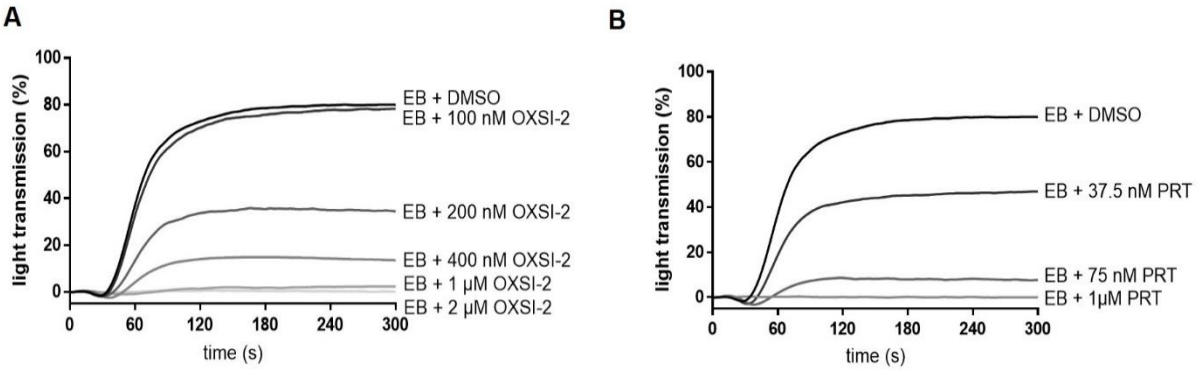

Figure 27. OXSI-2 and PRT318 inhibit platelet aggregation in a dose dependent manner.
WP were preincubated for 5 minutes with vehicle control (DMSO) or increased concentrations of OXSI-2 (A) or PRT318 (B), respectively prior to stimulation with EB. Platelet aggregation was monitored by light transmission aggregometry (LTA).

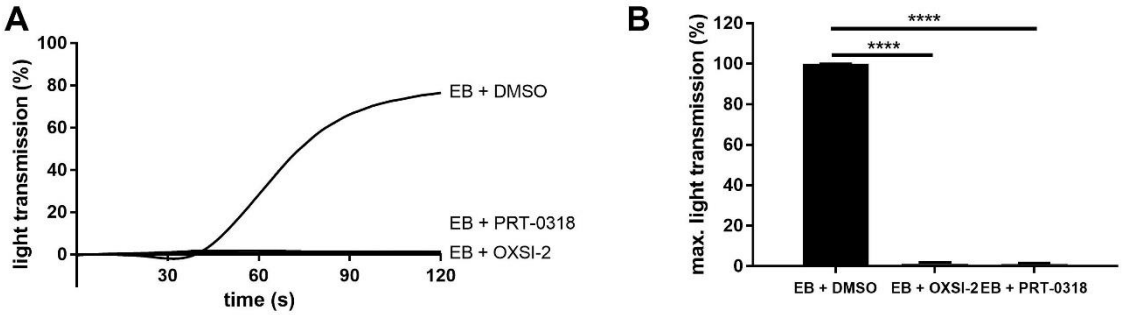

Figure 28. Syk inhibition abolished EB-induced platelet aggregation.
WP were preincubated for 5 minutes at 37°C with vehicle control (DMSO) or 2µM OXSI-2 or 1µM PRT318 prior to stimulation with EB. (A) representative aggregation curves of the effect of both Syk inhibitors on platelet aggregation. Platelet aggregation was monitored until 120s.(B) Results are normalized to 100% compared to EB and represented as mean ± S.D of 3 independent experiments with platelets from 3 healthy donors (****p<0.0001).

5.3.4.2 Effect of Syk inhibition on EB-stimulated tyrosine phosphorylation

Additionally, the general tyrosine phosphorylation pattern was checked in the presence and absence Syk inhibitors. Clearly, Syk inhibition prevented most of the tyrosine phosphorylation induced by EB (Figure 29).

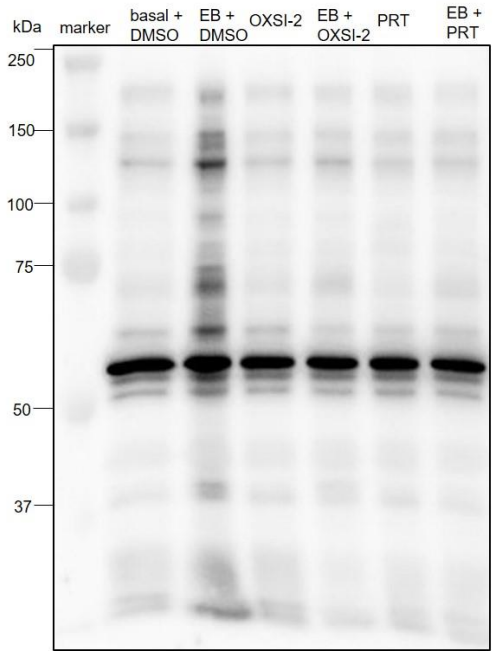

Figure 29. EB-induced tyrosine phosphorylation is dependent on Syk activation.
WP were treated as mentioned previously with DMSO, OXSI-2 or PRT318 prior to stimulation with EB. Platelet aggregation was stopped after 1 minute by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C for 10 minutes. General tyrosine phosphorylation was analyzed by western blot using a pan-tyrosine antibody.

5.3.4.3 Effect of OXSI-2 and PRT318 on EB-induced Syk phosphorylation

Furthermore, the kinetics of Syk phosphorylation at early stages (0, 15, 60 and 120 s) were analyzed. Syk Y525/526 was significantly inhibited by both inhibitors. However, Syk Y352 phosphorylation was not inhibited by OXSI-2 but rather it showed a prolonged phosphorylation. And pretreatment of platelets with PRT318 resulted in significantly enhanced and prolonged Y352 phosphorylation (Figure 30). In contrast, both Syk inhibitors strongly reduced EB-stimulated Syk S297 phosphorylation (Figure 30A and D)

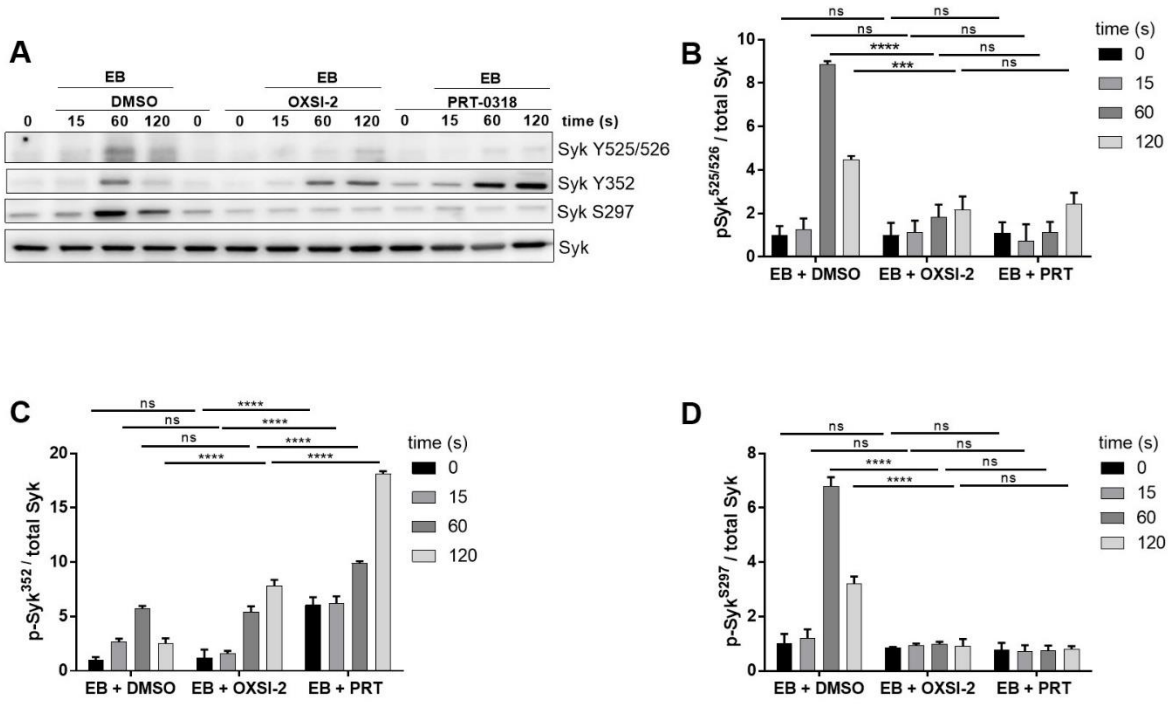

Figure 30. Syk inhibition differently affect the phosphorylation pattern of Syk itself.
WP were treated as mentioned previously with DMSO, OXSI-2 or PRT318 prior to stimulation with EB. Platelet aggregation was stopped after 15, 60 or 120 seconds by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C for 10 minutes. (A) Western blot analysis was performed using antibodies against Syk Y525/526, Y352 and S297. Quantitative analysis of Syk Y525/526 (B), Y352 (C) and S297 (D) are from at least 3 independent experiments with platelets from at least 3 different donors. They were analyzed compared to the total Syk and represented as mean ±S.D. ns: not significant, ***p<0.001, ****p<0.0001.

Furthermore, the phosphorylation of some of Syk downstream effectors was investigated in presence or absence of Syk inhibitors. Phosphorylation of PLCγ2 Y759 and Akt S473 was significantly inhibited by Syk inhibitors (Figure 31).

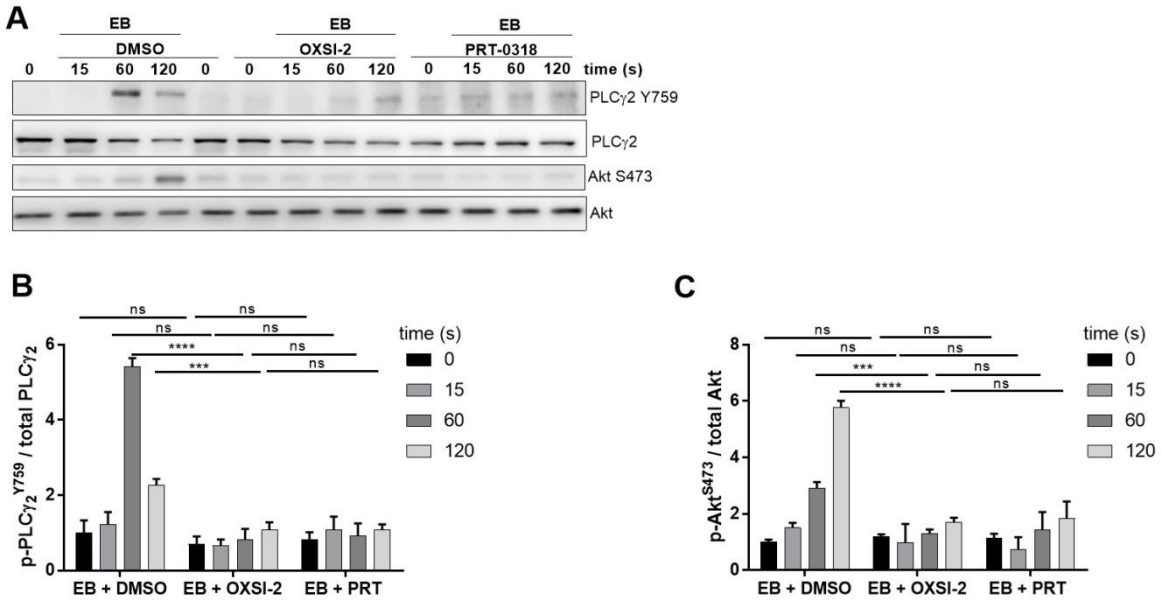

Figure 31. The phosphorylation of PLCγ2 Y759 and Akt S473 is tightly dependent on Syk activation. WP were treated as mentioned previously with DMSO, OXSI-2 or PRT318 prior to stimulation with EB. Platelet aggregation was stopped after 15, 60 or 120 seconds by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C for 10 minutes. (A) Western blot analysis was performed using antibodies against PLCγ2 Y759 and Akt S473. Quantitative analysis of PLCγ2 Y759 (B) and Akt S473 (C) are from at least 3 independent experiments with platelets from at least 3 different donors. They were analyzed compared to the corresponding total protein and represented as mean ±S.D. ns: not significant, ***p<0.001, ****p<0.0001.

5.3.5 Role of secondary mediators on EB-induced platelet activation

5.3.5.1 Effect of blockage of ADP and TxA_2 receptors on EB-induced platelet aggregation

Platelet adhesion receptor pathways variably require the activation-induced release of secondary mediators such as ADP and TxA_2 for a full response [155]. Therefore, the involvement of ADP and TxA_2 in EB-induced platelet aggregation was evaluated by simultaneously blocking of $P2Y_{12}$, $P2Y_1$ and TP receptors using AR-C69931, MRS2179 and SQ-29548, respectively. These conditions completely prevented platelet aggregation induced by EB (Figure 32).

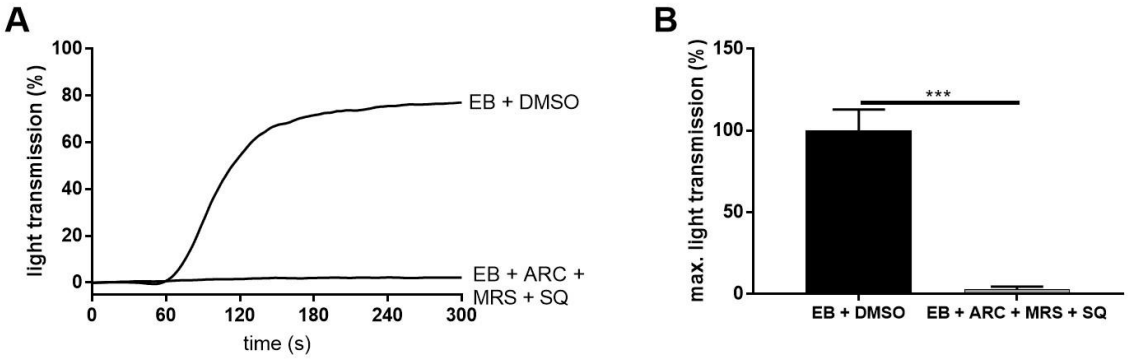

Figure 32. ADP and TxA_2 regulates EB-induced platelet aggregation.

WP were preincubated for 5 minutes at 37°C with vehicle control (DMSO) or simultanously with $P2Y_{12}$ antagonist, AR-C69931 (0.1 µM), $P2Y_1$ antagonist, MRS2179 (1 µM) and TxA_2 receptor antagonist, SQ-29548 (1 µM) prior to stimulation with EB. (A) representative aggregation curves of platelet aggregation mediated by EB. Platelet aggregation was monitored until 120s.(B) Results are normalized to 100% compared to EB and represented as mean ± S.D of 3 independent experiments with platelets from 3 healthy donors ($p<0.001$).

5.3.5.2 Effect of blockage of ADP and TxA_2 receptors on EB-mediated signaling pathway

Moreover, the phosphorylation responses of Syk stimulated by EB were analyzed. Western blot data showed that the phosphorylation of Syk on Y525/526 was only partially inhibited, and after 5 minutes of activation this phosphorylation was significantly enhanced (Figure 33A, B). In contrast, the phosphorylation of Syk on tyrosine 352 showed no significant changes in presence of these compounds compared to the activation pattern at earlier time points (1 and 2 minutes) and even an enhancement of the phosphorylation after 5 minutes. These data show that Y352 phosphorylation is not inhibited by ADP and TxA_2 receptors antagonists (Figure 33A, C). Furthermore, Syk S297 phosphorylation was significantly reduced by these inhibitors until 2 minutes of activation but some S297 phosphorylation was still detectable after this time (Figure 33A, D).

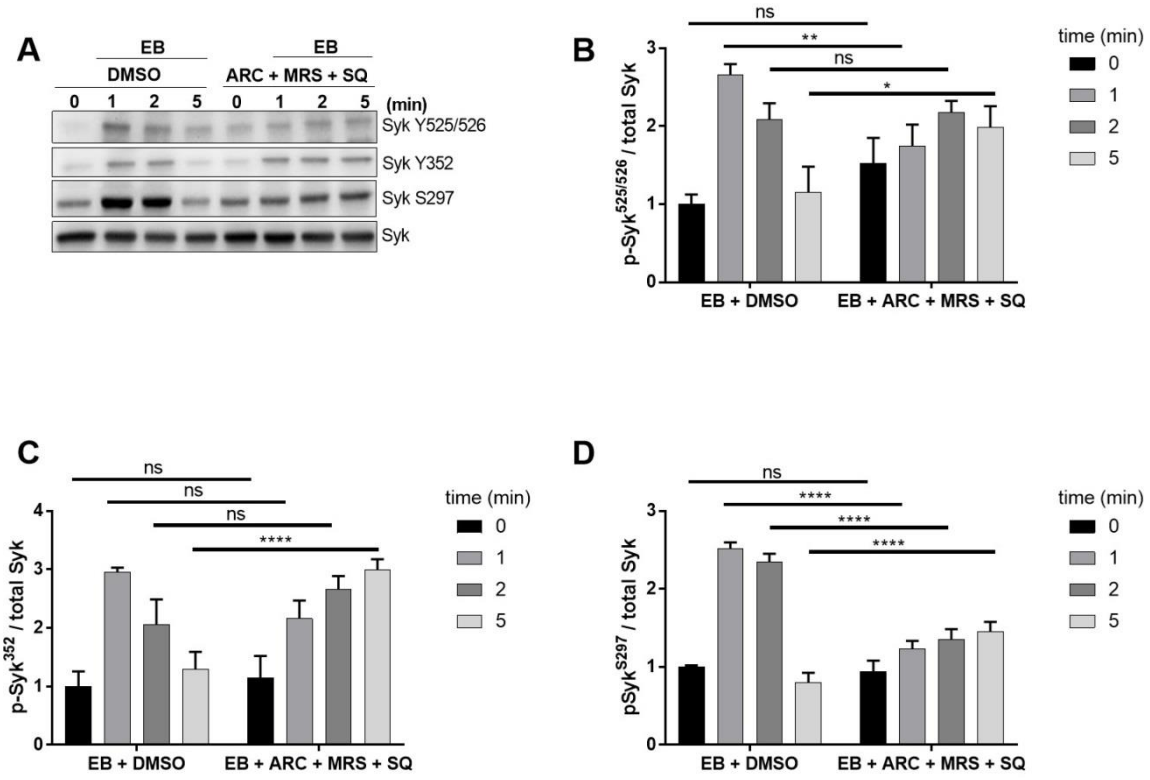

Figure 33. Syk activation is partially dependent on ADP and TxA_2.
WP were treated as mentioned previously with DMSO or simultanously with ADP and TxA_2 receptors antagonists prior to stimulation with EB. Platelet aggregation was stopped after 1, 2 or 5 minutes by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C for 10 minutes. (A) Western blot analysis was performed using antibodies against Syk Y525/526, Y352 and S297. The corresponding quantitation shown as ratio compared to total Syk, Y525/526 (B), Y352 (C) and S297 (D). Data are from at least 3 independent experiments with platelets from at least 3 different donors and presented as mean ±S.D. ns: not significant, *$p<0.05$, **$p<0.01$, ****$p<0.0001$.

In addition, the phosphorylation of Syk downstream effectors were investigated, PLCγ2 Y759 (Figure 34A, B) and Akt S473 (Figure 34A, C). The data showed that the phosphorylation on both sites was completely inhibited by blocking the receptors of ADP and TxA_2.

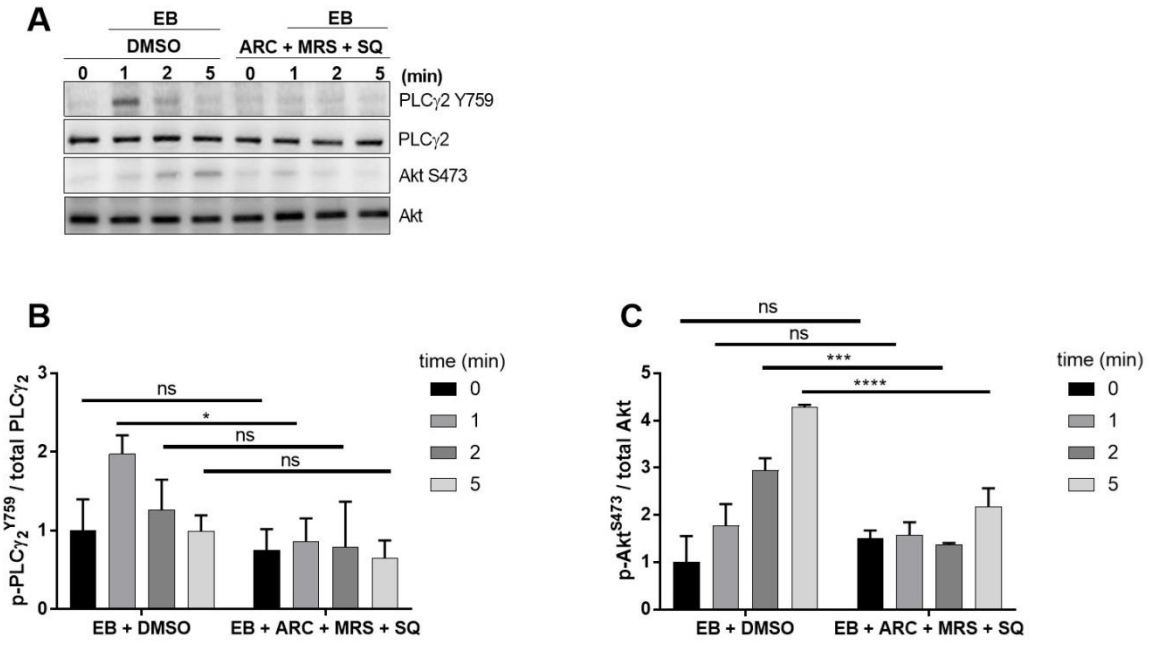

Figure 34. Activation of Syk downstream effectors is dependent on the secondary mediators ADP and TxA_2. WP were treated as mentioned previously with DMSO or simultanously with ADP and TxA_2 receptors antagonists prior to stimulation with EB. Platelet aggregation was stopped after 1, 2 or 5 minutes by adding directly Laemmli buffer into cuvettes. Samples were boiled at 95°C for 10 minutes. (A) Western blot analysis was performed using antibodies against PLCγ2 Y759 and Akt S473. Quantitative analysis of PLCγ2 Y759 (B) and Akt S473 (C) are from at least 3 independent experiments with platelets from at least 3 different donors. Data are presented as ratio compared to the corresponding total protein and represented as mean ±S.D. ns: not significant, *p<0.05 ***p<0.001, ****p<0.0001.

5.3.6 No involvement of the integrin $\alpha_{IIb}\beta_3$ in EB-induced Syk activation

To study the involvement of the main integrin on the platelet surface in Syk activation in the GPIbα pathway, we had the opportunity to get blood from a patient with Glanzmann thrombasthenia (GT), which lacks the integrin $\alpha_{IIb}\beta_3$. WP from a day control and from GT patients were stimulated for 1 minute with EB. Platelet aggregation took place only with WP from the healthy control, however platelets from GT did not present any aggregation. A similar result to platelet preincubated with tirofiban (Figure 19). Western blot analysis were performed in order to check the phosphorylation pattern of Syk on Y352 and Y525/526. The data show clearly that the phosphorylation profile of platelet Syk from a GT patient is similar to a healthy control (Figure 35).

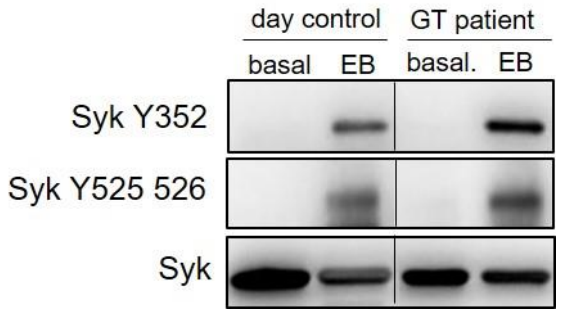

Figure 35. EB-induced Syk activation is not dependent on integrin $\alpha_{IIb}\beta_3$ outside-in signaling.
Washed human platelets from a healthy donor or from a patient with Glanzmann thrombasthenia were stimulated with EB. Platelet aggregation was stopped after 1 minute by adding Laemmli buffer. Then samples were boiled at 95°C for 10 minutes. Phosphorylation of Syk on tyrosine sites was analyzed by western blot compared to the total Syk.

