

STIM and Orai in hemostasis and thrombosis

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1. ABSTRACT

At sites of vascular injury, platelets rapidly adhere to the exposed subendothelial extracellular matrix, become activated and, together with the coagulation system, form a plug that seals the lesion. This process is essential to prevent blood loss, however, under pathological conditions it may lead to vessel occlusion. Agonist-induced elevation of intracellular Ca^{2+} levels is essential for platelet activation. It occurs through two different mechanisms: Ca^{2+} release from internal stores, involving phospholipase C (PLC)-dependent generation of inositol-1,4,5-trisphosphate (IP_3) and activation of IP_3 sensitive channels in the store membrane, and Ca^{2+} influx across the plasma membrane. *Store operated Ca^{2+} entry* (SOCE), triggered by store depletion, is the main influx pathway for extracellular Ca^{2+} in platelets, but the molecular mechanism underlying this pathway has long remained elusive. In the last years, however, the Ca^{2+} sensor *stromal interaction molecule 1* (STIM1) and the channel protein Orai1 emerged as the key players in platelet SOCE. This review summarizes the current knowledge about the role of these proteins in platelet physiology and thrombus formation and discusses their suitability as antithrombotic targets.

2. INTRODUCTION

Platelets are small anucleated cell fragments that originate from the cytoplasm of bone marrow megakaryocytes and circulate in the bloodstream for about ten days. In human blood, $150 - 450 \times 10^6$ platelets per ml are present, in mice around 1×10^9 platelets per ml. In the intact vasculature, platelets never undergo firm adhesion and are finally cleared from the circulation by macrophages in liver and spleen. However, when contacting damaged vessel walls, circulating platelets rapidly decelerate and adhere to the newly exposed subendothelial extracellular matrix (ECM). Sustained interaction of platelet receptors with components of the ECM triggers platelet activation, characterized by rearrangements of their cytoskeleton resulting in a change from discoid to spheric shape, fusion of intracellular alpha and dense granules with the plasma membrane and release of secondary platelet agonists (so-called second wave mediators), most notably adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin and thromboxane A_2 (TxA_2). These agonists along with locally produced thrombin induce intracellular signaling events through receptors coupled to heterotrimeric G proteins, thereby enhancing platelet

activation and recruitment of additional platelets from the bloodstream to the site of injury. Finally, this sophisticated interplay of extra- and intracellular signaling events leads to functional upregulation of adhesion receptors of the integrin class, most notably integrin $\alpha\text{IIb}\beta\text{3}$ (also called glycoprotein (GP)IIb/IIIa) resulting in the establishment of stable platelet-ECM contacts and, through the binding of plasma fibrinogen, in platelet aggregation. In conjunction with an increase of the procoagulant activity of the cells, these events lead to the formation of a stable thrombus that seals the wound (1-2) (Figure 1). This process, called primary hemostasis, is essential to limit excessive blood loss after tissue trauma. On the other hand, in diseased vessels, uncontrolled thrombus formation can lead to acute vessel occlusion and ischemia or infarction of vital organs. Myocardial infarction, triggered by erosion or rupture of an atherosclerotic plaque in coronary arteries, and stroke, elicited by carotid thrombi that embolize to the brain, are two of the leading causes of disability and mortality worldwide (3). Therefore, a better understanding of platelet function and thrombus formation is required in order to develop new therapeutic strategies to prevent and treat those diseases.

Calcium (Ca^{2+}) serves as an essential second messenger in virtually all eukaryotic cell types, regulating fundamental cellular processes such as exocytosis, motility, apoptosis and gene transcription (4-6). Each cell type expresses a distinct set of Ca^{2+} sensitive effector proteins and regulators of intracellular free Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) to assemble a Ca^{2+} signaling system that fulfills the cell's individual needs for spatial and temporal Ca^{2+} dynamics. In platelets, changes in $[\text{Ca}^{2+}]_i$ contribute to a variety of responses including granule release (7-8), cytoskeletal rearrangements required for shape change (8-9), coagulant activity (10-11) and inside-out activation of integrins, indispensable for platelet aggregation (12-13). Various agonists, such as collagens, ADP, TxA_2 or thrombin evoke a rise in $[\text{Ca}^{2+}]_i$ that consists of two parts: release of compartmentalized Ca^{2+} from intracellular stores and influx of extracellular Ca^{2+} across the plasma membrane. This Ca^{2+} influx from the extracellular space potentiates and extends the Ca^{2+} signals derived from store release and further stimulates platelet activation (14). Already in 1991, it was found that the main Ca^{2+} entry mechanism in human platelets is *store-operated Ca^{2+} entry* (SOCE) (15-16), where Ca^{2+} influx is triggered by the depletion of Ca^{2+} from the intracellular stores (17). However, it took additional 15 years until the key components of the underlying molecular machinery were identified: the endoplasmic reticulum (ER)-resident Ca^{2+} sensor *stromal interaction molecule 1* (STIM1) and the store operated Ca^{2+} channel Orai1 (also called CRACM1).

The identification of the molecular basis of SOCE enabled genetic analyses and genetic manipulations of this process in mouse models that markedly increased our knowledge about Ca^{2+} signaling in platelet activation and thrombus formation. This review summarizes the major advances in understanding Ca^{2+} signaling in platelets in health and disease with a particular focus on the STIM and Orai proteins.

3. ROLE OF INTRACELLULAR Ca^{2+} IN THROMBUS FORMATION

Exposure of highly thrombogenic subendothelial ECM components upon vessel wall injury leads to rapid recruitment of platelets that become tethered to and "roll" along the site of injury in a stop-and-go manner (Figure 1). Under high shear flow conditions, such as found in arterioles or stenosed arteries, this process is mediated by the interaction of two molecules: the multimeric plasma glycoprotein von Willebrand factor (vWF), that becomes immobilized on the exposed subendothelial collagen, and the platelet receptor GPIb, itself part of the GPIb-V-IX complex (18). This interaction is, however, characterized by a fast dissociation rate and therefore does not allow stable platelet adhesion by itself (18). Thus, the principal function of this interaction is to reduce the velocity of platelets and to enable other platelet receptors, such as collagen receptors, to establish contacts with the thrombogenic surface.

Besides this mandatory role for platelet adhesion, GPIb also acts as a signal transducer that can directly induce weak activation of the principal adhesion receptor in platelets, the integrin $\alpha\text{IIb}\beta\text{3}$ (19). GPIb-dependent binding of platelets to vWF-coated surfaces under flow has been shown to induce transient Ca^{2+} spikes, which initiated transient platelet arrest and integrin $\alpha\text{IIb}\beta\text{3}$ activation (20-21). These signals seem to derive from intracellular stores, as addition of the extracellular Ca^{2+} chelator EGTA had no inhibitory effect on the magnitude or duration of the Ca^{2+} spikes (20, 22).

GPIb-dependent deceleration of platelets allows the establishment of contacts between collagen and the immunoglobulin-like receptor GPVI, the major activating platelet collagen receptor (2, 23) (Fig.1). Binding of GPVI to collagen does not mediate stable adhesion, but induces intracellular signaling events, which, through a strong and sustained rise in $[\text{Ca}^{2+}]_i$, finally leads to cellular activation, release of second wave mediators, most notably ADP and TxA_2 , and exposure of negatively charged phosphatidylserine (PS) on the platelet surface (24-25). The latter provides a platform for local thrombin generation by the coagulation system which, together with the released second wave mediators, further activates the platelet (26). These agonists signal through different G-protein coupled receptor (GPCR) pathways also resulting in Ca^{2+} -release and SOCE thereby amplifying and sustaining the initial GPVI induced Ca^{2+