5.3.7 Effect of cAMP/PKA and cGMP/PKG on EB-mediated platelet activation

5.3.7.1 Effect of iloprost and riociguat on EB-induced platelet aggregation

A major aim in this study was to evaluate the possible effects of endogenous platelet inhibitory pathways mediated by iloprost/cAMP/PKA and riociguat/cGMP/PKG. Therefore, platelets were preincubated with increased levels of iloprost or riociguat at 37°C. Platelet aggregation was inhibited in a dose dependent manner (Figure 36).

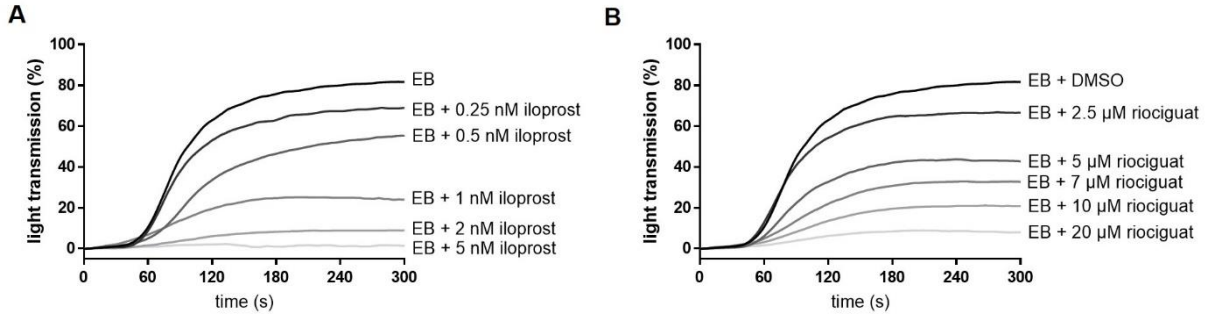

Figure 36. Iloprost and riociguat inhibit platelet aggregation in a dose dependent manner.
WP were preincubated for 3 or 2 minutes with increased concentrations of iloprost (A) or riociguat (B), respectively prior to stimulation with EB.

For the following experiments, WP were preincubated with 2 nM iloprost or 20 µM riociguat for 3 or 2 min, respectively.

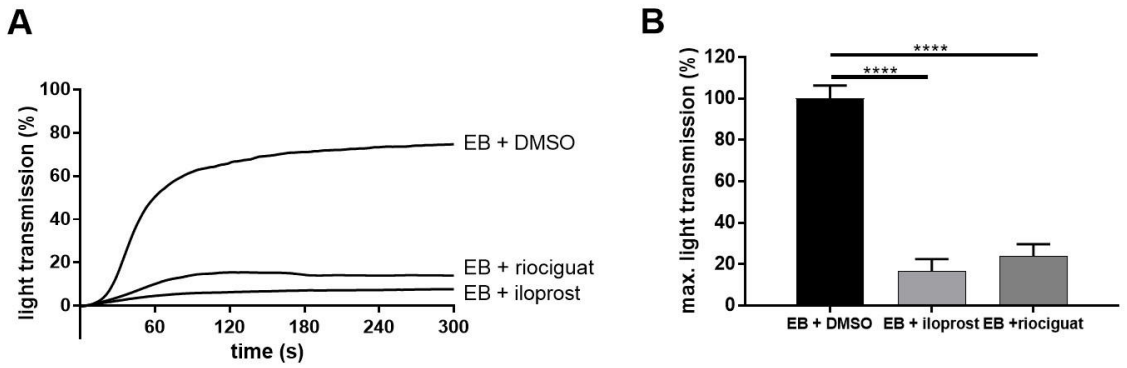

Figure 37. cAMP/iloprost and cGMP/riociguat strongly inhibit EB-induced platelet aggregation.
(A) WP were preincubated with vehicle control for 2 minutes or 2nM iloprost for 3 minutes or 20 µM riociguat for 2 minutes prior to stimulation with EB. (B) Results are normalized to 100% compared to EB and represented as mean ± S.D of 3 independent experiments with platelets from 3 healthy donors (****p<0.0001).

Under these conditions, platelet aggregation was strongly inhibited by iloprost and riociguat. However, riociguat showed a weaker effect than iloprost (Figure 37).

5.3.7.2 Effect of iloprost and riociguat on EB-mediated signaling pathway

Next, the effects of PKA and PKG pathways on EB-induced Syk activation and phosphorylation of selected downstream effectors (LAT, PLCγ2 and Akt) were studied. In contrast to the strong inhibition of platelet aggregation, iloprost and riociguat did not inhibit but enhanced and prolonged phosphorylation of the Syk activation marker Y525/526 (hyperphosphorylation), detected at early and late time points of EB stimulation (Figure 38A, B). Despite significant inhibition of phosphorylation of Syk Y352 at early time points (1 min), iloprost and riociguat prolonged Y352 phosphorylation (Figure 38A, C). Additionally, an important regulatory site of Syk was investigated, S297. This transient EB-stimulated phosphorylation was markedly inhibited by iloprost and less so by riociguat (Figure 38A, D).

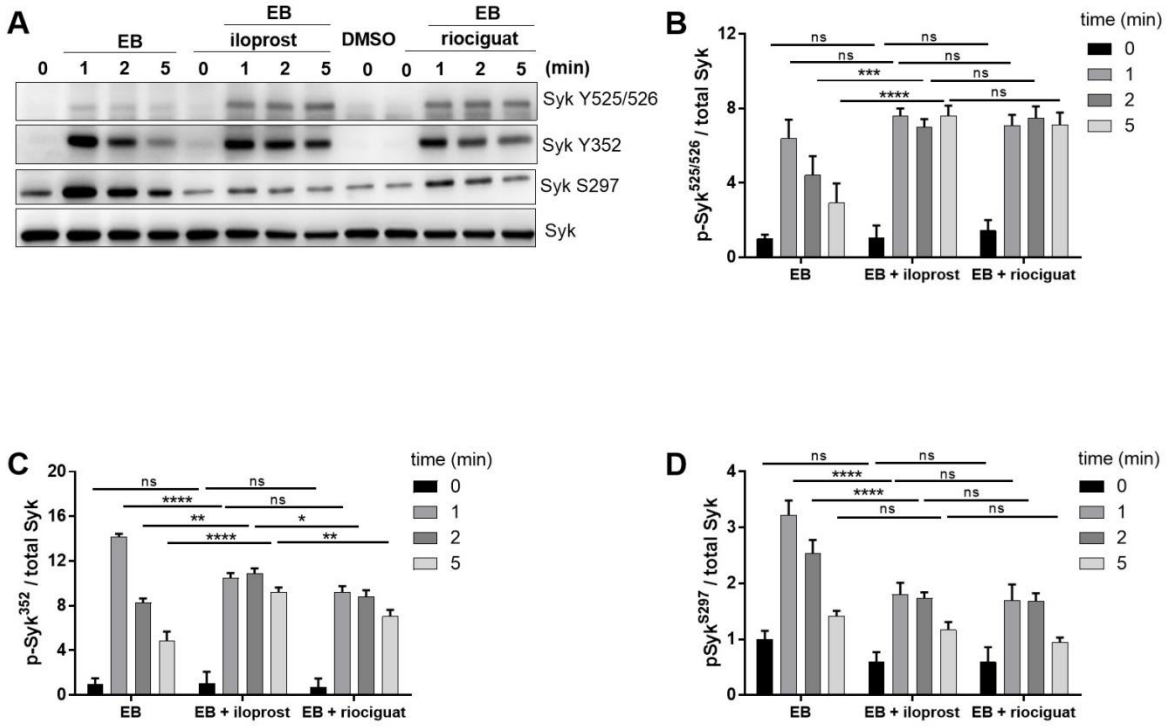

Figure 38. cAMP and cGMP inhibit Syk phosphorylation on serine 297 but not the tyrosine sites 525/526 and 352.
WP were treated as mentioned previously with vehicle control, iloprost or riociguat prior to stimulation with EB. Platelet aggregation was stopped after 1, 2 or 5 minutes by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C for 10 minutes. (A) Western blot analysis was performed using antibodies against Syk Y525/526, Y352 and S297. The corresponding quantitation shown as ratio compared to total Syk, Y525/526 (B), Y352 (C) and S297 (D). Data are from at least 3 independent experiments with platelets from at least 3 different donors and presented as mean ±S.D. ns: not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Since these data indicate that the strong inhibitory pathways PKA/PKG do not prevent Syk activation under these conditions we sought to confirm this at the level of the Syk substrates, LAT Y191 (Figure 39A, B) and PLCγ2 Y759 (Figure 39A, C). Both sites were not inhibited but strongly enhanced and prolonged until 5 minutes of activation.

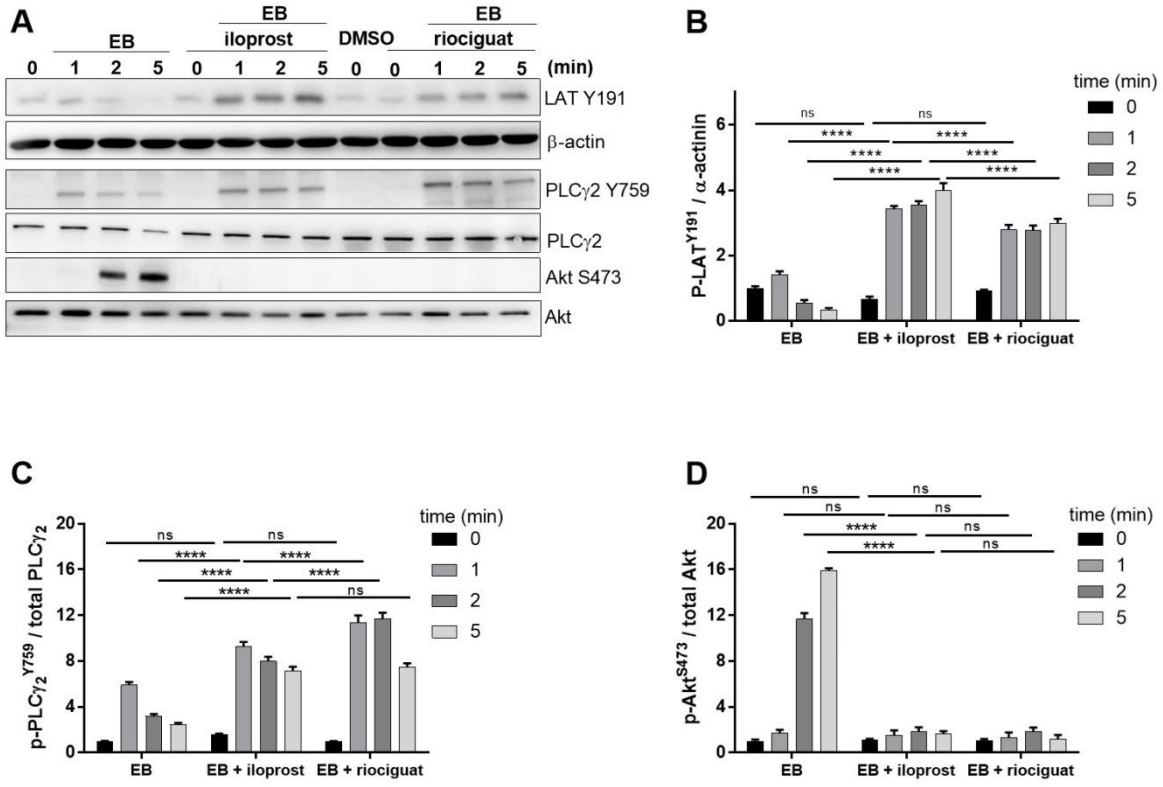

Figure 39. cAMP and cGMP enhance the phosphorylation of direct Syk substrates (LAT, PLCγ) but abolish phosphorylation of the downstream effector Akt.
WP were treated as mentioned previously with vehicle control, iloprost or riociguat prior to stimulation with EB. Platelet aggregation was stopped after 1, 2 or 5 minutes by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C for 10 minutes. (A) Western blot analysis was performed using antibodies against LAT (Y191), PLCγ2 Y759 and Akt S473. Quantitative analysis of LAT Y191 (B), PLCγ2 Y759 (C) and Akt S473 (D) are from at least 3 independent experiments with platelets from at least 3 different donors. They are represented as ratio compared to β-actin for LAT and to the total protein. Data are from at least 3 different experiments with platelets from at least 3 healthy volunteers and represented as mean ±S.D. ns: not significant, ****p<0.0001.

In contrast, delayed Akt phosphorylation at S473 was abolished by iloprost and riociguat (Figure 39A, D), which resembles the aggregation response.

5.3.8 Effect of protein kinase C (PKC) inhibition on EB-mediated platelet activation

5.3.8.1 Effect of PKC inhibition on EB-induced platelet aggregation

The striking results seen with PKA and PKG effects led us to search for possible mechanisms and mediators, which can regulate Syk activity. A previous study showed that PKC can catalyze the phosphorylation of Syk at S291 in murine B cells (equivalent to S297 in human Syk)[156]. Therefore, the next step was to study the effect of PKC inhibition on Syk phosphorylation and activation by using a general inhibitor of all PKC isoforms, GF109203X (short GFX).

The titration of GFX inhibited dose-dependently EB-mediated platelet aggregation (Figure 40).

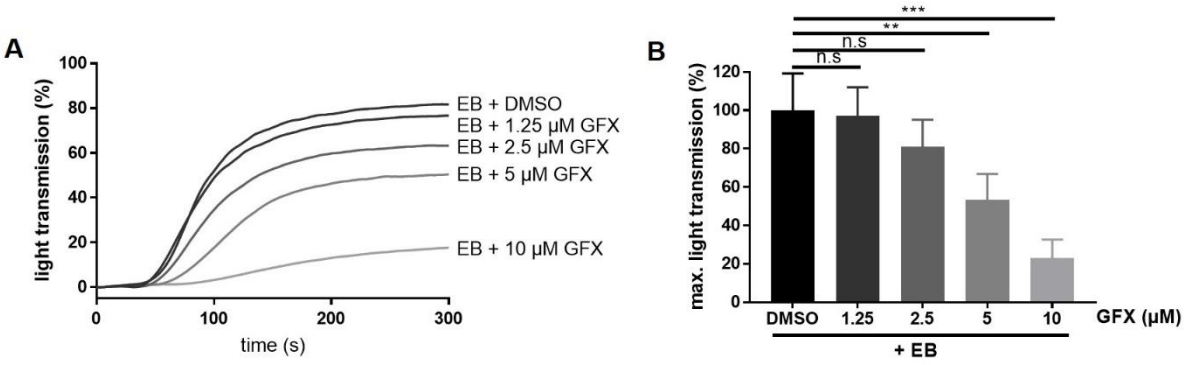

Figure 40. The pan-PKC inhibitor (GFX) inhibits EB-mediated aggregation in a dose dependent manner.
WP were preincubated with vehicle control (DMSO) or with increased concentrations of GFX for 5 minutes at 37°C prior to stimulation with EB. (A) Representative aggregation curves showing the effect of the PKC inhibitor. Platelet aggregation was monitored until 5 minutes. B) Results are normalized to 100% compared to the vehicle control and represented as mean ± S.D of 3 independent experiments with platelets from 3 healthy donors. n.s: not significant, **p<0.01, ***p<0.001.

EB-mediated platelet aggregation was partially inhibited by 5 µM GFX (Figure 41). These data support the concept that PKCs are implicated in platelet aggregation.

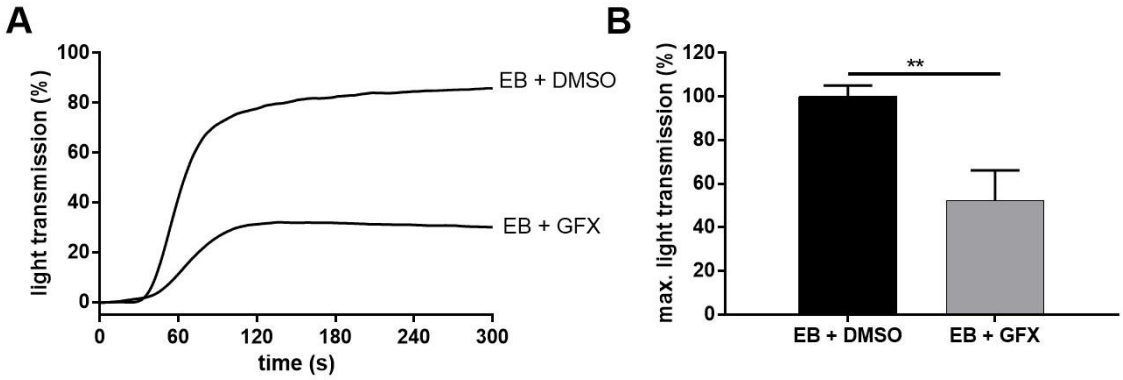

Figure 41. Pan-PKC inhibitor partially inhibits EB-induced platelet aggregation.
WP were preincubated for 5 minutes at 37°C with vehicle control (DMSO) or 5 µM GFX, a pan PKC inhibitor prior to stimulation with EB. (A) representative aggregation curves of the effect of PKC inhibition on platelet aggregation. Platelet aggregation was monitored until 5 minutes.(B) Results are normalized to 100% compared to EB and represented as mean ± S.D of 3 independent experiments with platelets from 3 healthy donors (p<0.01).

5.3.8.2 Effect of PKC inhibition on EB-mediated signaling pathway

Then, the effect of PKC inhibition on EB-induced Syk phosphorylation and activation was studied. Compared to the activation pattern without PKC inhibitor, the phosphorylation of Syk on Y525/526 and Y352 with GFX was not inhibited, but significantly increased. This phosphorylation remained significantly elevated even after 5 minutes. LAT and PLCγ2 phosphorylation on Y191 and Y759, respectively, was also enhanced and prolonged, a similar profile as observed for Syk kinase activity.

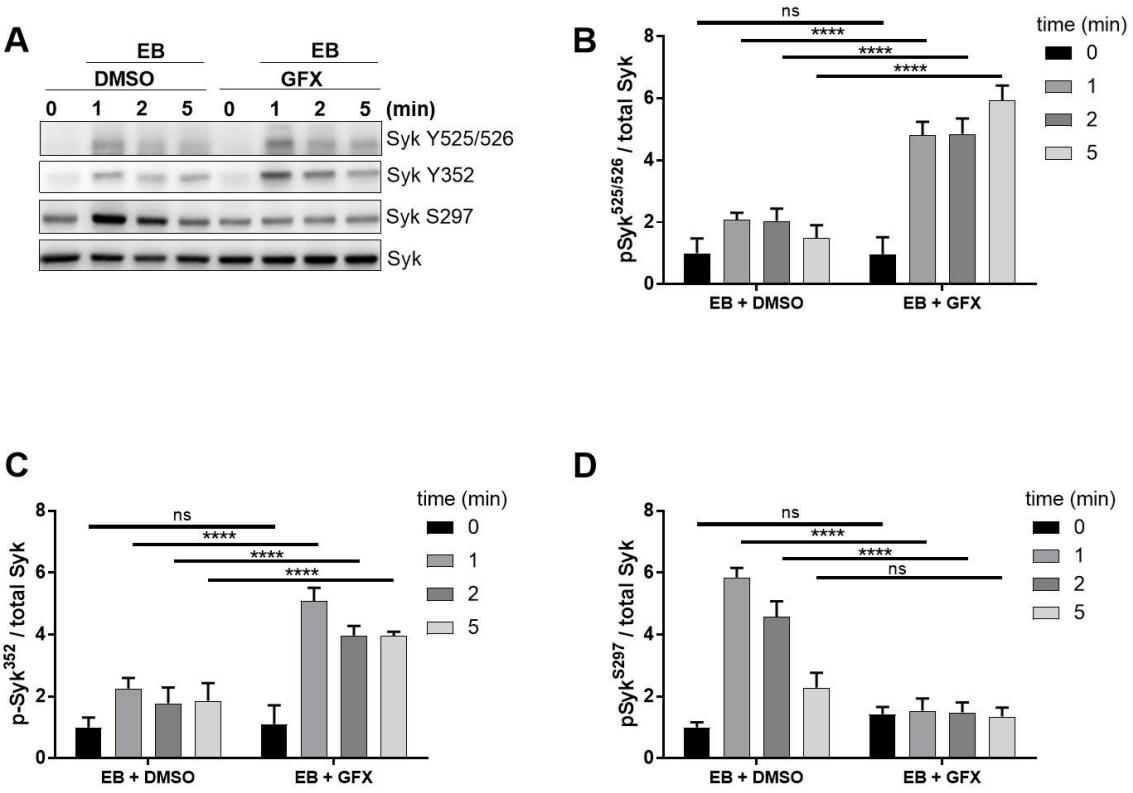

Figure 42. PKC inhibition stimulates Syk tyrosine phosphorylation but inhibits Syk phosphorylation on S297.
WP were treated as mentioned previously with vehicle control or GFX prior to stimulation with EB. Platelet aggregation was stopped after 1, 2 or 5 minutes by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C for 10 minutes. (A) Western blot analysis was performed using antibodies against Syk Y525/526, Y352 and S297. The corresponding quantitation shown as ratio compared to total Syk, Y525/526 (B), Y352 (C) and S297 (D). Data are from at least 3 independent experiments with platelets from at least 3 different donors and presented as mean ±S.D. ns: not significant, ****p<0.0001.

Furthermore, PKC inhibition led to a complete inhibition of Syk S297 phosphorylation (Figure 42A, D).

To investigate the activity of Syk , the phosphorylation of the direct Syk substrates were evaluated; the minimal EB-minimal induced LAT Y191 phosphorylation was strongly increased and enhanced by GFX in a transient manner (Figure 43A, B), the phosphorylation of PLCγ2 Y759 was also enhanced by EB (Figure 43A, C).

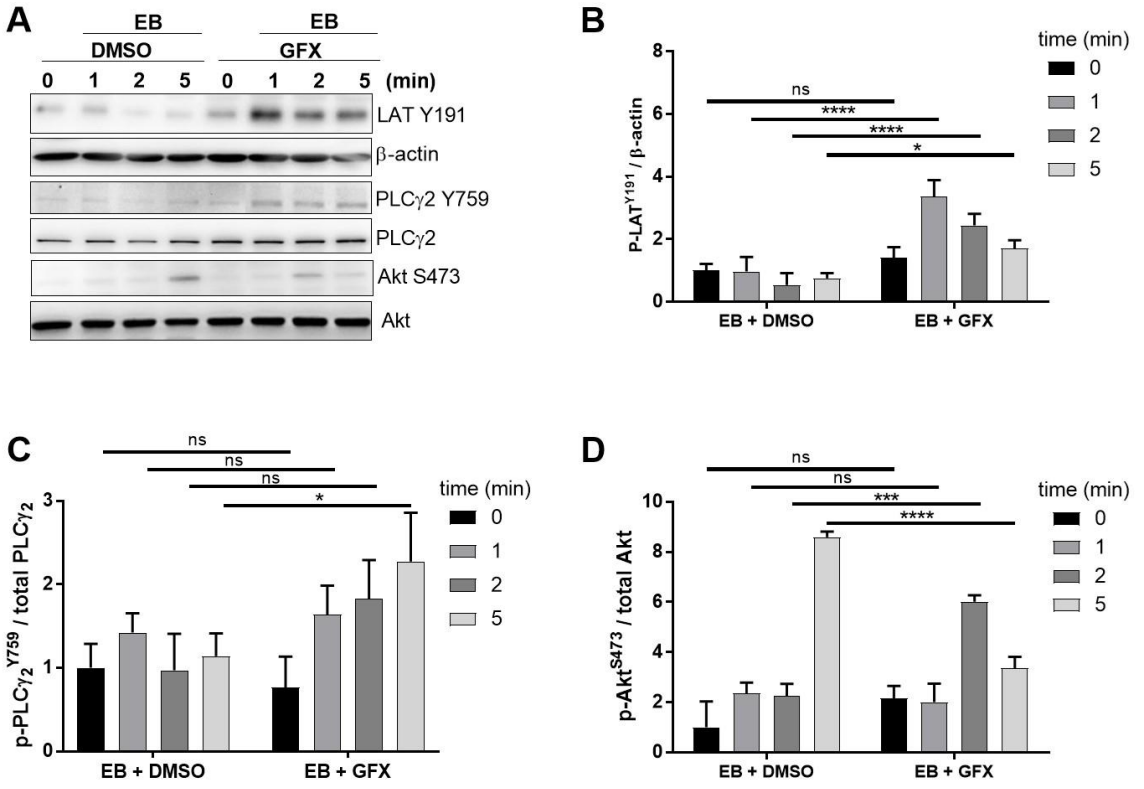

Figure 43. PKC inhibition increases the EB-stimulated phosphorylation of direct Syk substrates but inhibits Akt phosphorylation.
WP were treated as mentioned previously with vehicle control or GFX prior to stimulation with EB. Platelet aggregation was stopped after 1, 2 or 5 minutes by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C for 10 minutes. (A) Western blot analysis was performed using antibodies against LAT (Y191), PLCγ2 Y759 and Akt S473. Quantitative analysis of LAT Y191 (B), PLCγ2 Y759 (C) and Akt S473 (D) are from at least 3 independent experiments with platelets from at least 3 different donors. They are represented as ratio compared to β-actin for LAT and to the total protein. Data are from at least 3 different experiments with platelets from at least 3 healthy volunteers and represented as mean ±S.D. ns: not significant, ****p<0.0001, ***p<0.001, *p<0.05, n.s not significant.

Additionally, Akt S473 phosphorylation was stimulated at an earlier point in the presence of GFX (2 minutes of activation) compared to the control (where the phosphorylation appears only after 5 min). However, this increased phosphorylation after 5 min of stimulation was significantly inhibited by GFX (Figure 43A, D).

5.3.9 Inositol monophosphate (IP1) regulation in EB-induced platelet activation

To validate a functional response in platelets directly after Syk-mediated PLCγ2 phosphorylation/activation, EB-induced IP1 accumulation was measured in the presence of lithium chloride (LiCl) (paragraph 4.11.2), as marker of EB-mediated IP3 production according to published methods[157]. A clear 3-fold increase of IP1 accumulation in EB-treated platelets was detected, which was abolished by pre-incubation with echicetin monomers as control and by the Syk inhibitor PRT318 (Figure 44A). Iloprost and riociguat only partially inhibited this EB-mediated IP1 accumulation, which was more strongly reduced when ADP/TP receptors were blocked, but this combination of these inhibitors did not produce additive effects (Figure 44B).

Also, preincubation of platelets with tirofiban did not reduce IP1 accumulation, but the combination of iloprost with tirofiban showed the inhibition of EB-induced IP1 production as observed with iloprost alone (Figure 44B).

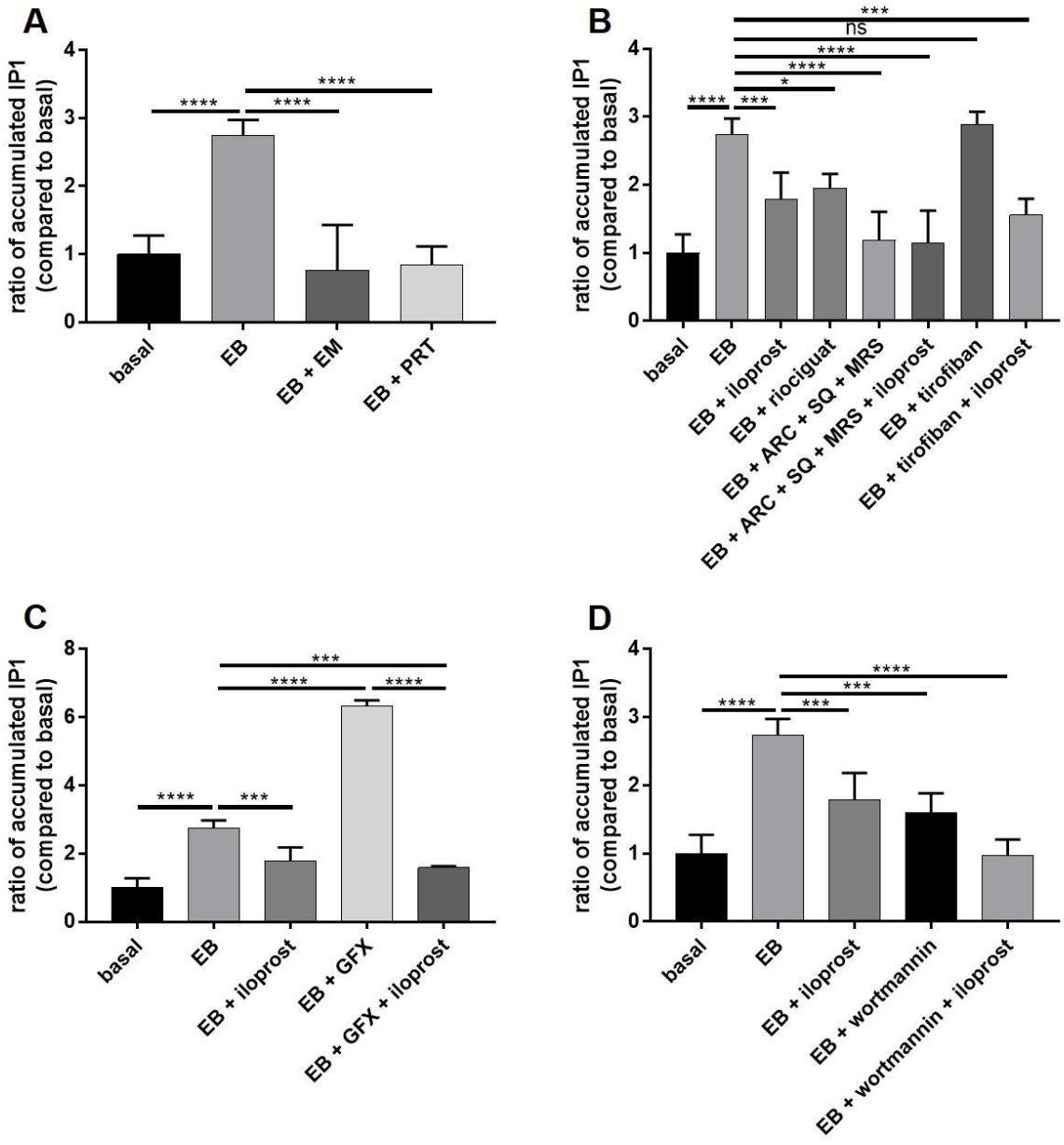

Figure 44. Differential regulation of EB-evoked IP1 accumulation in platelets treated with different inhibitors. WP were preincubated as described before and shown here with echicetin (25 µg/ml, 3 min), PRT318 (1 µM, 5 min), iloprost (2 nM, 3 min), riociguat (20 µM, 2 min), ARC+ SQ + MRS (0.1, 1, 1 µM respectively, 5 min), tirofiban (1.25 µg/ml, 1 min), GFX (5 µM, 5 min) and wortmannin (100 nM, 5 min) prior to stimulation with EB in presence of 1 mM LiCl. Aggregation was stopped after 5 minutes using the lysis buffer provided by the manufacturer. Data are representative of 3 different experiments from 3 healthy donors. Results are means ± S.D. ns: not significant, *p<0.05, ***p<0.001, ****p<0.0001.

Surprisingly, PKC inhibition showed a 2-fold increase in the IP1 accumulation compared to EB effect. But iloprost (PKA pathway) strongly inhibited this PKC inhibitor effect and regulated the

IP1 level back to basal levels (Figure 44C). Furthermore, we were interested to test the effect of the PI3K in the IP3 production process using wortmannin, a potent PI3K inhibitor, which inhibited the IP1 accumulation only partially compared to the EB-stimulated platelets. The combination of iloprost induced an additive inhibition of the IP1 accumulation by decreasing it to basal levels (Figure 44D).

5.3.10 Regulation of endogenous Ca^{2+} release induced by EB

Platelet agonists trigger the activation of phospholipases (PLCβ or PLCγ) and subsequent increase of the messengers inositol 1,4,5 triphosphate (IP3) and 1,2 diacylglycerol (DAG), which activate IP3 receptors or protein kinase C (PKC), respectively. IP3 receptors increase the intracellular Ca^{2+}-concentration by release of Ca^{2+} from intracellular stores[30]. Therefore, the regulation of EB-induced Ca^{2+} release was investigated in the presence of the different inhibitors used in the previous experiments. A clear 3-fold increase of Ca^{2+}-release induced by EB was detected, which was completely inhibited by the Syk inhibitor, PRT318. Iloprost partially inhibited the Ca^{2+}-release only at high dose (5 nM), and riociguat (20 µM) showed less pronounced inhibition of Ca^{2+}-release (Figure 45A). In addition, neither inhibition of the integrin $\alpha_{IIb}\beta_3$ (using tirofiban) nor blocking the ADP/TP receptors (using ARC, SQ, MRS) in the presence or absence of iloprost affected the Ca^{2+}-release evoked by EB (Figure 45B).

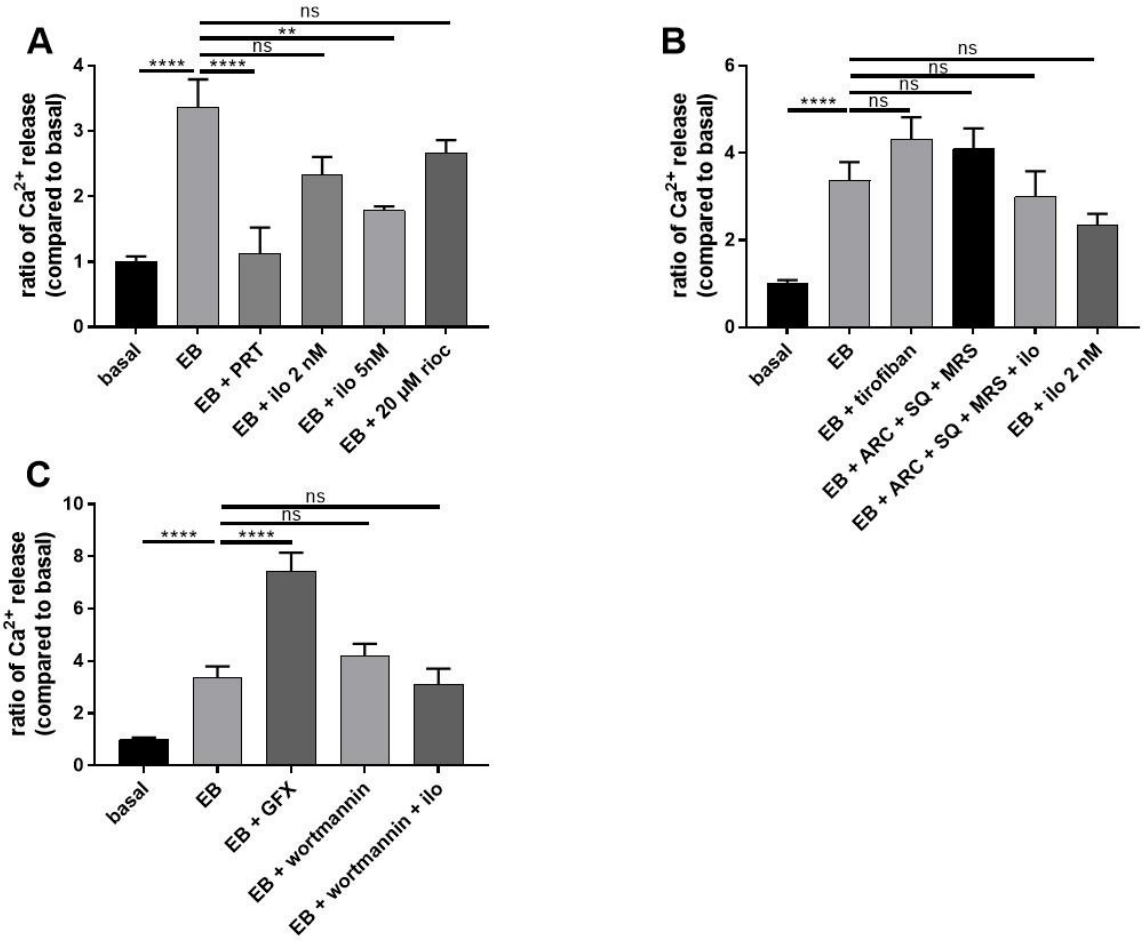

Figure 45. Differential regulation of EB-evoked Ca^{2+}-release by various inhibitors.
WP were preincubated for 30 minutes at 37°C with Fluo-3am and then WP were preincubated with echicetin (25 µg/ml, 3 min), PRT318 (1 µM, 5 min), iloprost (2 or 5 nM, 3 min), riociguat (20 µM, 2 min), ARC+ SQ + MRS (0.1, 1, 1 µM respectively, 5 min), tirofiban (1.25 µg/ml, 1 min), GFX (5 µM, 5 min) and wortmannin (100 nM, 5 min) prior to stimulation with EB. Ca^{2+} release was monitored for 2 minutes. Data are representative of 3 different experiments from 3 healthy donors. Results are means ± S.D. ns: not significant, **p<0.01, ****p<0.0001.

Furthermore, inhibiting the PKC by GFX showed a significant increase in the Ca^{2+}-release. In contrast, inhibiting the PI3K did not present any significant effect compared to EB stimulated platelets. Combining iloprost and wortmannin did not further inhibit the Ca^{2+}-release (Figure 45C).

5.4 Investigation of GPVI-mediated platelet signaling using convulxin

5.4.1 Convulxin induces platelet aggregation and activates Syk downstream signaling

Convulxin (cvx) is widely used as specific and potent GPVI agonist, its use is also well established and known to induce a rapid and full platelet aggregation and GPVI-downstream tyrosine phosphorylation including Syk activation[128, 158]. Therefore, the analysis of the GPVI-Syk pathway was also established here in order to directly compare the GPIbα- signaling with the potent GPVI- mediated signaling pathways.

Convulxin-induced Syk tyrosine phosphorylation (Y525/526 and Y352) and S297 phosphorylation were studied in a time dependent manner (Figure 46A, B). Syk phosphorylation of the 2 tyrosine sites and the one serine site showed a transient activation, which reached a maximum after 1 minute of stimulation and then decreased progressively after 2 and 5 minutes.

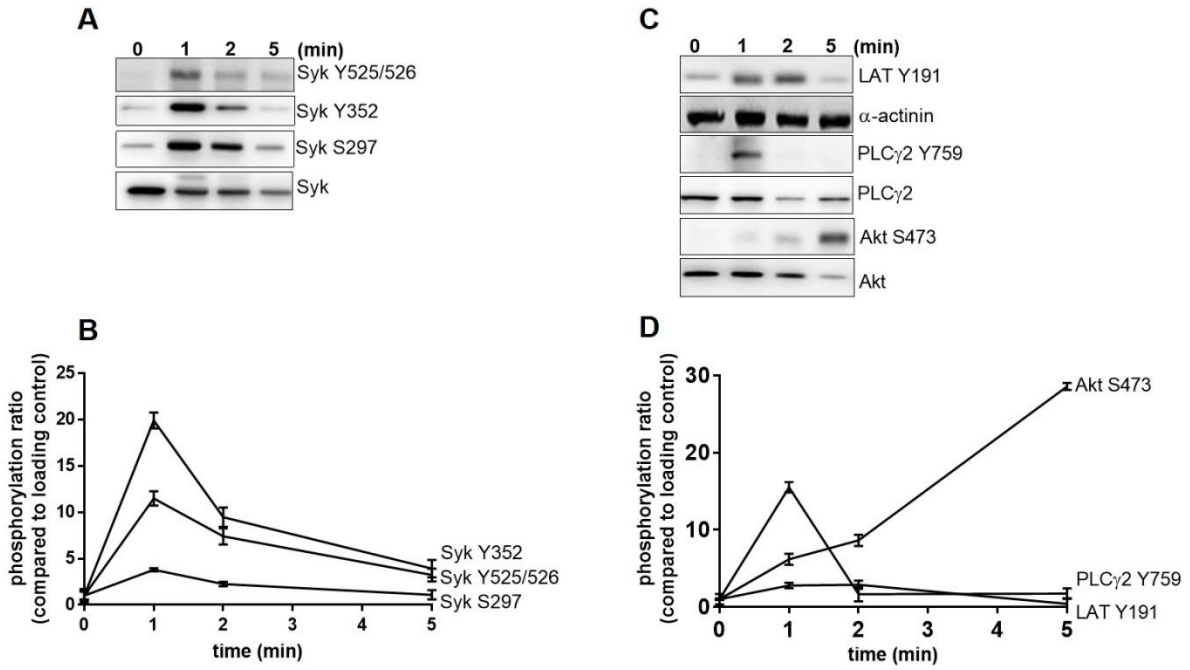

Figure 46. Convulxin-induced platelet activation triggers transient Syk phosphorylation and phosphorylation of Syk downstream effectors.
Convulxin-mediated platelet aggregation (50ng/ml) was stopped after 1, 2 or 5 minutes by adding Laemmli buffer. Platelets lysates were cooked at 95°C for 10 minutes. Tyrosine and serine phosphorylation patterns were detected by western blot. (A and C) Phosphorylation profiles of Syk tyrosine sites (525/526 and 352), serine site (Ser297) and PLCγ2 Y759, LAT Y191 and Akt S473, respectively. (B and D) Data from at least 3 independent experiments with at least 3 different donors were analyzed compared the corresponding loading controls. They are represented as mean ±S.D.

Additionally, from the direct Syk substrates, LAT Y191 showed a stable phosphorylation for 1 and 2 minutes and then declined, whereas PLCγ2 Y759 showed a fast activation and subsequent strong decrease. In contrast, serine phosphorylation of a Syk downstream effector,

Akt, showed a delayed phosphorylation on S473 site (after 5 minutes of stimulation) (Figure 46C, D).

5.4.2 Effect of Syk inhibition on cvx-mediated platelet activation

5.4.2.1 Effect of OXSI-2 and PRT318 on cvx-induced platelet aggregation

To test the role of Syk in cvx-induced platelet activation, potent Syk inhibitors were used, OXSI- 2 and PRT318 abolished cvx-mediated platelet aggregation (Figure 47).

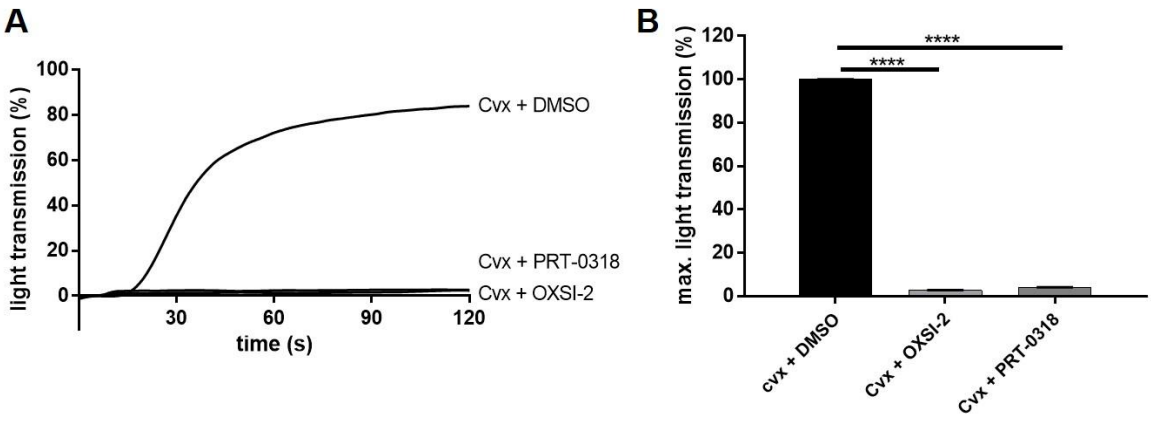

Figure 47. Convulxin induces platelet aggregation in a Syk-dependent manner.
WP were preincubated for 5 minutes at 37°C with vehicle control (DMSO) or 0.1µM OXSI-2 or 1µM PRT318 prior to stimulation with 50ng/ml convulxin. (A) representative aggregation curves of the effect of both Syk inhibitors on platelet aggregation. Platelet aggregation was monitored until 120s.(B) Results are normalized to 100% compared to EB and represented as mean ± S.D of 3 independent experiments with platelets from 3 healthy donors (****p<0.0001).

5.4.2.2 Effect of Syk inhibition on cvx-mediated Syk phosphorylation

Syk phosphorylation patterns were studied in presence of both Syk inhibitors at early time points (0, 15, 60 and 120 s). After 15s of stimulation, Syk Y525/526 phosphorylation was partially inhibited by OXSI-2 and stronger by PRT318 (Figure 48A, B). However, Y525/526 phosphorylation was enhanced and prolonged at 60 and 120 s in presence of Syk inhibitors. Syk Y352 phosphorylation was not inhibited but strongly enhanced and prolonged by both Syk inhibitors (Figure 48A, C).

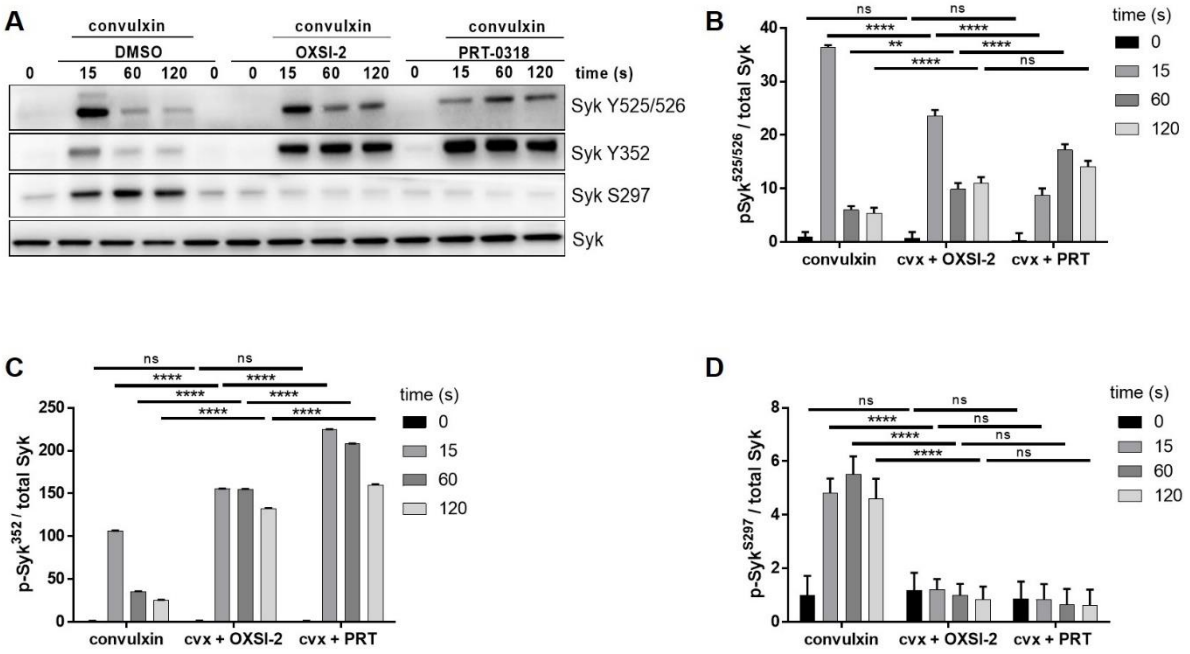

Figure 48. Differential regulation of Syk phosphorylation by Syk kinase inhibitors.
WP were treated as mentioned previously with DMSO, OXSI-2 or PRT318 prior to stimulation with cvx. Platelet aggregation was stopped after 15, 60 or 120 seconds by adding directly Laemmli buffer into cuvettes. Samples were boiled at 95°C for 10 minutes. (A) Western blot analysis was performed using antibodies against Syk Y525/526, Y352 and S297. Quantitative analysis of Syk Y525/526 (B), Y352 (C) and S297 (D) are from at least 3 independent experiments with platelets from at least 3 different donors. They were analyzed compared to the total Syk and represented as mean ±S.D. ns: not significant, **p<0.01, ****p<0.0001.

However, Syk S297 phosphorylation was downregulated compared to the control by both Syk inhibitors (Figure 48A, D).

In order to validate the inhibition of Syk in WP, Syk direct substrates (LAT and PLCγ) and one of Syk downstream effectors (Akt) were studied. LAT Y191 phosphorylation was significantly inhibited by OXSI-2 at 15s but less at later points whereas significant inhibition of LAT Y191 phosphorylation by PRT318 was observed (Figure 49A, B).

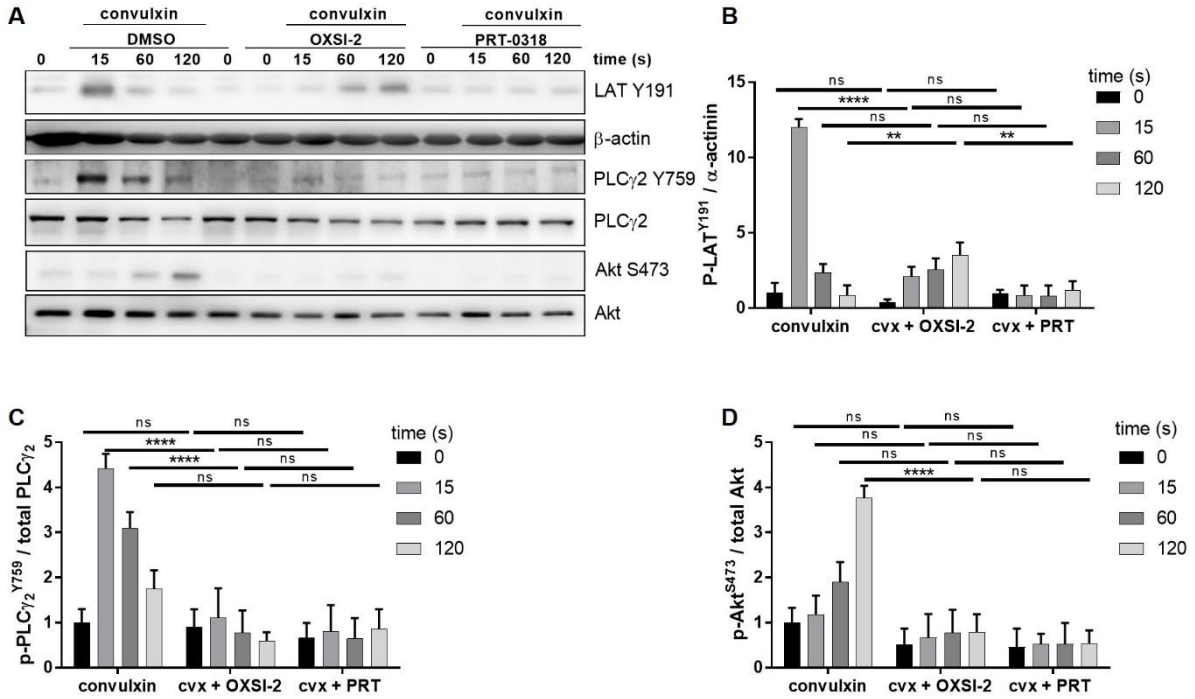

Figure 49. Syk activity was inhibited by the inhibitors OXSI-1 and PRT
WP were treated as mentioned previously with DMSO, OXSI-2 or PRT318 prior to stimulation with cvx. Platelet aggregation was stopped after 15, 60 or 120 seconds by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C for 10 minutes. (A) Western blot analysis was performed using antibodies against PLCγ2 Y759 and Akt S473. Quantitative analysis of PLCγ2 Y759 (B) and Akt S473 (C) are from at least 3 independent experiments with platelets from at least 3 different donors. They were analyzed compared to the corresponding total protein and represented as mean ±S.D. ns: not significant, **p<0.01, ****p<0.0001.

The phosphorylation of PLCγ2 Y759 and Akt S473 was significantly inhibited by both Syk inhibitors (Figure 49A, C, D).

5.4.3 Role of secondary mediators in cvx-induced platelet activation and downstream signaling

5.4.3.1 Role of secondary mediators for cvx-mediated platelet aggregation

A higher concentration of GPVI agonists can overcome the blockage by secondary mediators such as ADP and TxA_2 [155]. Therefore, the next aim was to investigate the role of ADP and TxA_2, separately, in cvx-mediated platelet aggregation and Syk-mediated activation.

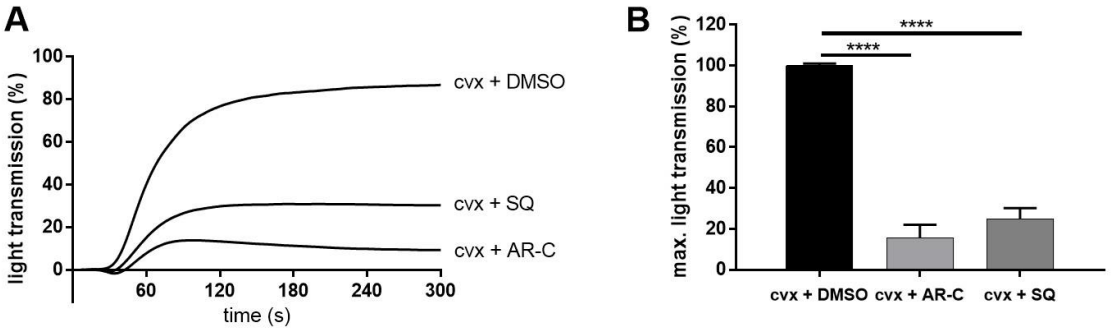

Figure 50. ADP and TxA_2 are essential for cvx-mediated platelet aggregation.
WP were preincubated for 5 minutes at 37°C with vehicle control (DMSO) or with $P2Y_{12}$ inhibitor, AR-C69931 (0.1µM) or with TxA_2 receptor antagonist, SQ-29548 (1µM) prior to stimulation with 5ng/ml convulxin. (A) representative aggregation curves of the effect of each antagonist on platelet aggregation. Platelet aggregation was monitored until 5 minutes.(B) Results are normalized to 100% compared to cvx and represented as mean ± S.D of 3 independent experiments with platelets from 3 healthy donors (****p<0.0001).

Blocking the ADP receptor ($P2Y_{12}$) with AR-C inhibited about 80% platelet aggregation, however, blocking the TP receptors with SQ inhibited about 70% compared the vehicle control (Figure 50).

5.4.3.2 Role of secondary mediators for cvx-mediated platelet activation

Next, it was interesting to investigate the effects of ADP and TxA_2 on GPVI- mediated Syk phosphorylation. Syk Y525/526 as well Y352 phosphorylation was not inhibited by blocking ADP and TxA_2 receptors but the phosphorylation was rather increased and prolonged (Figure 51A, B, C).

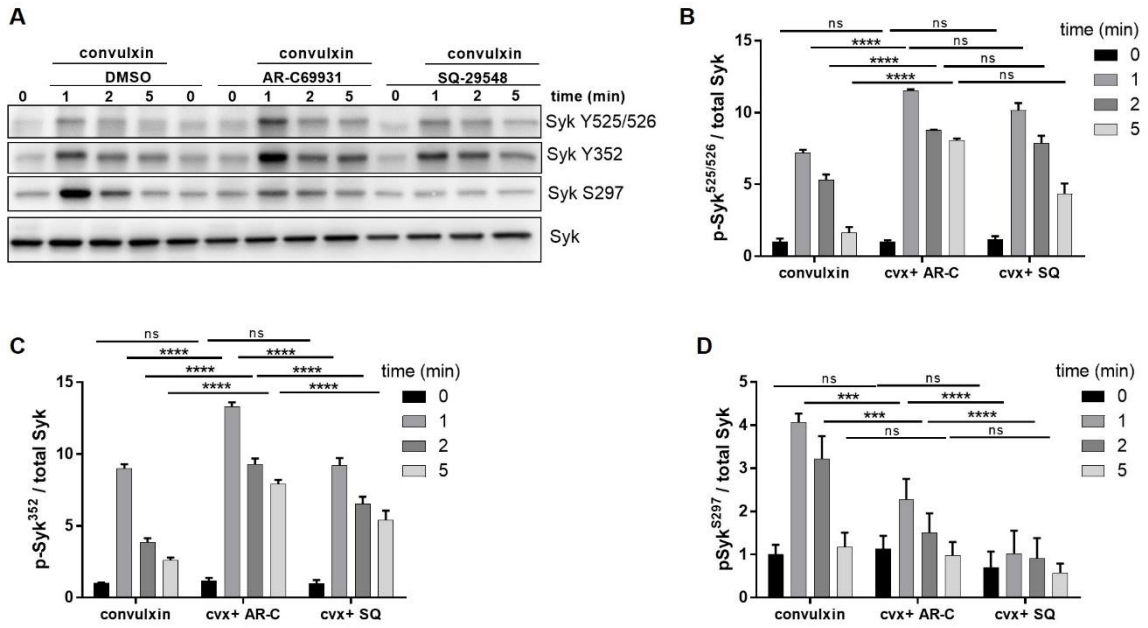

Figure 51. Syk tyrosine phosphorylation (Y525/526 and Y352) is independent of ADP and TxA_2 compared to Syk S297
WP were treated as mentioned previously with DMSO, AR-C or SQ prior to stimulation with cvx. Platelet aggregation was stopped after 1, 2 or 5 minutes by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C

for 10 minutes. (A) Western blot analysis was performed using antibodies against Syk Y525/526, Y352 and S297. Quantitative analysis of Syk Y525/526 (B), Y352 (C) and S297 (D) are from at least 3 independent experiments with platelets from at least 3 different donors. They were analyzed compared to the total Syk and represented as mean ±S.D. ns: not significant, ***p<0.001, ****p<0.0001.

In contrast, the analysis of Syk S297 phosphorylation revealed downregulation, apparently more strongly with TP receptor blockers than with ADP receptor blockers (Figure 51A, D).

Furthermore, PLCγ2 Y759 showed to be differentially regulated by ADP and TxA_2. Blocking the ADP receptor $P2Y_{12}$, did not inhibit this phosphorylation but enhanced PLCγ2 phosphorylation after 5 minutes of stimulation. However, PLCγ2 Y759 phosphorylation was significantly inhibited when the TxA_2 receptors were blocked (Figure 52A, B).

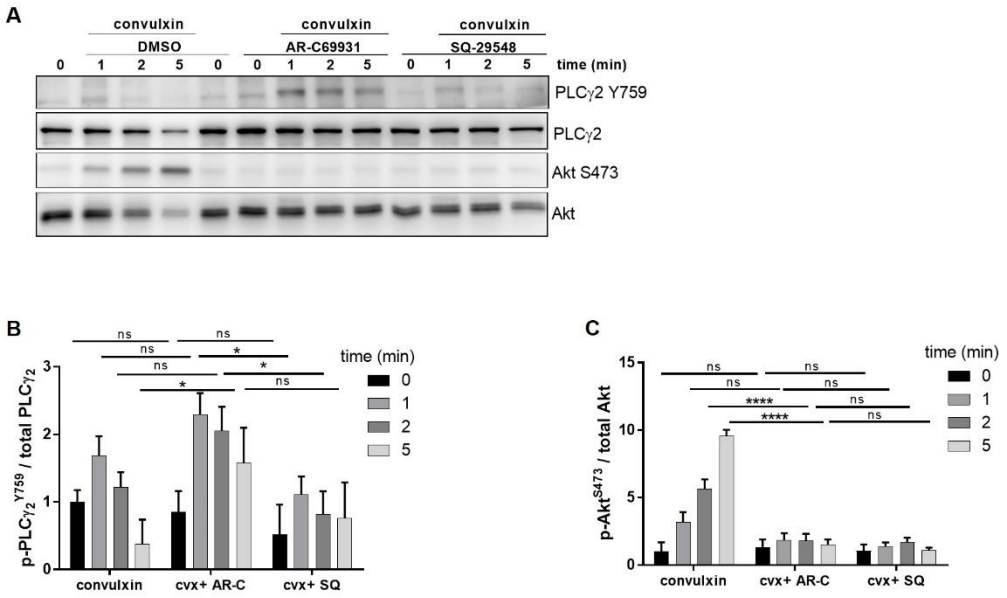

Figure 52. PLCγ2 phosphorylation is differentially regulated by ADP and TxA_2-mediated pathways compared to Akt phosphorylation, which is completely dependent on the activation of the secondary mediators.
WP were treated as mentioned previously with DMSO, AR-C or SQ prior to stimulation with cvx. Platelet aggregation was stopped after 1, 2 or 5 minutes by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C for 10 minutes. (A) Western blot analysis was performed using antibodies against PLCγ2 Y759 and Akt S473. Quantitative analysis of PLCγ2 Y759 (B) and Akt S473 (C) are from at least 3 independent experiments with platelets from at least 3 different donors. They were analyzed compared to the corresponding total protein and represented as mean ±S.D. ns: not significant, *p<0.05, ****p<0.0001.

On the other hand, the delayed phosphorylation of the major PI3K substrate, Akt, was completely inhibited by blocking ADP and TxA_2 receptors (Figure 52A, C).

5.4.4 Effect of cAMP/PKA and cGMP/PKG on cvx-mediated platelet activation

5.4.4.1 Effect of iloprost and riociguat on cvx-induced platelet aggregation

Next, the crosstalk between the GPVI-activation and the PKA/PKG-inhibitory signaling pathways was analyzed. Therefore, washed human platelets were preincubated with 2 nM iloprost for 3 minutes or 20 µM riociguat for 2 minutes. Cvx-mediated platelet aggregation was strongly inhibited by iloprost or riociguat compared to the vehicle control (Figure 53).

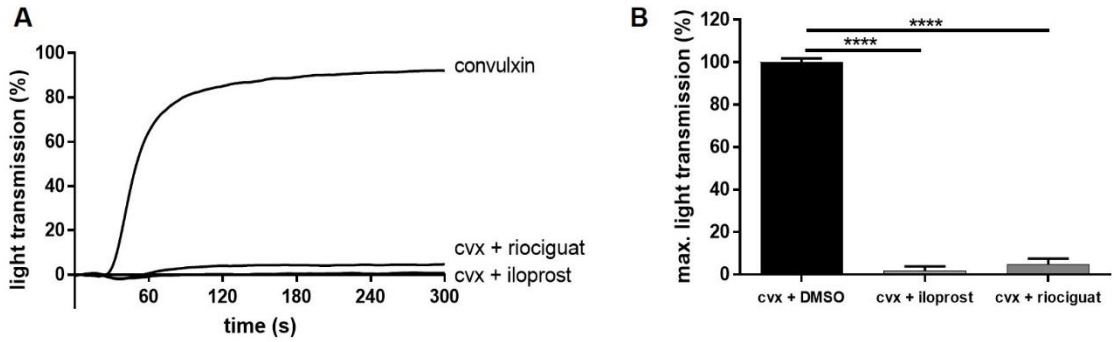

Figure 53. cAMP/iloprost and cGMP/riociguat abolish platelet aggregation induced by convulxin.
(A) WP were preincubated with vehicle control for 2 minutes or 2nM iloprost for 3 minutes or 20 µM riociguat for 2 minutes prior to stimulation with 50ng/ml cvx. (B) Results are normalized to 100% compared to cvx and represented as mean ± S.D of 3 independent experiments with platelets from 3 healthy donors (p<0.0001).

5.4.4.2 Effect of iloprost and riociguat on cvx-induced platelet activation

Similar to EB-mediated Syk activation, the regulation of cvx-mediated Syk activation by cAMP/PKA and cGMP/PKG-mediated pathways was analyzed. Cvx-stimulated phosphorylation of Syk Y525/526 and Y352 was not inhibited by iloprost and riociguat. Instead, Syk Y525/526 phosphorylation was enhanced and prolonged until 5 minutes of activation and Y352 phosphorylation was also enhanced (at 2 minutes) and prolonged compared to vehicle control (Figure 54A, B).

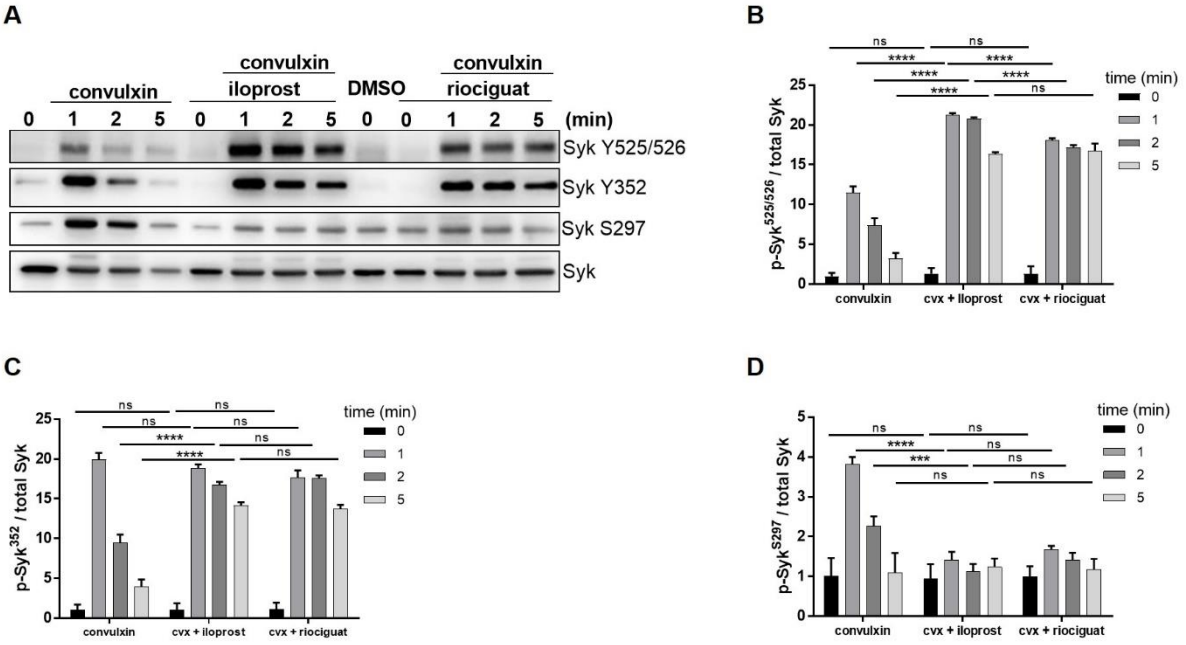

Figure 54. Iloprost and riociguat inhibit Syk phosphorylation on S297 but enhance phosphorylation the tyrosine sites 525/526 and 352.
WP were treated as mentioned previously with vehicle control, iloprost or riociguat prior to stimulation with cvx. Platelet aggregation was stopped after 1, 2 or 5 minutes by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C for 10 minutes. (A) Western blot analysis was performed using antibodies against Syk Y525/526, Y352 and S297. The corresponding quantitation shown as ratio compared to total Syk, Y525/526 (B), Y352 (C) and S297 (D). Data are from at least 3 independent experiments with platelets from at least 3 different donors and presented as mean ±S.D. ns: not significant, ***p<0.001, ****p<0.0001.

On the other hand, the transient cvx-induced Syk S297 phosphorylation was significantly inhibited by the cAMP/PKA (iloprost) or cGMP/PKG (riociguat) pathway (Figure 54A, D).

To confirm increased Syk activity as a result of Syk hyperphosphorylation, tyrosine-phoshorylation of LAT, PLCγ2 and Akt were analyzed. Iloprost and riociguat did not inhibit but enhanced and prolonged cvx-induced LAT Y191 and PLCγ2 Y759 (Figure 55A, B, C).

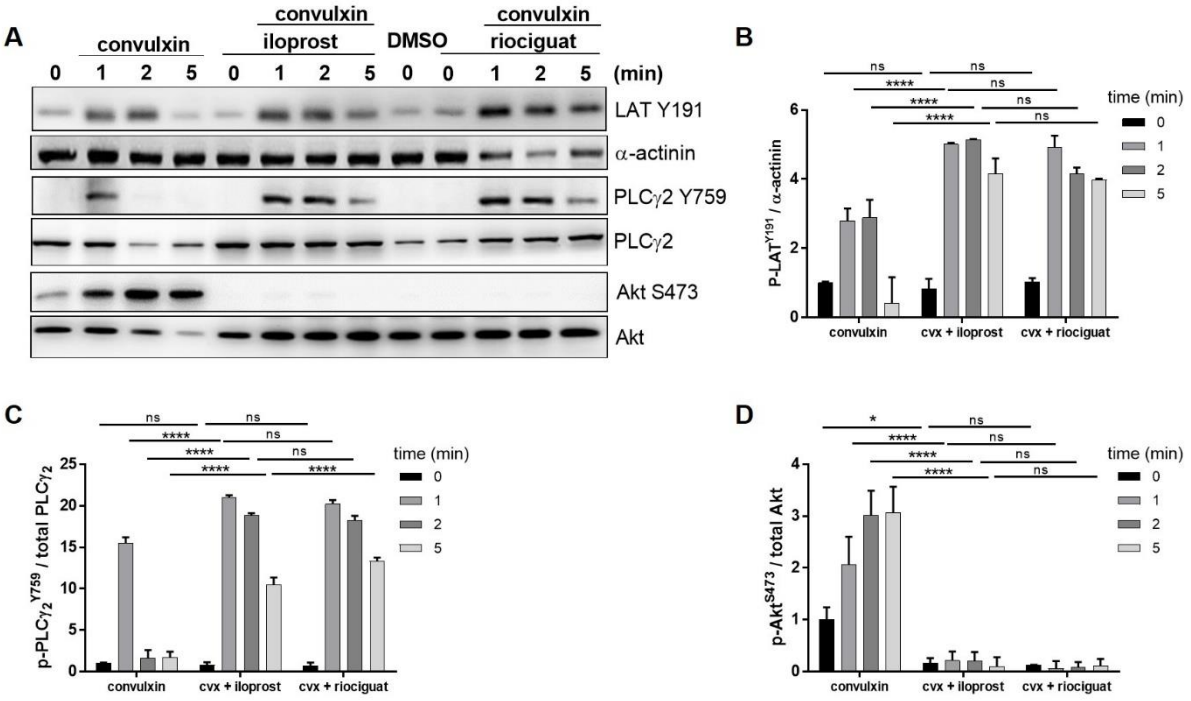

Figure 55. Iloprost (cAMP) and riociguat (cGMP) enhance the phosphorylation of Syk direct substrates (LAT, PLCγ) but abolish serine phosphorylation of the downstream effector Akt.
WP were treated as mentioned previously with vehicle control, iloprost or riociguat prior to stimulation with cvx. Platelet aggregation was stopped after 1, 2 or 5 minutes by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C for 10 minutes. (A) Western blot analysis was performed using antibodies against LAT (Y191), PLCγ2 Y759 and Akt S473. Quantitative analysis of LAT Y191 (B), PLCγ2 Y759 (C) and Akt S473 (D) are from at least 3 independent experiments with platelets from at least 3 different donors. They are represented as ratio compared to α-actinin for LAT and to the total protein. Data are from at least 3 different experiments with platelets from at least 3 healthy volunteers and represented as mean ±S.D. ns: not significant, *p<0.5, ****p<0.0001.

Despite Syk hyperphosphorylation and increased Syk activity, Akt phosphorylation on S473 was completely abolished by iloprost and riociguat (Figure 55A, D).

5.4.5 Further validation of cAMP/PKA-mediated platelet Syk hyperphosphorylation by immunoprecipitation

To validate Syk phosphorylation signals and its phospho-regulation, Syk was immunoprecipitated from lysates of washed human platelets. Syk was successfully bound to the Syk monoclonal antibodies, whereas no signal for Syk (72 kDa) was detected with the control mouse IgG_{2a} (Figure 56). However, a non-specific band was detected at around 50 kDa

by both Syk and the control IgGs which most probably refers to the heavy chain of the mouse immunoglobulins present in all samples (Syk and control IgGs derived from mice sera).

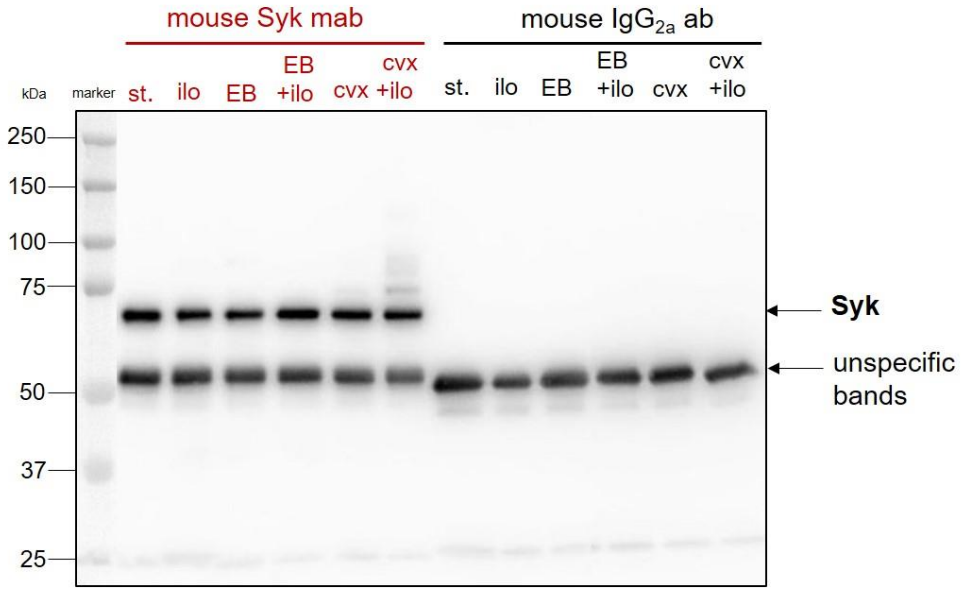

Figure 56. Syk immunoprecipitation from lysates of human platelets.
WP were preincubated with or without iloprost (2 nM; 3 min) prior to stimulation with EB or cvx (50 ng/ml). Platelets were lysed using NP40 lysis buffer after 120 s of stimulation. Syk was immunoprecipitated as mentioned in paragraph 4.10. Proteins were separated by electrophoresis on 8% SDS-gel. Immunoblotting was performed using monoclonal mouse anti-Syk antibodies (1:1000) and signal was detected by ECL using goat anti-mouse polyclonal antibodies (1:5000).

The same blot was reprobed with phospho-Syk Y525/526 antibodies. Syk phosphorylation was increased after EB and more after convulxin stimulation. This phosphorylation was enhanced in presence of iloprost (Figure 57). A similar result was obtained for the phosphorylation of Syk Y352 (Figure 58).

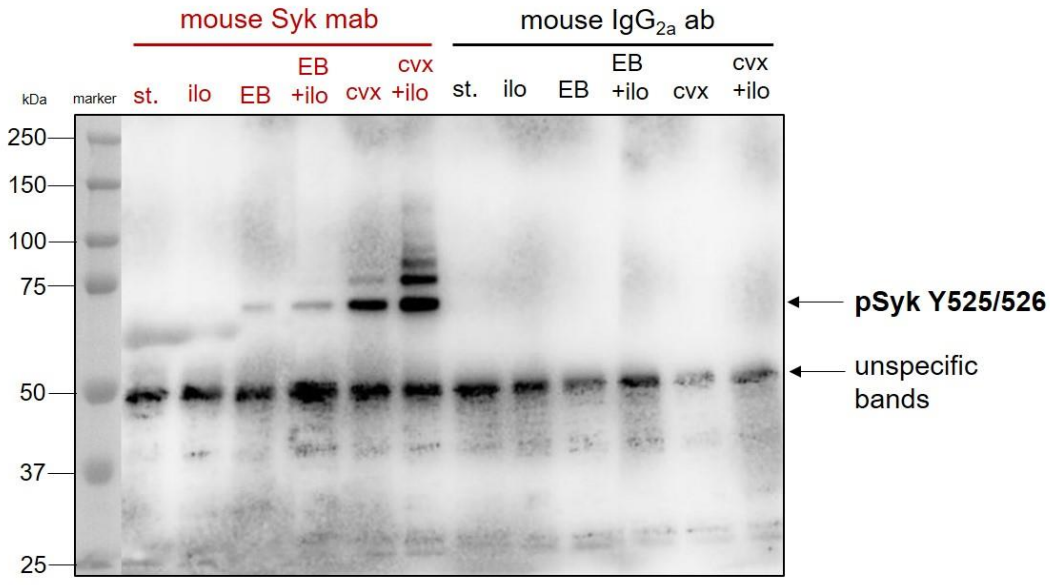

Figure 57. Immunoprecipitation of Syk and Syk pY525/526 detection.

Blot with total Syk antibody was stripped and reprobed overnight with monoclonal rabbit anti-pSyk Y525/526 antibodies (1:1000) and signal was detected by ECL using goat anti-rabbit polyclonal antibodies (1:5000).

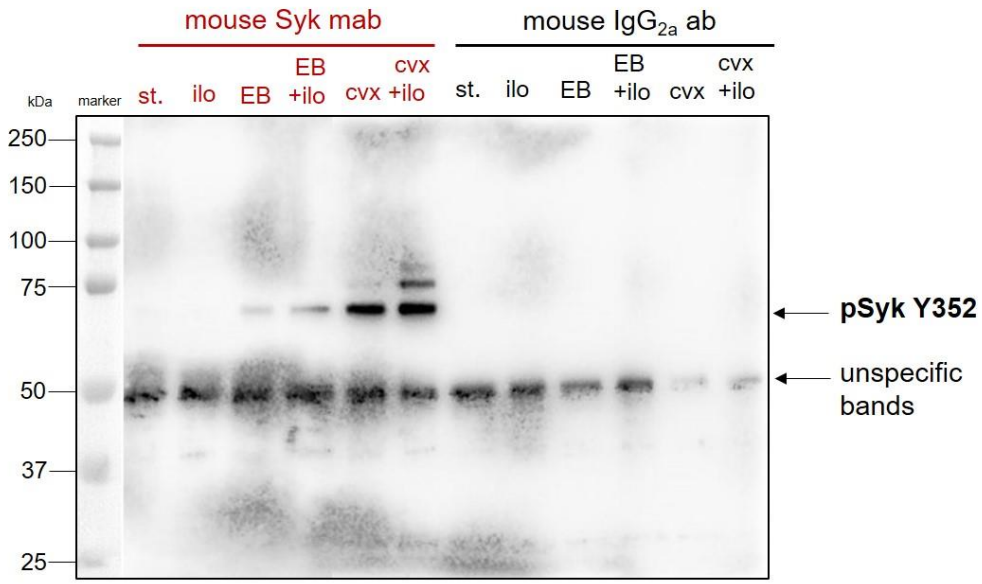

Figure 58. Immunoprecipitation of Syk and Syk pY352 detection.
Blot with pSyk Y525/526 antibody was stripped and reprobed overnight with monoclonal rabbit anti-pSyk Y352 antibodies (1:1000) and signal was detected by ECL using goat anti-rabbit polyclonal antibodies (1:5000).

In all IP-Syk blots (Figure 56-58), some additional bands appear with higher MW than for Syk (72 kDa) when platelets were stimulated with convulxin +/- iloprost and they are recognized by the Syk monoclonal antibody. These data could be explained that Syk is regulated by different posttranslational modifications such as ubiquitination, which affect the mobility in SDS PAGE gel.[159-161]

To minimize unspecific signals upon stripping and reprobing the same blot, another experiment was performed to check the regulation of Syk S297 phosphorylation by immunoprecipitation.

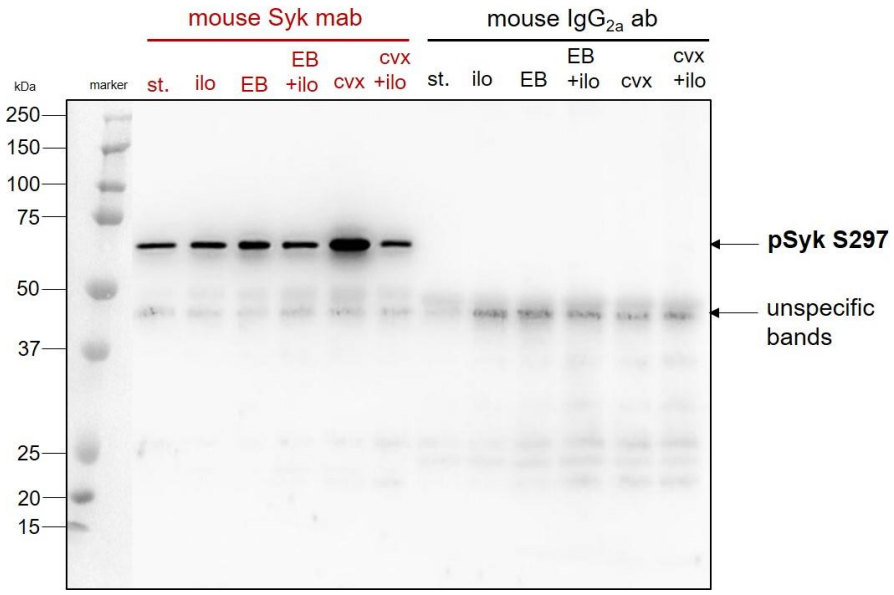

Figure 59. Immunoprecipitation of Syk and Syk pS297 detection.
WP were preincubated with or without iloprost (2nM; 3 min) prior to stimulation with EB or cvx (50 ng/ml). Platelets were lysed using NP40 lysis buffer after 120 s of stimulation. Syk was immunoprecipitated as mentioned in paragraph 4.10. Proteins were separated by electrophoresis on 10% SDS-gel. Immunoblotting was performed using polyclonal rabbit anti-pSyk S297 antibodies (1:1000) and signal was detected by ECL using goat anti-rabbit polyclonal antibodies (1:5000).

A basal phosphorylation level of Syk S297 was observed under control conditions (stirring) and when platelets were preincubated with iloprost without further stimulation. EB induced Syk S297 phosphorylation, which was inhibited by iloprost. A higher stimulation level was detected after cvx stimulation, which was also inhibited by iloprost (Figure 59).

5.4.6 Effect of PKC inhibition on cvx-induced platelet activation

5.4.6.1 Effect of PKC inhibition on platelet aggregation

Syk hyperphosphorylation profiles were not only observed in GPIbα-mediated signaling pathways but also in GPVI. In addition to the potential role of PKC in regulating S297[156], a previous study reported Syk tyrosine hyperphosphorylation in the presence of GFX[162]. Therefore, we aimed to study the involvement the PKCs in cvx-induced platelet aggregation and cvx-induced Syk regulation.

GFX inhibited cvx-mediated platelet aggregation in a dose dependent manner. A concentration of 5 µM GFX was used for all the following experiments as also described by others before[162]. GFX at both 5 and 10 µM only partially inhibited cvx-induced aggregation (Figure 60).

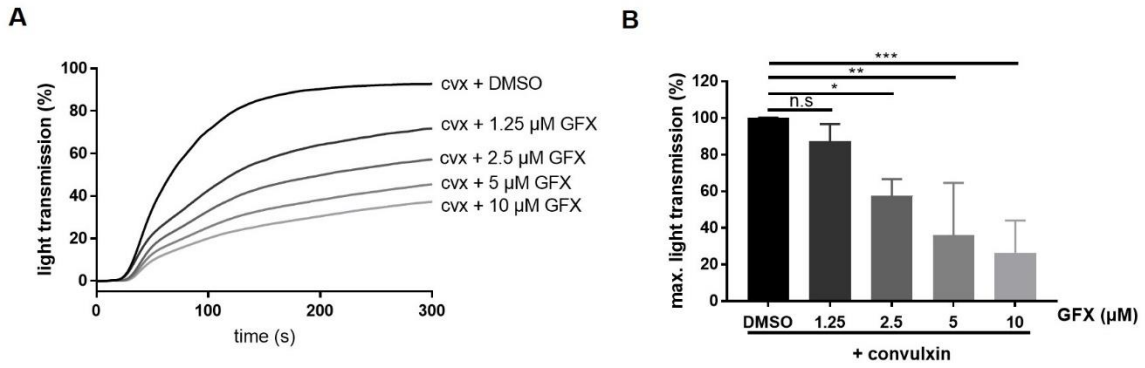

Figure 60. PKC inhibitor (GFX) inhibits cvx-mediated aggregation in a dose dependent manner.
WP were preincubated with vehicle control (DMSO) or with increased concentrations of GFX for 5 minutes at 37°C prior to stimulation with 50ng/ml cvx. (A) Representative aggregation curves showing the effect of the PKC inhibitor. Platelet aggregation was monitored until 5 minutes. B) Results are normalized to 100% compared to the vehicle control and represented as mean ± S.D of 3 independent experiments with platelets from 3 healthy donors. n.s: not significant, *p<0.0.5 **p<0.01, ***p<0.001.

Further experiments were performed with 5 µM GFX, where the platelet aggregation was only partially inhibited (around 70% inhibition compared to the vehicle control (Figure 61).

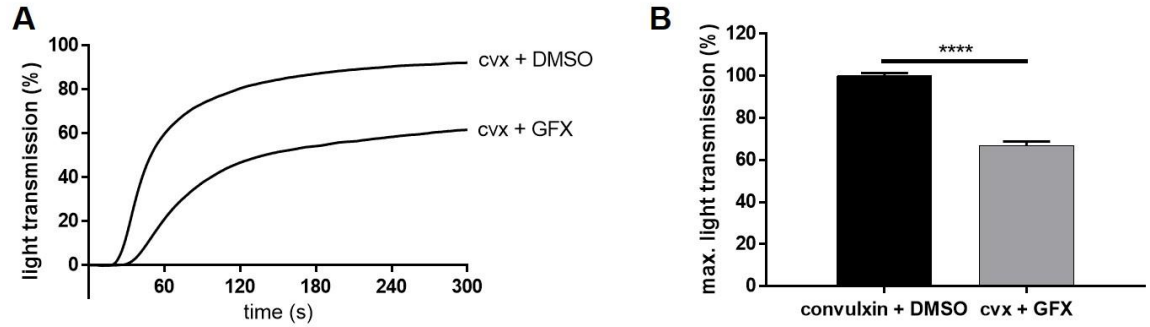

Figure 61. Cvx-mediated platelet aggregation partially depends on PKCs.
WP were preincubated for 5 minutes at 37°C with vehicle control (DMSO) or 5 µM GFX prior to stimulation with 50ng/ml cvx. (A) Representative aggregation curves showing the effect of the PKC inhibitor. Platelet aggregation was monitored until 5 minutes. B) Results are normalized to 100% compared to the vehicle control and represented as mean ± S.D of 3 independent experiments with platelets from 3 healthy donors (****p<0.0001).

5.4.6.2 Effect of PKC inhibition on Syk and its downstream effectors

As first analysis, phosphorylation of Syk Y525/526 as well as of Syk Y352 was studied, which showed comparable results. Significant enhancement of these phosphorylations was detected after 1 minute of stimulation. This phosphorylation seemed to be transient but the phosphorylation was significantly higher compared to the control even after 5 minutes of stimulation (Figure 62A, B, C).

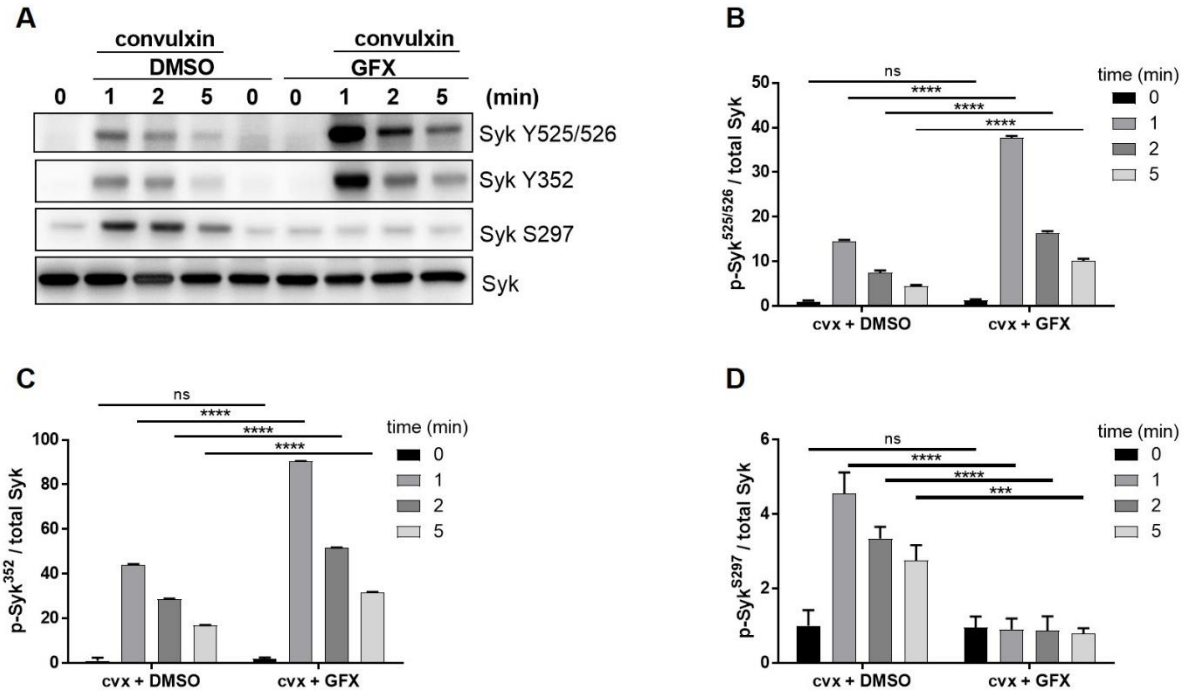

Figure 62. PKC inhibition enhances Cvx-induced Syk tyrosine phosphorylation and inhibits Syk phosphorylation on S297.
WP were treated as mentioned previously with vehicle control or GFX prior to stimulation with cvx. Platelet aggregation was stopped after 1, 2 or 5 minutes by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C for 10 minutes. (A) Western blot analysis was performed using antibodies against Syk Y525/526, Y352 and S297. The corresponding quantitation shown as ratio compared to total Syk, Y525/526 (B), Y352 (C) and S297 (D). Data are from at least 3 independent experiments with platelets from at least 3 different donors and presented as mean ±S.D. ns: not significant, ***p<0.001, ****p<0.0001.

Similar to Syk S297 regulation by PKC in GPIbα-signaling, the phosphorylation on Syk S297 was strongly reduced by the PKC inhibitor (Figure 62A, D).

Then, the aim was to analyze the phosphorylation of direct Syk substrates and the PI3K substrate, Akt. A similar phosphorylation profile of the Syk substrates LAT Y191 and PLCγ2 Y759 was observed as for the analyzed Syk tyrosine sites, (Figure 63A, B, C).

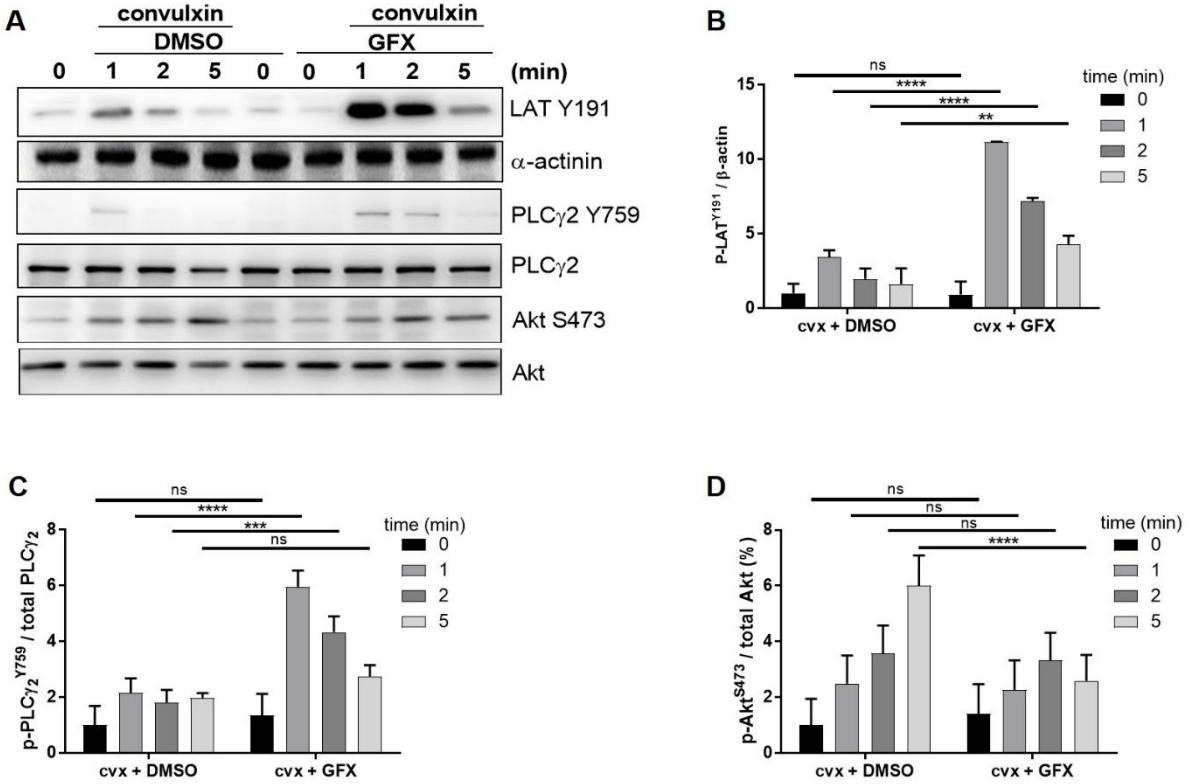

Figure 63. PKC inhibition enhances Cvx-induced LAT and PLCγ2 tyrosine phosphorylation but partially inhibits Akt S473 phosphorylation.
WP were treated as mentioned previously with vehicle control or GFX prior to stimulation with cvx. Platelet aggregation was stopped after 1, 2 or 5 minutes by adding directly Laemmli buffer into cuvettes. Samples were cooked at 95°C for 10 minutes. (A) Western blot analysis was performed using antibodies against LAT (Y191), PLCγ2 Y759 and Akt S473. Quantitative analysis of LAT Y191 (B), PLCγ2 Y759 (C) and Akt S473 (D) are from at least 3 independent experiments with platelets from at least 3 different donors. They are represented as ratio compared to β-actin for LAT and to the total protein. Data are from at least 3 different experiments with platelets from at least 3 healthy volunteers and represented as mean ±S.D. ns: not significant, **p<0.01, ***p<0.001, ****p<0.0001.

However, Akt S473 phosphorylation showed a different profile. At times 1 and 2 minutes, there was no significant effect compared to the vehicle control, but after 5 minutes of stimulation, Akt phosphorylation was clearly inhibited (Figure 63A, D).

5.4.7 IP1 regulation in cvx-induced platelet activation

To monitor the PLCγ2 activation in response to convulxin, the IP1 accumulation was checked as marker for IP3, which is produced by PLCγ2. Platelets stimulated with cvx had an 10-fold increased IP1 accumulation, which (different from EB response) was not affected by the presence of the echicetin monomers. However, this level of IP1 accumulation was totally inhibited in presence of the Syk inhibitor (PRT318) and strongly inhibited by iloprost and riociguat. Surprisingly, the inhibition of the protein kinase C by GFX enhanced cvx-mediated IP1 accumulation about 3 times (Figure 64).

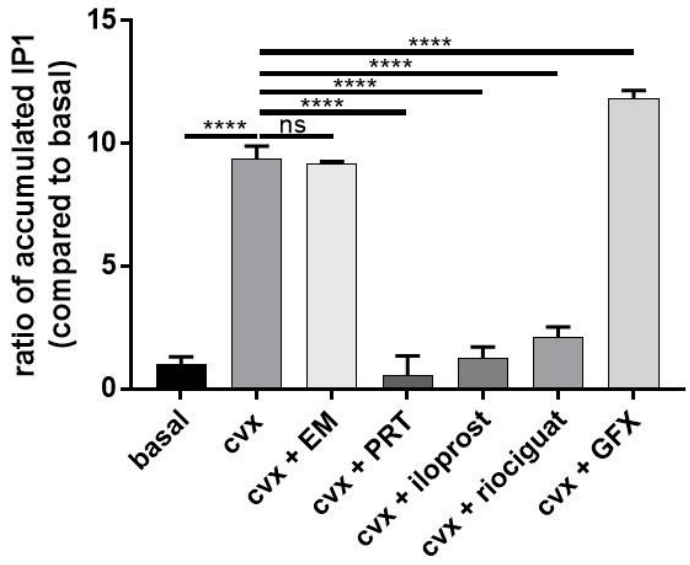

Figure 64. Differential regulation of the IP1 accumulation induced by convulxin.
WP were preincubated as described before with echicetin (25 µg/ml, 3 min), PRT318 (1 µM, 5 min), iloprost (2 nM, 3 min), riociguat (20 µM, 2 min) and GFX (5 µM, 5 min) prior to stimulation with 50 ng/ml cvx in presence of 1 mM LiCl. Aggregation was stopped after 5 minutes using the lysis buffer provided by the manufacturer. Data are representative of 3 different experiments from 3 healthy donors. Results are means ± S.D. ns: not significant, ****p<0.0001.

5.4.8 Regulation of platelet endogenous Ca2+ release induced by convulxin

In response to increased levels of produced IP3, Ca^{2+} is released from the dense tubular system (DTS). Therefore, intracellular Ca^{2+} release was investigated in response to cvx under the following different treatments. Platelets stimulated with convulxin induced a 15-fold increase of Ca^{2+} release, which was completely inhibited by the Syk inhibitor (PRT318). A partial inhibition of Ca^{2+} release was observed in the presence of iloprost (2 nM), which was significantly more inhibited at a higher concentration of iloprost (5 nM). Riociguat showed also a partial inhibition of Ca^{2+} release (Figure 65A).

The inhibition of the integrin $\alpha_{IIb}\beta_3$ (using tirofiban) inhibited the Ca^{2+} release evoked by cvx, however, inhibiting the receptors of ADP and TxA_2 inhibited more strongly this release, which was not further inhibited by adding iloprost (Figure 65B).

Of interest, PKC inhibition enhanced the cvx-mediated Ca^{2+} release which was partially inhibited by the PI3K inhibitor (wortmannin) (Figure 65C).

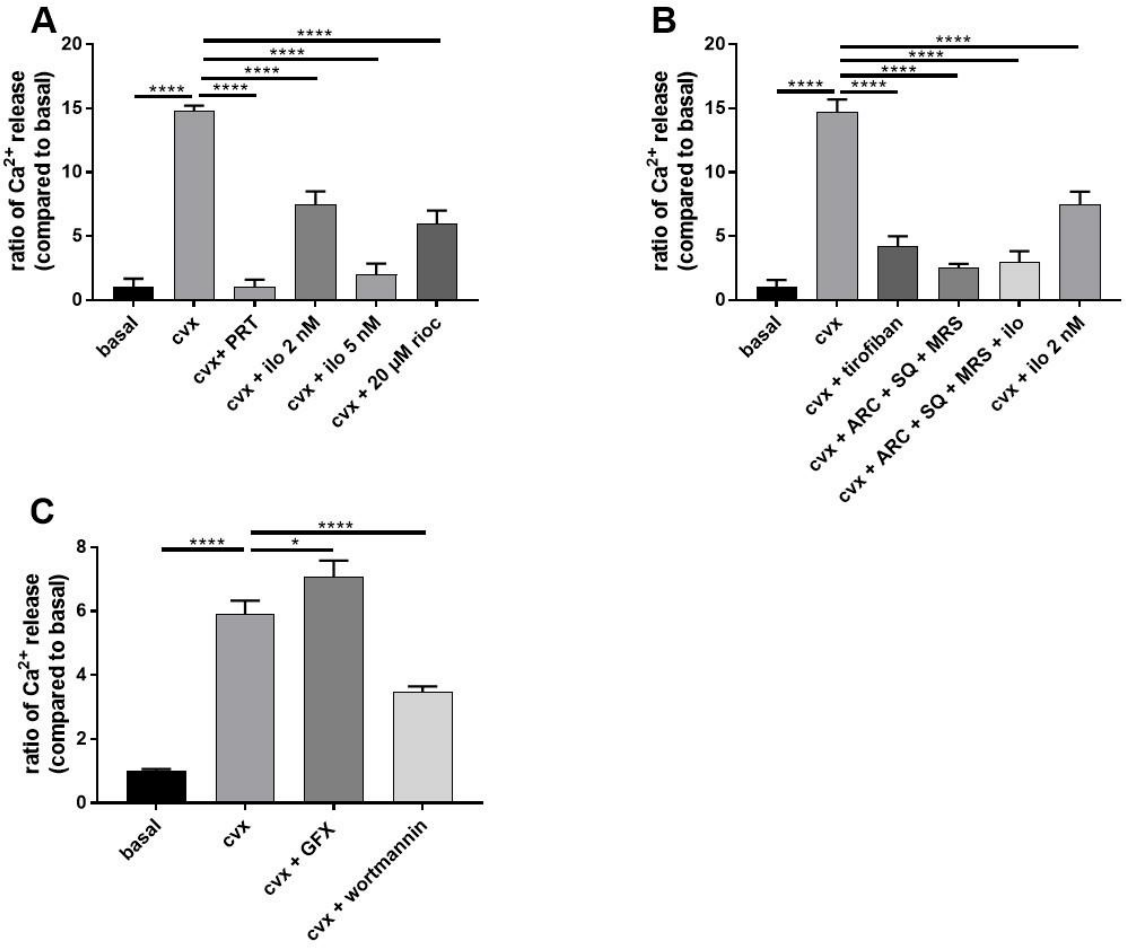

Figure 65. Differential regulation of cvx-mediated Ca^{2+} release.
WP were preincubated for 30 minutes at 37°C with Fluo-3, AM and then WP were preincubated with PRT318 (1 µM, 5 min), iloprost (2 or 5 nM, 3 min), riociguat (20 µM, 2 min), ARC+ SQ + MRS (0.1, 1, 1 µM respectively, 5 min), tirofiban (1.25 µg/ml, 1 min), GFX (5 µM, 5 min) and wortmannin (100 nM, 5 min) prior to stimulation with 50 ng/ml cvx. Ca^{2+} release was monitored for 2 minutes. Data are representative of 3 different experiments from 3 healthy donors. Results are means ± S.D. ns: not significant, *p<0.05, ****p<0.0001.

6 Discussion

6.1 GPIbα-mediated activation mechanisms

6.1.1 Specificity of echicetin beads

The data of my dissertation establish the signaling pathway of human platelet GPIbα by using a selective ligand, echicetin coated on polystyrene beads (EB). The EB-mediated signaling pathway induces SFK-dependent Syk activation, leading to Ca^{2+} release and full platelet aggregation. These effects are antagonized by echicetin as native single protein (EM). Echicetin was successfully purified from *Echis carinatus sochureki* crude venom after two successive purification steps: affinity chromatography (isolating echicetin) by using polyclonal antibodies against echicetin and DEAE weak anion exchange chromatography (IEX). Western blot analysis and silver staining confirmed the isolation of two different forms of proteins eluted in two separated peaks. Additionally, mass spectrometry validated that echicetin is found in the first peak in an equal ratio of α- and β- subunits. In the second peak, β- subunit is present and α- subunit only in small amount. However, according to the MS data one sequence was found not matching to the *Echis carinatus sochureki* species, which indicate a non-specific protein or the presence of some impurities maybe due to the isolation process of the crude venom. Therefore, only proteins from peak 1 were used in all the experiments.

Echicetin binds selectively to GPIbα without unspecific activation effects. It does not affect platelet aggregation induced by collagen, ADP, TRAP-6 and thrombin. It competes the binding of vWF to its main receptor (GPIbα) inhibiting vWF/ristocetin-mediated platelet agglutination and aggregation in washed platelets. The coating of echicetin on polystyrene beads with a separating distance less than 7 nm, resembles to the repetitive domains of the vWF and triggers GPIbα- receptor clustering inducing full platelet aggregation including IP3 production followed by Ca^{2+} release; these activation aspects are strongly antagonized by EM. Moreover, EB induced platelet aggregation only in wild-type mice but not in mice expressing the IL4/GPIbα fusion protein in which most of the extracellular GPIbα has been replaced by a human interleukin-4 receptor α-subunit domain (IL-4Rα)[95]. These data established that EB and EM can be used to probe the participation of GPIbα for the regulation of intracellular pathways of human and murine platelets.

6.1.2 Role of SFKs in EB/GPIbα-specific signaling pathway

Using EB as selective GPIbα ligand helped to dissect the signaling pathway specifically mediated by GPIbα. The SFKs (src and Lyn) are activated by the GPIbα subunit upon EB binding,which is inhibited by using the SFK inhibitors, PP2 and dasatinib in a non-ATP and an ATP competing mechanisms, respectively. Both inhibitors are widely used in cancer research and treatment as potent and selective SFK inhibitors. These data and other data published[40] demonstrate that SFKs play an essential role in human platelets since PP2 and dasatinib

prevented the platelet aggregation mediated by some of the main platelet receptors (GPIbα, GPVI and $P2Y_{1/12}$ receptors. In contrast to imatinib, another tyrosine kinase inhibitor known as weak SFK inhibitor did not show any significant effect.

Following the activation of SFKs, the signal propagates rapidly and activates the spleen tyrosine kinase, Syk, which has an essential role in the primary activation mechanisms. I assessed Syk activation by studying the phosphorylation of Syk itself and two of its direct substrates, the linker adaptor protein for T cells (LAT Y191) and the phospholipase Cγ2 (PLCγ2 Y759). From the Syk tyrosine phosphosites that I measured, the sites Y525/526 are located in the kinase domain and the sites Y352 and S297 in the interdomain B. Syk activation is initiated when the tyrosine sites of Syk (Y525/526 and Y352) are phosphorylated by the SFKs and by autophosphorylation or by the dually phosphorylated ITAM chains which recruit Syk to the membrane [109, 163].

The rapid phosphorylation of Syk on several phospho-sites upon EB stimulation (Y352, Y525/526, S297) was transient and decreased with time suggesting that a strong dephosphorylating process followed the initial activation. Previous studies [164] showed that dephosphorylation of murine Syk Y346 (Y352 in human platelets) by the protein tyrosine phosphatase TULA-2, suppressed its activation in murine platelets. The functional role of TULA-2 (also known as Ubiquitin-associated and SH3 domain-containing protein B) for Syk is discussed later (paragraph 6.3).

6.1.3 ITAM involvement in GPIbα-mediated platelet activation

Trying to understand the involvement of the ITAM-mediated Syk activation in the GPIbα-specific pathway, previous studies described the presence of two ITAM-containing proteins in human platelets, the Fc receptor γ-chain (FcRγ) and the low affinity IgG receptor FcγRIIa[11, 165]. The FcRγ chain is considered essential for the GPVI expression on the platelet surface and GPVI function[57]. However only the FcRγ chain has been described in murine platelets [61, 62]. In our previous study[95], we could demonstrate that EB activate strongly murine platelets. Therefore, it is more likely that the FcRγ chain is involved rather than the FcγRIIa. Quantitative proteomic studies[166] with human platelets demonstrated the expression of the FcRγ with 8,170 copies, FcγRIIa with 990 copies and Syk with 4,900 copies. Furthermore, an ongoing phosphoproteomic study (to be published) from our group in collaboration with the ISAS Dortmund detected multiple tyrosine-phosphorylated proteins in EB-stimulated human platelets including dually Y-phosphorylated FcRγ (Y56 and Y76) with 1.8 fold increased phosphorylation, however, tyrosine phosphorylation of FcγRIIa was not detected.

These data together indicate that EB activation of the human GPIbα complex stimulates Syk phosphorylation via an SFK-dependent mechanism, which is antagonized by EM. Syk

activation by SFKs involves direct phosphorylation of Syk Y352 (see also below) and probably phosphorylation of the platelet ITAM protein FcRγ.

6.1.4 Role of Syk in GPIbα-specific signaling pathways and signal propagation

In order to validate the functional role of Syk in the GPIbα-signaling in human platelets, we used two different Syk inhibitors, OXSI-2 and PRT-060318. Both inhibitors have been described as Syk and platelet function inhibitors in GPVI signaling[153, 167]. PRT318 prevented heparin-induced thrombocytopenia (HIT) and thrombosis in a mouse model[154]. In my studies, both compounds inhibited significantly EB-mediated platelet aggregation as well many tyrosine phosphorylation sites including phosphorylation of Syk Y525/526 located in the activation loop, as expected. Interestingly Syk S297 phosphorylation was also inhibited, indicating that EB stimulation of S297 phosphorylation required Syk activity. However, Syk Y352 phosphorylation was not inhibited, suggesting that Syk Y352 is phosphorylated by SFKs rather than by Syk autophosphorylation. Furthermore, Syk inhibition abolished the phosphorylation of the direct substrate PLCγ2 (Y759) and a downstream effector Akt (S473) indicating that EB activate human platelets by Syk-dependent process. A representative scheme of EB/GPIbα is shown in Figure 66 to summarize the activation process downstream of GPIbα based on the obtained results.

6.1.5 Role of the secondary mediators ADP/TxA_2 and integrin $\alpha_{IIb}\beta_3$ in GPIbα

Other receptors such as GPVI and CLEC-2 activate platelets by a Syk-dependent mechanism and require the release of ADP and TxA_2 synthesis to further activate the platelets[155]. Therefore, we aimed to study the involvement of the secondary mediators in EB-mediated platelet activation. By blocking $P2Y_{12}$, $P2Y_1$ and TP receptors (TxA_2 receptors) simultaneously, we noticed a full inhibition of aggregation. In contrast, Syk Y525/526 phosphorylation was only partially inhibited and Syk Y352 phosphorylation not at all. On the other side, Syk S297 phosphorylation was significantly inhibited but gradually increased again after 5 minutes of stimulation. PLCγ2 and Akt were strongly inhibited by blocking the receptors of the secondary mediators all together. These data demonstrate that the initial Syk tyrosine phosphorylation/activation is not dependent on secondary mediators, in contrast to further downstream events of Syk including Syk S297 phosphorylatiom, IP3 production and aggregation.

Additionally to ADP and TxA_2, other pathways such as the activation of the integrin $\alpha_{IIb}\beta_3$ signaling influence various platelet activation events including ITAM (FcγRIIA)-dependent Syk stimulation[63, 168]. In order to evaluate the possible involvement of the outside-in signaling we compared Syk tyrosine phosphorylation between EB-stimulated platelets from healthy controls and from a Glanzmann's Thrombasthenia patient. The data obtained demonstrate that Syk tyrosine phosphorylation is independent from the integrin $\alpha_{IIb}\beta_3$ outside-in pathway.

6.1.6 GPIbα-mediated crosstalk between platelet activation and inhibition by cAMP/PKA and cGMP/PKG-mediated pathways

Platelet activation mechanisms are firmly regulated by a network of intracellular protein kinases and phosphatases controlling several substrates and effectors which are responsible for the regulation of the diverse platelet functions. The regulation of GPIbα and GPVI signaling by cAMP/PKA and cGMP/PKG pathways were not investigated so far. In this present study, the selective PKA pathway activator (iloprost) and PKG pathway activator (riociguat) inhibited EB-induced platelet aggregation dose dependently in washed human platelets. The conditions of use of these clinically used drugs are well established and their specificity was validated by monitoring their established substrates[147, 169, 170]. The maximum inhibitory concentrations abolished platelet aggregation but surprisingly not Syk tyrosine phosphorylation. EB stimulated Y525/526 phosphorylation was strong and even enhanced/prolonged (hyperphosphorylation) by both iloprost and riociguat; Syk Y352 phosphorylation was initially (60 sec) significantly decreased but then this phosphorylation was also increased/prolonged when compared to the control. The enhanced phosphorylation was also seen for Syk downstream substrates LAT and PLCγ2. In contrast, Syk S297 phosphorylation was differently regulated, a significant downregulation of the phosphorylation was observed, similar to Akt phosphorylation and platelet aggregation. This result maybe is explained by the high dependency of Syk S297 and Akt to the secondary mediators ADP and TxA_2 and their signaling since their effects are significantly inhibited by PKA and PKG pathways[24, 25].

Signal transmission via the protein tyrosine kinases (PTKs) is one of the essential ways to propagate the signal within the cell. There is already much known about the PTKs. However, less is known about the protein tyrosine phosphatases (PTPs) which play an important role in modulating the signals. The major role of PTPs is to dephosphorylate their targets, however, these enzymes are very selective and tightly controlled[171], they regulate many important aspects of cellular functions (differentiation, adhesion, migration and secretion) and diseases (cancer and autoimmune diseases)[102, 172]. They are present in platelets in two different classes, classical PTPs (the receptor-like CD148, PTP-1B, Shp1/2, MEG2) and the non-classical PTPs (PTEN, LMW-PTP, TULA-2, PP1, PP2A). Each of these PTPs plays a distinct role at different levels such as downstream the GPCRs, GPVI, $\alpha_{IIb}\beta_3$, as well at the level of platelet aggregation[173-175]. PTPs functions in platelets were also validated by the using mouse models[102].

Our preliminary phosphoproteomic data (not published) showed that the phosphorylation of the direct Syk phosphatase (Ubiquitin-associated and SH3 domain-containing protein B/ T-cell Ubiquitin ligand-2) TULA-2 [176], Y19 and Thr34 was downregulated by iloprost and by sodium nitroprusside (another NO donor). Additionally EB-induced TULA-2 phosphorylation on Y19

and Thr34 was significantly inhibited by iloprost and SNP. *In vivo* studies demonstrated Syk Y352 and Y525/526 hyperphosphosphorylation which was also validated by the increased phosphorylation of Syk direct downstream substrates PLCγ2, ERK, SLP-76 by using TULA-2 knock-out mice platelets and osteoclasts[164, 176, 177]. Therefore, one possible explanation for Syk hyperphosphorylation would be the inhibition of TULA-2 by PKA or PKG or another mediator which block the dephosphorylation of Syk tyrosine sites. However, the effects of the mentioned phosphorylation of TULA-2 on its activity are not known, and other mechanisms exist.

6.1.7 Role of PKCs in GPIbα-mediated platelet activation

The mechanism behind the increase and prolonged phosphorylation of Syk Y525/526 and Y352 is still under investigation in our laboratory. Principally, this could be due to either increased kinase activity/ phosphorylation or inhibited dephosphorylation or even both.

Interestingly, a previous study[162] showed a convulxin mediated- Syk Y525/526 hyperphosphorylation in the presence of a pan-PKC inhibitor (GFX). This finding may be a link or an explanation for my detected tyrosine hyperphosphorylation which I investigated. The inhibition of PKCs by such a pan-PKC inhibitor induced a partial inhibition of platelet aggregation. Syk tyrosine hyperphosphorylation of Y525/526 and Y352 was clearly observed and validated by the increased and prolonged phosphorylation of its direct downstream substrates LAT and PLCγ2.

Interestingly, Syk S297 phosphorylation was strongly inhibited by GFX in contrast to Syk tyrosine phosphorylation. A previous study showed a direct link between PKC and Syk via the phosphorylation of Syk on S291[156] or S297[110] in murine and human B lymphocytes, respectively. Moreover, Syk S297 phosphorylation was significantly increased upon BCR stimulation and recruited the 14-3-3 adaptor protein which specifically bound to phosphorylated Syk S297. The study proposed that this attenuates Syk recruitment to the membrane resulting in decreased Syk activation[110]. This is certainly an attractive hypothesis but different results (nature of the pS297 Syk adapter proteins, functional effects) were obtained with other systems [178, 179]. Clearly, a possible interaction between PKA/PKG and PKC has to be further investigated, which may explain the observed Syk hyperphosphorylation and Syk relocalisation in the cytoplasm (more details are presented in the last part of the discussion, paragraph 6.3).

6.1.8 Regulation of IP3 and Ca^{2+} release in GPIbα-mediated signaling

The elevation intracellular Ca^{2+} concentrations is essential for a full platelet activation in hemostasis and thrombosis. Two ways can lead to this increase; Ca^{2+} is released from intracellular stores such as the dense tubular system, lysosome-like acidic organelles and mitochondria or extracellular Ca^{2+} enters the intracellular space through plasma membrane

channels. The regulation mechanisms behind Ca^{2+} mobilization and especially behind the internalization of extracellular Ca^{2+} are not fully understood. However, it is well established that the release from intracellular stores involves phospholipases (such as the different isoforms PLCβ found downstream the GPCRs and PLCγ2 found downstream GPIbα, GPVI, CLEC-2, integrin $\alpha_{IIb}\beta_3$) which generate inositol-1,4,5-triphosphate (IP3) and 1,2-diacyl-glycerol (DAG) from phosphoinositide-4,5-bisphosphate (PIP2). IP3 induces Ca^{2+} release while DAG is involved in extracellular Ca^{2+} entry and PKC activation[180].

My results showed that selective EB binding to GPIbα triggers an increased IP3 production (measured by IP1 accumulation) and intracellular Ca2+ release. This GPIbα-induced IP3 production and Ca^{2+} release are completely dependent on GPIbα (response abolished by EM) and completely dependent on Syk (abolished by PRT318) but not integrin dependent $\alpha_{IIb}\beta_3$ (no effect in the presence of tirofiban). In contrast to the well-known strong Ca^{2+}release inhibition by PKA and PKG in response to the GPCR-mediated platelet activation (ADP, thrombin and TxA_2)[181, 182], GPIbα-mediated IP3 and Ca^{2+} release were only partially inhibited by cAMP/cGMP. These data indicate that iloprost and riociguat inhibit only partially EB-mediated specific GPIbα activation at the level of Ca^{2+} response and are more likely to cause a stronger inhibition downstream of the production of IP3 and Ca^{2+} release. A proposed model of EB-mediated GPIbα activation and its crosstalk with the inhibitory pathways mediated by PKA and PKG is presented in Figure 66 according to the findings of the presented work.

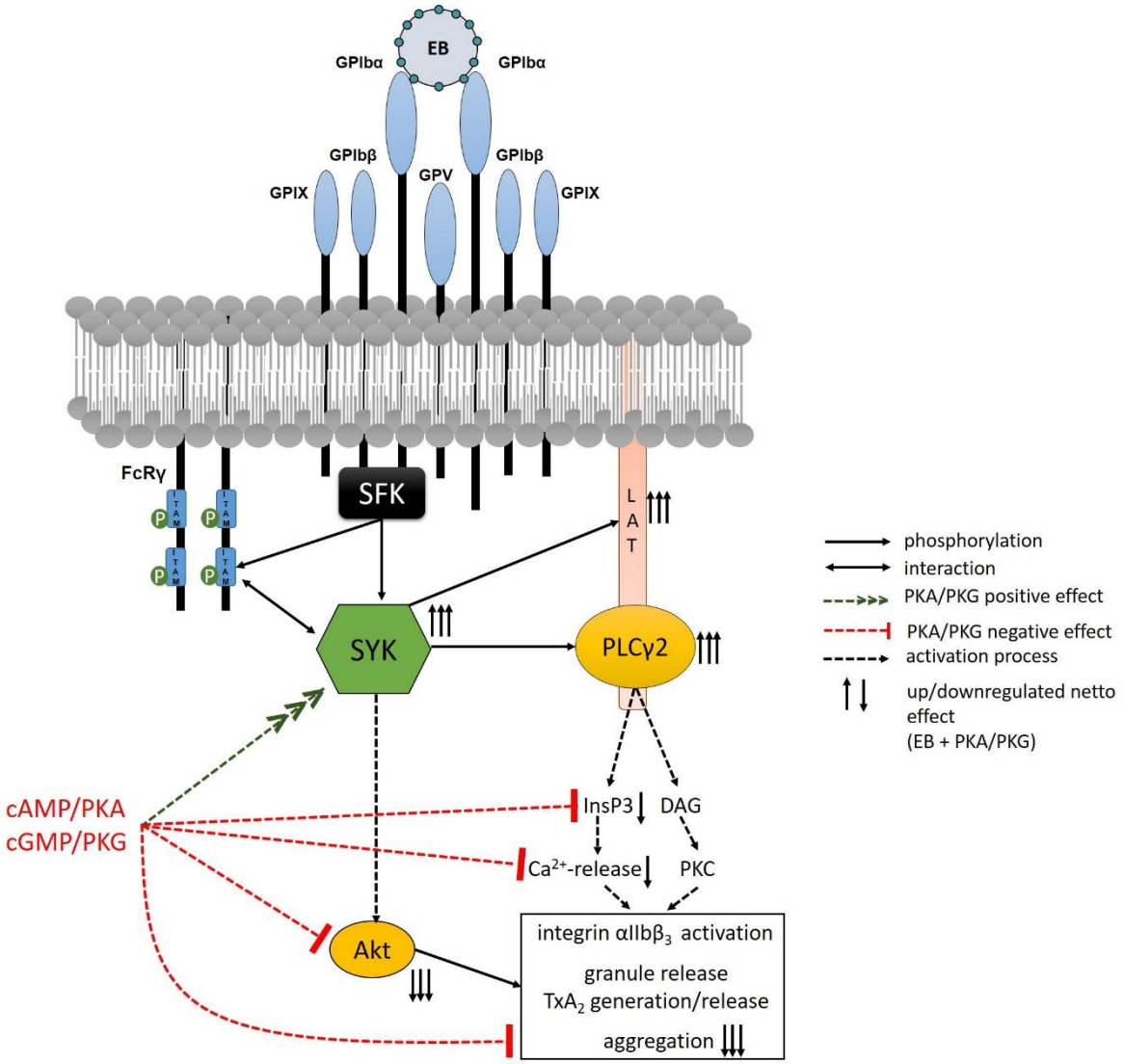

Figure 66. cAMP/PKA and cGMP/PKG pathways cause dichotomous regulation of GPIbα-mediated Syk stimulation and activation of human platelets.
Selective binding of the snake venom toxin echicetin as multimeric complex (echicetin beads, EB) to the extracellular domain of GPIbα leads to GPIbα crosslinking and initiates a signaling cascade starting from activation of src family kinases (SFK). This GPIbα activation resulted in tyrosine phosphorylation of ITAM-containing FcRγ-chains and SFK-dependent phosphorylation and recruitment of the spleen tyrosine kinase (Syk), via its SH2 domains to tyrosine phosphorylated ITAMs produce full Syk activation. Syk-dependent phosphorylation and activation of the adaptor protein (LAT), phospholipase Cγ2 and others lead to increased levels of IP3 (IP3; measured here by its metabolite IP1) and DAG, which are responsible for Ca^{2+} release and PKC activation (dotted black arrows). Additionally, Syk mediates indirectly the phosphorylation of Akt, one of Syk downstream effectors (dotted black arrow). Altogether, this leads to integrin activation, granule release and TxA_2 synthesis and subsequent platelet aggregation. The major platelet inhibitory pathways represented by cAMP/PKA and cGMP/PKG strongly enhance EB-induced Syk phosphorylation/activation (dotted green arrows) and enhance Syk-mediated tyrosine phosphorylation of LAT and PLCγ2 whereas IP3 increase and Ca^{2+} release was partially inhibited, Akt phosphorylation strongly inhibited (dotted red bars). The net effect of this crosstalk between platelet activation by EB and inhibition by cAMP/PKA and cGMP/PKG is marked with up or down black arrows (arrow number reflects the intensity of the effect). Syk and its direct substrates PLCγ2 and LAT are strongly activated (three arrows direction up), IP3 and its subsequent Ca^{2+} release are partially inhibited (one arrow down). The phosphorylation of Akt is strongly inhibited by PKA and PKG-elevating agents, similar to the overall aggregation (three arrows direction down).

Surprisingly PKC inhibition in GPIbα signaling induced a significant increase of IP3 compared to the agonist response, which was inhibited by iloprost in GPIbα activation mechanisms. This inhibition by iloprost might indicate that PKA regulates the PKC-regulated IP3 production and Ca^{2+} release. This regulation might occur at different levels of Ca^{2+} secretion process, such as PLCγ recruitment to the signalosome which can result in modulating its hydrolysis and its subsequent IP3 production and Ca^{2+} release. However, this observation needs to be further investigated. GFX effect on GPIbα-mediated activation effects is similar to GPVI; I will discuss this in more in detail in paragraph (6.2.4) since it has been studied previously in GPVI-mediated signaling.

Wortmannin, an inhibitor of the PI3Ks, inhibited the IP3 production stronger than iloprost, however both together inhibited the total generated IP3 upon EB activation. These data show that IP3 production does not always reflect Ca^{2+} release responses; GPIbα-mediated IP3 showed to be tightly dependent on ADP, TxA_2, and PI3Ks. Thus, Ca^{2+} release showed to be insensitive to these mediators (ADP, TxA_2 and PI3Ks).

These experiments were performed in washed human platelets in absence of extracellular Ca^{2+}, therefore we assume that the measured Ca^{2+} derives from intracellular stores. All the data together show the complexity of the mechanisms behind Ca^{2+} release and its regulation.

6.2 GPVI-mediated activation mechanisms and regulation

6.2.1 GPVI-mediated platelet activation and SFKs/Syk involvement

In my present study, a major aim was to compare the EB/GPIbα-mediated responses with the well-established convulxin/GPVI-specific signaling pathway. Similar to the literature[183], my data showed that convulxin induces a full platelet aggregation involving a time dependent increase in the tyrosine phosphorylation of Syk and its substrates LAT and PLCγ2. Syk S297 was studied here also for the first time in platelets, and a strong phosphorylation was observed within 15 s of cvx stimulation. The PI3 kinase substrate, Akt, has a similar regulation as observed in GPIbα, it revealed a late phosphorylation at S473. GPVI requires the ITAM-containing FcRγ chain for its expression and its activation. GPVI-FcRγ chain together trigger a robust and fast intracellular signaling via SFKs activation (Fyn and Lyn)[45, 98] resulting in Syk activation and its subsequent LAT, PLCγ2 phosphorylation. Based on these results, a model of the GPVI activation by convulxin is represented in Figure 67.

Syk is essential for a full GPVI-mediated platelet aggregation and response[183, 184] and Syk inhibition prevented Syk downstream events (PLCγ2 phosphorylation, IP3 and Ca^{2+}), but not Syk Y352 phosphorylation (observed also by other groups[153]) and only partially inhibited Y525/526 phosphorylation. In contrast, Cvx induced Syk S297 phosphorylation is completely

dependent on Syk activation by autophosphorylation of the kinase domain containing the tyrosine residues Y525/526 inducing a kinase active status.

6.2.2 Role of the secondary mediators ADP/TxA_2 and integrin $\alpha_{IIb}\beta_3$ in GPVI-mediated platelet activation

It has been reported that blocking secondary mediators such as ADP and TxA_2 prevents platelet aggregation mediated by low convulxin concentrations[155]. I now show that cvx-mediated platelet aggregation is only partially dependent on the secondary mediators ADP and TxA_2. Importantly, Syk tyrosine phosphorylation and activation was not dependent on ADP or TxA_2-activating pathways. However, Syk S297 phosphorylation and Akt S473 phosphorylation were highly dependent on these secondary mediators. These data are supported by published phosphoproteomic data from our group[147] that ADP stimulated Syk S297 phosphorylation in washed platelets. At the same time, PLCγ2 activation showed to be more dependent on the TxA_2 and not on the ADP-mediated feedback. This result might be the explanation of the significant inhibition of cvx-mediated Ca^{2+} release seen by blocking the secondary mediators (ADP and TxA_2 together). Furthermore, the inhibition of the integrin $\alpha_{IIb}\beta_{3-}$ mediated Ca^{2+} release is a result of the inhibition of the integrin outside-in signaling by tirofiban, which blocks the activation of the src-dependent PLCγ activation and consequently Ca^{2+} release. These data show that ADP and TxA_2 are involved in the platelet activation amplification mechanisms but not in the primary Syk phosphorylation and activation.

6.2.3 GPVI-mediated crosstalk between platelet activation and inhibition by cAMP/PKA and cGMP/PKG-mediated pathways

Despite the well-established work on the activation mechanisms mediated by GPVI, the crosstalk between GPVI-activation and inhibitory pathways needs further investigation. Our data showed that GPVI-induced Syk, LAT and PLCγ2 tyrosine phosphorylation, were not inhibited and even enhanced by cAMP- and cGMP- elevating agents, whereas platelet aggregation, Akt phosphorylation and Syk S297 phosphorylation were strongly inhibited. Compared to the interaction of GPIbα and cAMP/cGMP pathways, the crosstalk of the inhibitory pathways with GPVI activation seemed to be very similar. Moreover, it has been also reported that the cAMP pathway does not inhibit src, Syk and PLCγ2 tyrosine phosphorylation in GPVI-signaling[185], and that CLEC-2-mediated mouse platelet activation was weakly inhibited by cAMP but not by cGMP inhibitory pathway[186].

Previous work on the GPVI-mediated signaling pointed to the important role of the phosphatase TULA-2[187] in negatively regulating Syk activity upon GPVI stimulation and more specifically by dephosphorylating Syk Y346 in mice platelets[164] (equivalent to Syk Y352 in human platelets). Similarly to the GPIbα-PKA/PKG crosstalk (already discussed), it can be suggested that increased TULA- 2 phosphorylation (on Y19 and Thr34), which is inhibited by

the PKA and PKG pathway, is involved in the regulation of TULA-2 phosphatase. However, this clearly needs further investigation as other mechanism are possible.

PKA/PKG inhibitory pathways inhibit the overall platelet aggregation but definitely not the primary activation mechanisms mediated by SFKs and Syk. However, PKA and PKG might work on the level of phosphorylation of Syk substrates/binding proteins which can modulate Syk functions.

6.2.4 Role of PKCs in GPVI-mediated platelet activation

It was already discussed that PKCs are tightly involved in the GPIbα-mediated signaling pathway and Syk regulation. A similar mechanism may be involved in GPVI signaling. The interaction and involvement of PKCs in Syk activation and regulation in other models (B cell lines) were previously reported by several groups[156, 188]. Inhibition of PKCs partially decreases platelet aggregation stimulated by cvx. However, with PKC inhibition a hyperphosphorylation profile of both Syk tyrosine sites was observed. Previous work showed only a hyperphosphorylation profile by GFX for Y525/Y526 and not for Y352[162]. The inhibition of Syk S297 phosphorylation by GFX is additional evidence that PKCs regulate Syk directly via phosphorylation of S297 in washed human platelets as has been described for lymphocytes[110]. Taking all the presented data so far, the hypothesis can be developed that Syk tyrosine hyperphosphoylation is, at least partially, due to PKC inhibition, an effect also observed with the PKA/PKG pathways, perhaps via inhibition of PKCs. Alternatively, the major Syk tyrosine phosphatase (TULA-2) is activated by PKCs which is reversed by PKA/PKG.

The regulation of Syk S297 phosphorylation during cvx-induced platelet activation was addressed by a master student (Stephanie Dorschel) in our group using a novel method, phos-tag SDS-PAGE, which she also established in our laboratory. The phos-tag method provides a precise overview, simultaneously, on various phosphorylation sites of a protein compared to the non-phosphorylated ones[189]. Since the phosphorylation of each site results in a characteristic mobility shift of the protein in phos-tag SDS PAGE detectable by antibodies, it is possible to estimate the phosphorylation stoichiometry (complete phosphorylation, all protein shifted; no phosphorylation, no shift). With this method, Stephanie Dorschel demonstrated also by the Phos-tag method cvx-induced Syk tyrosine hyperphosphorylation of Y525/526 and Y352 in the presence of PKC inhibition (GFX). Importantly, this phos-tag method showed that cvx caused a stoichiometric Syk S297 phosphorylation (complete shift detected), which was prevented (return to basal) by both PKC inhibition (GFX) and by the PKA pathway (iloprost). Furthermore, specific activation of PKCs by the phorbol 12, 13-dibutyrate (PDBu) strongly induced phosphorylation of Syk on S297 within 15s in human washed platelets, but not the tyrosine sites (Y525/526 and Y352) (data not shown). In phos-tag SDS-PAGE this PDBu treatment caused an almost complete shift of Syk (stoichiometric phosphorylation the site

S297). These findings, the published data of our group and the data of my thesis demonstrate that the Syk 297 (in mouse Syk 291) is phosphorylated by one of the regular PKCs present in platelets, which can be blocked by PKC inhibitors and by the PKA/PKG pathways. It will be of interest to determine the PKC subtype(s) responsible for this phosphorylation, especially that PKCs are activated downstream of Syk/PLCγ and by a distinct pathway involving the ADP-mediated signaling. Both pathways inducing PKC activation trigger a fast phosphorylation of Syk S297.

6.2.5 Regulation of IP3 and Ca^{2+} release in GPVI-mediated signaling

Convulxin, a selective GPVI- agonist, triggers an increased levels of IP3 production (measured by IP1 accumulation) and a high intracellular Ca^{2+} release. GPVI-mediated IP3 production is GPIbα independent (no effect by EM) and the subsequent Ca^{2+} release are mainly dependent on Syk activation (abolished by PRT318).

GPVI-mediated Ca^{2+}release was only partially inhibited by iloprost and riociguat despite the significant IP3 inhibition. These data demonstrate the involvement of multiple activated pathways upon GPVI activation such as the ADP and TxA_2 activating pathways which are negatively regulated by cAMP and cGMP, which might be an explanation for the stronger inhibition of the GPVI-mediated IP3 production compared to the GPIbα. These results show that iloprost and riociguat partially inhibit GPVI-activation at the level of Ca^{2+} response and a stronger inhibition downstream these responses. A representative model of the crosstalk between platelet activation (by GPVI) and platelet inhibition by PKA and PKG according to the results obtained in this thesis work is represented below in Figure 67.

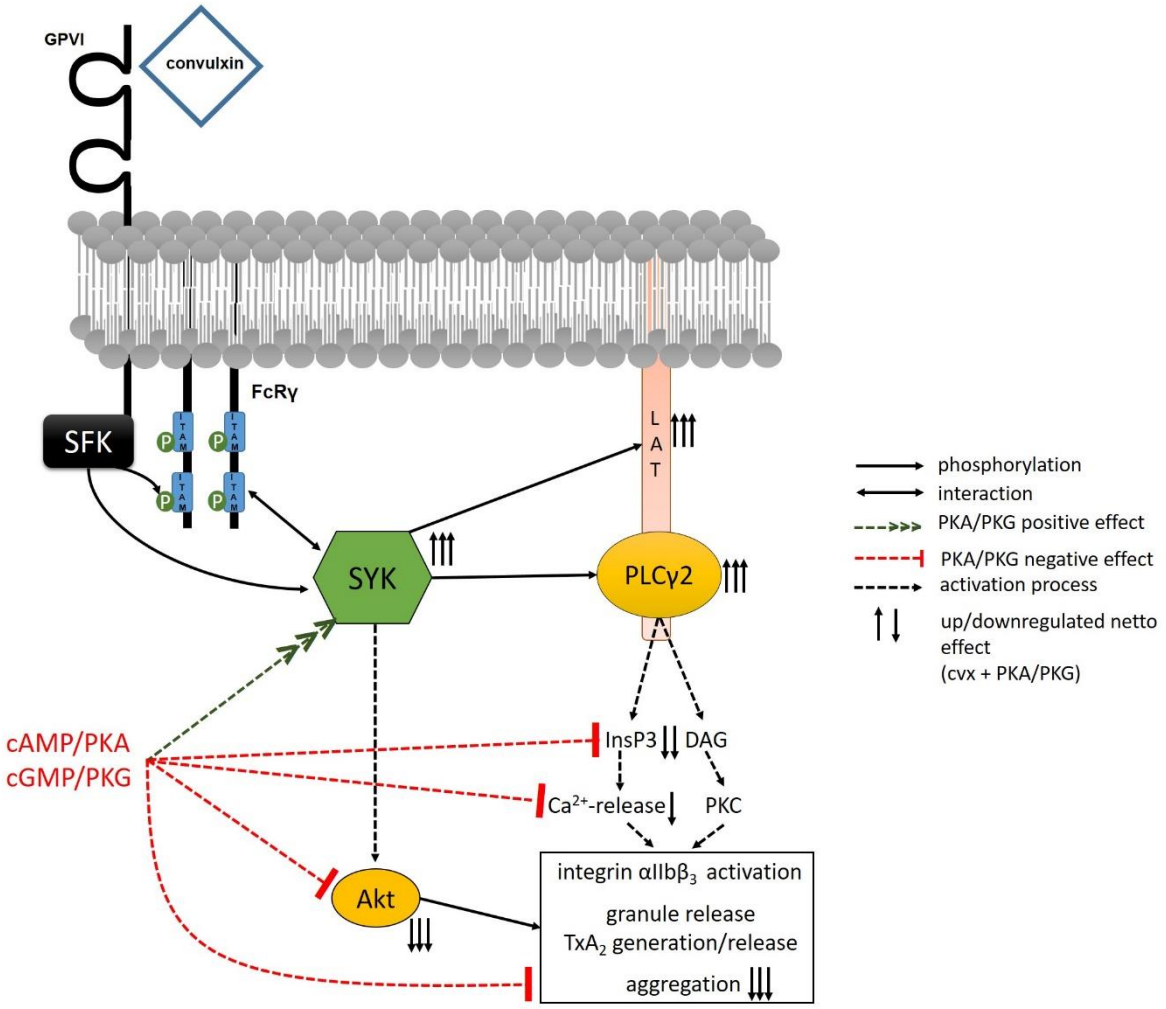

Figure 67. cAMP/PKA and cGMP/PKG pathways cause dichotomous regulation of GPVI-mediated Syk stimulation and activation of human platelets.
Selective binding of convulxin to GPVI leads to cluster GPVI dimers and initiates a signaling cascade starting from activation of src family kinases (SFK). This GPVI activation resulted in tyrosine phosphorylation of ITAM-containing FcRγ-chains and SFK-dependent phosphorylation and recruitment of the spleen tyrosine kinase (Syk), via its SH2 domains to tyrosine phosphorylated ITAMs produce full Syk activation. Syk-dependent phosphorylation and activation of the adaptor protein (LAT), phospholipase Cγ2 and others lead to increased levels of IP3 (IP3; measured here by its metabolite IP1) and DAG, which are responsible for Ca^{2+} release and PKC activation (dotted black arrows). Additionally, Syk mediates indirectly the phosphorylation of Akt, one of Syk downstream effectors (dotted black arrow). Altogether, this leads to integrin activation, granule release and TxA_2 synthesis and subsequent platelet aggregation. The major platelet inhibitory pathways represented by cAMP/PKA and cGMP/PKG strongly enhance convulxin-induced Syk phosphorylation/activation (dotted green arrows) and enhance Syk-mediated tyrosine phosphorylation of LAT and PLCγ2 whereas IP3 increase was significantly inhibited and Ca^{2+} release was only partially inhibited and Akt phosphorylation was strongly inhibited (dotted red bars). The net effect of this crosstalk between platelet activation by convulxin and inhibition by cAMP/PKA and cGMP/PKG is marked with up or down black arrows (arrow number reflects the intensity of the effect). Syk and its direct substrates PLCγ2 and LAT are strongly activated (three arrows direction up), IP3 strongly inhibited (two arrows down) and its subsequent Ca^{2+} release is partially inhibited (one arrow down). The phosphorylation of Akt is strongly inhibited by PKA and PKG-elevating agents, similar to the overall aggregation (three arrows direction down).

Surprisingly PKC inhibition in GPVI resulted in similar effects as observed with GPIbα (significant increase of IP3 and Ca^{2+} compared to the agonist response). It has been reported that each PKC isoform found in platelet play a distinct role in GPVI-mediated signaling[190].

However, there is variable literature, and the regulation of the different PKCs are not clearly understood. The inhibition of the isoforms α and β suppresses Ca^{2+} release and the deficiency in PKCθ increases significantly Ca^{2+} release[190]. It might be that under our conditions, the involvement of PKCθ plays a role in this increase of Ca^{2+} release in presence of GFX, since it has been reported that PKCθ is the principal isoform mediating the early effect of PKC in downregulating GPVI-induced platelet Ca^{2+} signaling and procoagulant activity[191].

Furthermore, PI3K plays an important role in GPVI-mediated platelet activation[115, 157, 192]. PI3K only partially inhibits GPVI-mediated Ca^{2+} release. This data suggest that PI3k only modulates these effects in GPVI-mediated signaling, which agrees with previous studies that demonstrated only a partial role of PI3k on all GPVI activation mechanisms[115, 192].
Unfortunately, due to limited number of samples that we can measure by ELISA IP1, only few samples were assessed, therefore the relation of IP1 and Ca^{2+} in GPVI cannot be clearly interpreted. However, in contrast to GPIbα, GPVI-mediated Ca^{2+} release seems to be highly dependent on secondary mediators and the integrin activation and only partially on the PI3Ks, which also can explain once more the strong inhibition mediated by iloprost and riociguat.
According to these data and findings, GPIbα and GPVI-mediated signaling pathways seem to be very similar but definitely not identical. The recruitment of SFKs, Syk and phosphorylation profiles of the tyrosine kinases is common for both pathways, however the recruitment of the secondary mediators for platelet aggregation is variable. The regulation of the activation pathways by PKA and PKG and the involvement of PKCs in both pathways and especially in Syk functions seem to be very similar. Hence, the major difference was observed at the level of Ca^{2+} release, where in GPVI, it is very dependent on the secondary mediators which is not the case in GPIbα- pathway. These data open new doors for further investigations and undergoing specialized studies at the level of platelet functional aspects.

6.3 Syk differential activation and regulation mechanisms

The essential role of Syk tyrosine phosphorylation for the activation of this kinase in immune cells and platelets was discovered and well described by many different approaches [193-196] although there is limited information how Syk is regulated in intact cells. As discussed before, cytosolic Syk is thought to be activated by two distinct, overlapping mechanisms designated as ITAM-dependent or tyrosine-phosphorylation-dependent switches (“OR-Gate” activation). Both mechanisms separate the kinase domain from the 2 SH2 domains and relieve the autoinhibition of Syk which involves the tyrosine phosphorylation of 2 tyrosine pairs within the interdomain linker (Y348/ Y352) and the kinase domain itself (Y525/ Y526)[195-198]. Syk activation is initiated when these Y-sites are phosphorylated by SFKs or when dually Y-phosphorylated ITAM-containing membrane proteins recruit the two Syk-SH2 domains. This

hypothesis is also supported by the structural data on the full-length Syk[198]. Syk activation is then completed by its autophosphorylation, especially of the sites Y525/526[195, 197]. Very recently, Mansueto et al. reevaluated the mechanism of Syk activation using various recombinant Syk proteins/mutants without tags and with/without pre-phosphorylation and present a revised model[199] (Figure 68).

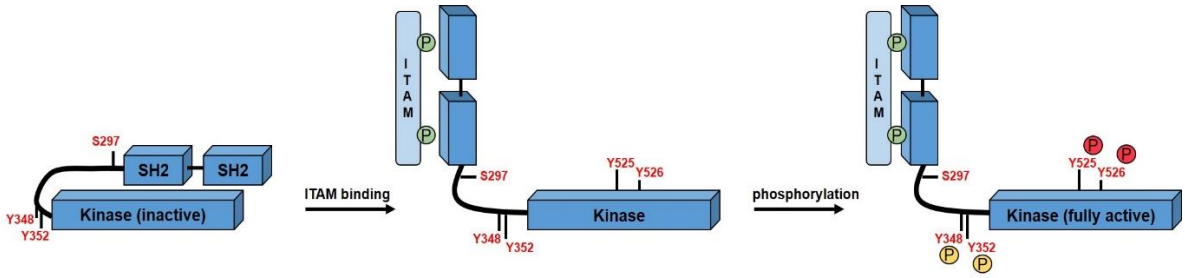

Figure 68. Suggested scheme of Syk activation mechanism.
Non-activated, autoinhibited Syk has a low binding affinity and a closed conformation (left pattern). Upon a receptor stimulation ITAM binding primes Syk (middle pattern) for a faster SFK-mediated Syk Y352 and Y348 phosphorylation. This leads to the phosphorylation in the activation loop (Y525/526) (third pattern). Both mechanisms, together, ensure a full kinase activation. Symbol of phosphorylation in green reflects the initial phosphorylation event, followed by the one marked in yellow and at the end in red. Scheme adapted from Mansueto M. et al (2019)[199]

They showed that ITAM binding primes Syk by making Y352 accessible for rapid SFK-mediated phosphorylation. This then facilitates Y348 phosphorylation which is tightly linked to activation loop phosphorylation at the sites Y525/Y526). Interestingly, Y525/526 and Y348 Syk (Y>F) mutants were clearly impaired in their kinase activity (but not abolished). However, other studies and especially phosphoproteomic studies described many additional tyrosine and also serine/threonine phosphorylation sites spread throughout the Syk molecule[110, 194]. These sites are not directly involved in Syk activation but serve as docking sites for other proteins and adapter molecules[193, 194]. For example, the ubiquitin ligase c-Cbl binds to Y323 (murine Y317) phosphorylated Syk[200, 201] and is responsible for Syk ubiquitination which enhances Syk activity[202, 203], see also further discussion below. The Y348 phosphorylated site (murine Y342) has been described as binding site for the SH2 domain of the Rac regulator Vav 1/3. The tyrosine phosphorylation of Y348 and Y352 (murine Y342,Y346) enhanced signaling both by increased Syk activity and by generating docking sites for binding proteins / adapters[194]. This includes proteins such as PLCγ, Vav-1/2, the Src-family kinases Lck and Fgr, the p85 subunit of PI3K and Grb2 which have SH2 domains and the capacity to bind to one or both of these phosphotyrosines. Some proteins bind when only one or the other tyrosine is phosphorylated and others bind preferentially when both are modified. The interaction between Syk and PLCγ1/2 is mediated by the PLCγ-SH2 domain and requires the dually phosphorylated Syk (murine Y342/Y346). C-terminal Syk contains 3 tyrosine phosphorylation sites (Y629, Y630, Y631, 4 amino acids less in the murine form) which serve (especially 630) as ligand for the SH2-domain of the adapter protein family BLNK/SLP65 (Figure 69).

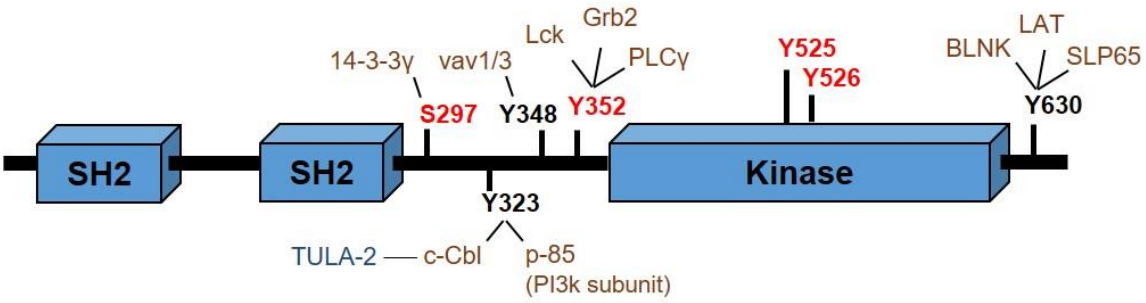

Figure 69. Representative scheme of Syk with some of its direct substrates and different phosphosites. Syk can interact with different proteins and kinases in order to form the signalosome, which is essential for the signal propagation. Only some are represented in this scheme (marked in brown above the corresponding phosphosite). Syk phosphatase, TULA-2 (marked in blue), binds to Syk via c-Cbl.

A more global study addressed both Syk phosphorylation sites and Syk interacting proteins (interactome) and investigated human Syk of resting and activated B cells by high- resolution mass spectrometry[110]. At total of 32 phosphorylation sites were reported with prevalence for tyrosine residues (15) followed by serine (11) and threonine (6), and most (23) of these sites are located within the interdomain linker/interdomain B (Syk residues 260-370), see also Figure 3 in the introduction. One of the most frequently detected P-site of Syk in this study was pS297, also within the interdomain B, which showed a strongly increased phosphorylation within 5 min in chicken DT40 B cells upon BCR stimulation. It was reported that the 14-3-3y adapter protein (also known as YWHAG; P61981) binds directly to this site pS297 which attenuated membrane binding of Syk and reduced its activity toward substrates (PLCγ2, SLP65), all lost in in Syk S297A mutants[110]. Parallel to this study, another group showed for murine B cells that PKC phosphorylates Syk at S291 (corresponding to S297 in human Syk)[178]. In this study, a reduced activation of transcription factors nuclear factor of activated T cells and Elk-1 was found in BCR-stimulated DT40 B cells expressing the S291A mutant of murine Syk. The compromised signaling response correlated with the inability of the S291A variant to associate with the chaperone prohibitin. However, no evidence of interaction of the pS291 sites with 14-3-3 proteins was observed[178]. In a related study but using a different cell line (the EBV-positive Burkitt lymphoma cell line Namalwa) Akt, 14-3-3 and importin 7 were shown to have a crucial role in the regulation of BCR signaling[179]. Overall, 14-3-3 proteins attenuated tyrosine phosphorylation of Syk and its substrate BLNK whereas Syk with 295A/297A mutations showed increased Syk Y525/Y526 phosphorylation. It was concluded that Akt and 14-3-3 proteins down-regulate the activity of several BCR-associated components, including BTK, BLNK and Syk[179]. There are marked differences/discrepancies between these three studies, perhaps due to different experimental systems (BCR stimulated chicken DT40 B cells, human Namalwa lymphoma cell, human versus murine Syk and other conditions). What these studies have in common is that Syk phosphorylation and activity is not only regulated by ITAM binding and/or tyrosine phosphorylation (Y348/Y352 interdomain B; Y525/526 activation loop) but also by many additional serine/threonine/tyrosine

phosphorylation sites and various adapter proteins. Interestingly, many but not all Syk adapter proteins are also Syk substrates[110, 193, 194]. It has also been recognized that the phosphorylation of many Syk substrates including BLNK/SLP-65, LAB/NTAL/LAT2, 3BP2, BCAP, BANK and GCET leads to the assembly of larger signaling complexes (signalosome)[194] . On the other side, Syk interacting proteins directly affect Syk properties and activity as was shown for the binding of the 14-3-3 adapter protein which attenuates Syk activity and tyrosine phosphorylation. A remarkable Syk binding protein which modifies Syk properties and activity is the ubiquitin ligase c-Cbl (known as E3 ubiquitin-protein ligase CBL). It binds to Y323 (murine Y317) phosphorylated Syk[200, 201] and is responsible for Syk ubiquitylation which enhances Syk activity[202, 203]. In c-Cbl–deficient mice, Syk is not ubiquitylated showing that c-Cbl is the responsible E3 ligase [203]. In contrast, others noted with c-Cbl-deficient mice increased phosphorylation of FcRγ chain, Syk, PLCγ2 together with an increased GPVI response [202]. There is substantial evidence that Syk, c-Cbl and TULA-2 interact and regulate Syk activity. The TULA-UBA domain binds to ubiquitin, while the TULA-SH3 domain facilitates interactions of Syk and TULA through Cbl which, as adapter, binds to both Syk and TULA-2 leading to down-regulation of signaling by Syk,[204, 205]. This is supported by the observation that a loss of TULA- 2 promotes Syk hyperphosphorylation and platelet hyperactivation[206, 207]. Another level of ubiquitylation/phosphorylation complexity was recently reported that multiple human platelet proteins (691 proteins, 1634 sites) including Syk are ubiquitylated upon GPVI activation by CRP[201]. Of special interest to this study, Syk showed with 17 ubiquitylation sites the most extensive intensity of modifications, concentrated at the interlinker domain B and at the kinase domain. While the function of the multiple site ubiquitylation is not clear, it is generally known that this modification can either target the protein for degradation by the proteasome or affect protein interactions or phosphorylation sites[208]. Clearly, a variety of mechanisms exist which modify (enhance or impair) the ITAM- and/or tyrosine phosphorylation- dependent activation of Syk and Syk interactions with important signaling proteins.

To sum up this part, the data of this thesis pinpoint on Syk regulation in GPIbα and GPVI-mediated signaling pathways. Syk activation was enhanced by PKA, PKG and PKC inhibition. However, an interesting result was observed with Syk S297 which is rapidly phosphorylated by ADP, EB and cvx-mediated platelet activation. According to our data, Syk S297 is likely mediated by PKCs and affected by PKA and PKG inhibitory pathways. Altogether, Syk S297 phosphorylation is an important target and modulator for the regulation of Syk activity that need to be further investigated.

7 Conclusions

This study established the specific mechanisms of human platelet activation induced by the vWF receptor (GPIbα) using echicetin beads and echicetin monomers as selective GPIbα agonists and antagonists, respectively. This made it possible to compare for the first time specific GPIbα-dependent signaling with the signaling of GPVI, the convulxin-activated collagen receptor, and their regulation by additional pathways.

EB/GPIbα-induced platelet activation resulted in a full platelet activation dependent on the activation of the src family kinases (SFKs) which phosphorylate ITAM containing membrane proteins at tyrosine residues. This then recruits the spleen tyrosine kinase (Syk) to the membrane forming a complex together with its direct downstream effectors such as LAT and PLCγ2. This pathway was assessed by the phosphorylation analysis of Syk and other proteins and was found to be very similar, but not identical, compared to the signaling stimulated by cvx/GPVI-mediated platelet activation. Both GPIb and GPVI signaling required, but to a different degree, the secondary mediators ADP/TxA_2 for a sustained platelet activation. However, primary platelet activation responses, for example Syk tyrosine phosphorylation and activation, was independent of the secondary mediators and the integrin $\alpha_{IIb}\beta_3$ outside-in signaling. GPIbα and GPVI- specific activation evoked the production of IP3 and consequently the release of intracellular Ca^{2+} stored in the intracellular dense tubular system.

Then and for the first time, the crosstalk between the activation mechanisms mediated by GPIbα/GPVI and the inhibitory signaling pathways regulated by cAMP/PKA and cGMP/PKG was investigated. The data, on the one hand, confirmed the literature that the PKA/PKG-pathways strongly inhibit GPVI-induced platelet aggregation which was now also observed with GPIbα-induced aggregation. On the other hand, Syk tyrosine hyperphosphorylation (sites Y352, Y525/526) and increased activation was observed when EB- or cvx-stimulated platelets were inhibited by cAMP- and cGMP-elevating mediators. This striking result is related to publish data on Syk tyrosine hyperphosphorylation when PKC was inhibited [162] . I not only validated these PKC data but also extended them to additional Syk substrates under my experimental conditions. There is evidence with murine and human B cells that PKC phosphorylates Syk at S291 (murine kinase) and S297 in human Syk)[156] which (when S297/S291 phosphorylated) binds directly to 14-3-3γ protein and other adapter proteins[110]. It was suggested that the Syk binding of 14-3-3γ proteins prevents the recruitment of Syk to the plasma membrane which prevents its subsequent activation and cellular effects. My data with human platelets demonstrate a strong, even stoichiometric but transient stimulation of Syk S297 phosphorylation by EB/GPIbα, cvx/GPVI and ADP. The increased S297 phosphorylation was abolished by the PKC inhibitor GFX and by the PKA / PKG system which caused Syk tyrosine hyperphosphorylation. These data support the present concept that EB/GPIbα and

cvx/GPVI rapidly stimulate one of the PKCs known to be present in platelets which then phosphorylates Syk S297 and which is inhibited by GFX and PKA/PKG. This is also supported by the data that a direct PKC activator (PDBu) stimulated Syk S297 phosphorylation. This strong and reversible phosphorylation of Syk S297 phosphorylation is located within the interdomain B which is a crucial site (hot spot) of Syk regulation by multiple pathways and modification (see Fig. 69 and discussion there). The observed Syk297 phosphorylation in response to GPIb and GPVI stimulation can be presently viewed as a PKC-dependent mechanism to limit Syk activation although the precise mechanisms needs to be elucidated. It is of considerable interest that PKCß was reported, also in B cells, to act as a feedback loop inhibitor of Btk activation, via Btk S180 phosphorylation [209]. Preventing Btk S180 phosphorylation caused a Btk-mediated hyperactivation of the B cell.

In addition to the tight regulation of Syk by multiple phosphorylation, modifications and binding proteins, many direct Syk substrates are known [210], others have been not yet identified, also in platelets. Since Syk substrates (such as PLCγ2, LAT1 and many others) are essential for the functions of Syk. It will be important to study their properties, regulation and interaction within the Syk network in more detail. Such direct Syk substrates were recently used to generate distinct Syk networks in cancer cells[211-213], which is also very relevant for human platelets. The impact of these Syk networks on the regulation of platelet activation in vitro and in vivo needs to be further investigated in order to elucidate the physiological and pathophysiological role of Syk in platelets, immune cells, thrombosis, inflammation and cancer. Therefore, understanding the complexity of Syk activation and its regulation by different phosphosites and interacting proteins will offer a greater insight into Syk functions. This will further increase the potential of Syk as promising diagnostic and pharmacological target.

8 Outlook and perspectives

The spleen tyrosine kinase (Syk) is an essential mediator for the activation of immune cells and as well in platelets. Syk plays an essential role for GPVI, CLEC-2 but also for GPIbα activation as demonstrated in the present study.

Recent developments in the field and this work show that Syk is a tightly regulated tyrosine kinase but the intracellular mechanisms of activation/down-regulation and the spectrum of targets (substrates) are only partially understood. My work with Syk and Syk signaling in human platelets, a primary human cell, has established data and conditions to elucidate important Syk functions in a highly relevant human model system.

In order to elucidate the relevance of Syk phosphorylation and regulation by ITAMs and/or by the different substrates, the next step is to investigate their relevance at the level of activation and inhibition of human platelets. In addition, studying the role of ITAMs in Syk activation *in vitro* using a specific Syk fluorescent kinase assay, which is commercially available, offers a promising insight. By establishing these methods, this will allow to further study the impact of different kinases or proteins on Syk regulation and phosphorylation on different sites. Since Syk is a potential therapeutic target, this will help to provide new, specific antagonists acting efficiently on different sites of Syk not only at the level of the ATP-binding kinase domain.

It will be possible to study the Syk interacting proteins during platelet activation (pulldown approach). Furthermore, with the established selective activation and inhibition of Syk and the proteomic/phosphoproteomic advances it will be possible to study the spectrum of protein tyrosine substrates of Syk. Of special interest are the cellular pathways and mechanism, for example Syk S297 phosphorylation, which affect the Syk interdomain B, a possible key for activating/inhibiting this tyrosine protein kinase. Based on the data of this study and the established methods, testing the binding of the 14-3-3γ protein to Syk S297 in human platelets has to be further investigated. It is very likely that such data are essential to enhance the understanding of Syk and its physiological and pathophysiological role in human cells, tissues and human diseases.

9 Abstract

The von Willebrand factor and collagen are the main physiological ligands of two platelet receptors, GPIb-IX-V and GPVI, respectively, which play an essential role in platelet adhesion and thrombus formation. However, these agonists also bind to the integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$, respectively, which prevents the analysis of specific GPIbα and GPVI signaling. C type lectin snake toxins are widely used as specific platelet receptor ligands. Echicetin is a selective GPIbα agonist when coated on polystyrene beads (EB), whereas convulxin (cvx) induces GPVI-specific signaling. Despite having this important role, intracellular GPIbα and GPVI signaling and their regulation by other pathways are not well defined. This limitation was addressed here using EB and cvx, in order to study the effects of GPIbα and GPVI activation on platelet aggregation, Syk tyrosine kinase, Syk substrates and the crosstalk with inhibitory pathways.

Echicetin purified from snake *Echis carinatus sochureki* venom was validated by mass spectrometry. Washed human platelets were stimulated with EB or cvx, in the presence or absence of echicetin monomers (EM), Src family kinase (SFK) inhibitors, Syk inhibitors and the cAMP-, cGMP-elevating agents, iloprost and riociguat respectively. Platelet aggregation was analyzed by light transmission aggregometry, protein phosphorylation by immunoblotting. Intracellular messengers inositol monophosphate (IP1) and $Ca^{2+}i$ were measured by ELISA and Fluo-3 AM/FACS, respectively.

GPIbα and GPVI-specific agonists, EB and convulxin, induced full platelet aggregation and strong phosphorylation of tyrosine and serine/threonine protein kinases and substrates such as Syk, $PLC\gamma_2$ (a direct Syk substrate) and Akt (a Syk downstream effector). These activation mechanisms were SFK- and Syk-dependent, integrin $\alpha_{IIb}\beta_3$ independent and differentially required the secondary mediators ADP, TxA_2. The activation of cAMP or cGMP pathways by iloprost or riociguat, respectively, strongly inhibited platelet aggregation and Akt phosphorylation but not EB/cvx-induced tyrosine phosphorylation of Syk and PLCγ2, which was often enhanced. For the first time in platelets, an important human model system for Syk regulation, a strong, stoichiometric but transient stimulation of Syk S297 phosphorylation by EB/GPIbα and cvx/GPVI was observed, which was abolished by PKC inhibition and by the PKA/PKG system. Both interventions caused Syk tyrosine hyperphosphorylation and increased activation. The Syk S297 phosphorylation site is located within the interdomain B, which is a hot spot for Syk regulation by multiple modifications.

The major inhibitory pathways PKA/PKG strongly inhibited EB/cvx-induced platelet aggregation and Akt phosphorylation but, in contrast, enhanced Syk and LAT/PLCγ2 tyrosine phosphorylation (hyperphosphorylation). These data suggest that Syk and Syk effector systems are differentially regulated by protein kinases PKA, PKG, and PKC. This is also

supported by the data that EB/cvx-induced Ca^{2+}-release was Syk-dependent, but only partially inhibited by PKA/PKG pathways.

Overall, the results of this project demonstrate EB and EM as specific agonists and antagonists, respectively, of GPIbα-mediated Syk activation modulating platelet aggregation. This is independent of the integrin alphaIIbbeta3 and GPVI activation. The cAMP/PKA and cGMP/PKG pathways do not inhibit but enhance GPIbα-/GPVI-initiated, SFK dependent Syk activation, but strongly inhibit further downstream responses including aggregation. GPIbα and GPVI affect distinct signaling pathways, which are similar but not identical. These data establish an important intracellular regulatory network induced by GPIbα and GPVI with Syk as central element. This essential kinase is controlled by multiple phosphorylation sites, modifications and binding proteins and has multiple substrates as effector system, which are not fully understood yet.

Zusammenfassung

Der Von Willebrand-Faktor und Kollagen repräsentieren die physiologischen Hauptliganden von zwei wichtigen Thrombozytenrezeptoren, GPIb-IX-V und GPVI, die eine wesentliche Rolle bei der Thrombozytenadhäsion und Thrombusbildung spielen. Jedoch binden diese Agonisten auch an die Integrine $\alpha_{IIb}\beta_3$ bzw. $\alpha_2\beta_1$, was die Analyse spezifischer GPIbα- und GPVI-Signale verhindert. C-Typ-Lektin Schlangentoxine werden häufig als spezifische Thrombozytenrezeptorliganden verwendet. Echicetin, immobilisiert auf Polystyrol- Beads (EB), ist ein selektiver GPIbα-Agonist, während Convulxin (Cvx) GPVI-spezifische Signale induziert. Trotz dieser wichtigen Rolle sind die intrazellulären GPIbα- und GPVI-Signale und ihre Regulation durch andere Signalwege nicht genau definiert. Diese Einschränkung wurde hier mit EB und Cvx behoben, um die Auswirkungen der GPIbα- und GPVI-Aktivierung auf die Thrombozytenaggregation, die Syk-Tyrosinkinase, die Syk-Substrate und das Ansprechen auf inhibitorischen Signalwegen zu untersuchen.

Aus Schlangen gereinigtes Echicetin-Gift von *Echis carinatus sochureki* wurde durch Massenspektrometrie validiert. Gewaschene humane Thrombozyten wurden mit EB oder Cvx in An- oder Abwesenheit von Echicetin-Monomeren (EM), Inhibitoren der Src-Familienkinase (SFK), Syk-Inhibitoren und den cAMP-, cGMP-erhöhenden Mediatoren, Iloprost bzw. Riociguat, stimuliert. Anschließend wurden die Thrombozytenaggregation durch Lichttransmissionsaggregometrie und die Proteinphosphorylierung durch Immunblotting analysiert. Die intrazelluläre Botenstoffe Inositmonophosphat (IP1) bzw. Ca^{2+}i wurden mittels ELISA bzw. Fluo-3 AM/FACS gemessen.

Die GPIbα- und GPVI-spezifische Agonisten, EB und Convulxin, induzierten eine vollständige Thrombozytenaggregation und eine starke Phosphorylierung von Tyrosin- und Serin/Threonin-Proteinkinasen und Substraten wie Syk, PLCγ_2 (eines der direkten Syk-Substrate) und Akt (eines der Syk-Downstream-Effektoren). Diese Aktivierungsmechanismen waren SFK- und Syk-abhängig, Integrin $\alpha_{IIb}\beta_3$-unabhängig und erforderten die sekundären Mediatoren ADP und TxA_2. Die Stimulierung von cAMP- oder cGMP-Signalwegen durch Iloprost oder Riociguat inhibierte deutlich die Thrombozytenaggregation und die Akt-Phosphorylierung, jedoch nicht die EB/Cvx-induzierte Tyrosinphosphorylierung von Syk und PLCγ2, die häufig verstärkt wurde. Zum ersten Mal wurde in Thrombozyten ein wichtiges menschliches Modellsystem für die Syk-Regulation, eine starke, stöchiometrische, aber transiente Stimulierung der Syk S297-Phosphorylierung durch EB/GPIbα und Cvx/GPVI, beobachtet, die durch PKC-Hemmung und durch PKA/PKG aufgehoben wurde. Im Gegensatz dazu, verursachten beide Interventionen eine Syk-Tyrosin-Hyperphosphorylierung und eine vermehrte Aktivierung. Die Syk S297-Phosphorylierungsstelle befindet sich innerhalb der Interdomäne B, die durch mehrere Modifikationen ein Zentrum für die Syk-Regulation darstellt.

Die inhibitorischen Signalwege, die durch PKA/PKG kontrolliert werden, hemmen die EB/Cvx-induzierte Thrombozytenaggregation und die Akt-Phosphorylierung, im Gegensatz dazu verstärkten sie die Syk- und LAT/PLCγ_2-Tyrosinphosphorylierung (Hyperphosphorylierung). Diese Daten zeigen, dass Syk- und Syk-Effektorsysteme durch Proteinkinasen PKA, PKG und PKC unterschiedlich reguliert werden. Dies wird gestützt durch die EB/Cvx-induzierte Ca^{2+}-Freisetzung, die Syk-abhängig war, jedoch nur teilweise durch PKA/PKG-Signalwege inhibiert wurde.

Insgesamt zeigen die Ergebnisse dieser Arbeit, dass EB und EM spezifische Agonisten bzw. Antagonisten der GPIbα-vermittelten Syk-Aktivierung sind, die die Thrombozytenaggregation modulieren. Dies ist unabhängig von der Aktivierung des Integrins alphaIIbb3 und GPVI. Die cAMP/PKA- und cGMP/PKG-Signalwege hemmen nicht die GPIbα-/GPVI-initiierte, SFK-abhängige Syk-Aktivierung jedoch weitere Downstream-Reaktionen, einschließlich der Aggregation. GPIbα und GPVI beeinflussen unterschiedliche Signalwege, die ähnlich, aber nicht identisch sind. Diese Daten bilden die Grundlage für ein wichtiges intrazelluläres regulatorisches Netzwerk, das durch GPIbα und GPVI mit Syk als zentralem Element induziert wird. Diese essentielle Kinase wird durch mehrere Phosphorylierungsstellen, Modifikationen und Bindungsproteinen gesteuert und weist mehrere Substrate als Effektorsystem auf, die noch nicht vollständig verstanden sind.

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11 Appendix

11.1 Abbreviations

Ab	antibodies
AC	adenylylcyclase
ADP	adenosine diphosphate
AM	acetoxymethyl
ATP	adenosine triphosphate
BSA	bovine serum albumin
BSS	Bernard-Soulier syndrom
Btk	bruton tyrosine kinase
Ca^{2+}	calcium cations
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CLEC-2	C-type lectin-like receptor 2
COX-1	cyclooxygenase 1
cvx	convulxin
DAG	diacylglycerol
DEAE	diethylaminoethanol
DMSO	dimethyl sulfoxide
DTS	dense tubular system
EB	echicetin beads
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid)
ELISA	enzyme-linked immunosorbent assay
g	Gram
	x g or g centrifugal speed force
GAP	GTPase activating proteins
GEF	Guanine nucleotide exchange factors
GMP	guanosine monophosphate
GP	glycoprotein
GPCRs	G-protein coupled receptors
GTP	guanosine triphosphate

IEX	ion exchange chromatography
IL-4R	interleukin- 4 receptor
IP	immunoprecipitation
IP1	inositol monophosphate
IP3	inositol 1,4,5-triphosphate
IP3 R1	inositol 1,4,5-triphosphate receptor 1
IRAG	or MRVI, IP3 receptor-associated cGK I substrate protein
ITAM	immunoreceptor tyrosine-based activation motif
kDa	kilo Dalton
LASP	LIM and SH3 domain protein
LAT	linker adaptor protein for T cells
LTA	light transmission aggregometry
MAPK	mitogen-activated protein kinase
MLCK	myosin light chain-chain kinase
MS	mass spectrometry
n.s.	not significant
NO	nitric oxide
P	*p*-value
$P2Y_1$	purinergic G-protein coupled receptor Y1
$P2Y_{12}$	purinergic G-protein coupled receptor Y12
PBS	phosphate-buffered saline
PDBu	phorbol 12, 13-dibutyrate
PDVF	polyvinylidene difluoride membranes
PGI_2	prostaglandin I_2, prostacyclin
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKB	protein kinase B
PKG	protein kinase G
PLCγ2	phospholipase C Gamma 2
PPP	platelet-poor plasma
PRP	platelet-rich plasma
PS	phosphatidylserine

PTK	protein tyrosine kinase
PTP	protein tyrosine phosphate
Rap1	Ras-proximate-1 or Ras-related protein 1
S or Ser	serine
SDS	sodium dodecyl sulfate
SFK	Src (sarcoma) family kinase
sGC	soluble guanylyl cyclase
SLP-76	SH2 domain containing leukocyte protein of 76 kDa
	lymphocyte cytosolic protein 2
SNP	sodium nitroprusside
Syk	Spleen tyrosine kinase
T	Tween 20®
	T or Thr threonine
TBS	tris-buffered saline
Temed	tetramethylethylenediamine
TKI	tyrosine kinase inhibitors
TMB	3, 3', 5, 5'- tetramethylbenzidine
Tris	tris (hydroxymethyl)aminomethane
TULA-2	protein T cell ubiquitin ligand 2
TxA_2	thromboxane A_2
VASP	vasodilator-stimulated phosphoprotein
vWF	von Willebrand factor
Y or tyr	tyrosine

11.2 List of tables

11.3 List of figures

11.4 Versicherung

Hiermit versichere ich, Stephanie Makhoul, geboren am 16.05.1990 in Rmeich, Libanon, gemäß § 11, Abs. 3d der Promotionsordnung vom 22.12.2003, die vorliegende Dissertation mit dem Titel ‚GPIb-V-IX and GPVI-specific intracellular signaling and their regulation by PKA/PKG-dependent inhibitory pathways in washed human platelets‘:

(zutreffendes ist angekreuzt)

☒ Ich habe die heute als Dissertation vorgelegte Arbeit selbst angefertigt und alle benutzten Hilfsmittel (Literatur, Apparaturen, Material) in der Arbeit angegeben.

☒ Ich habe oder hatte die jetzt als Dissertation vorgelegte Arbeit nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht.

☐ Ich hatte die heute als Dissertation vorgelegte Arbeit als Prüfungsarbeit für folgende Prüfung eingereicht:

Bezeichnung der Prüfung:_____

Prüfungsstelle: _____

☒ Ich hatte weder die jetzt als Dissertation vorgelegte Arbeit noch Teile einer Abhandlung bei einer anderen Fakultät bzw. einem anderen Fachbereich als Dissertation eingereicht.

☐ Ich hatte die folgende Abhandlung mit nachstehenden Ergebnis eingereicht:

Titel der Abhandlung:

Fakultät bzw. Fachbereich und Hochschule:

Ergebnis bzw. Beurteilung: _____

Mainz, den _____ _____

(Stephanie Makhoul)

11.5 Acknowledgments

11.6 Curriculum Vitae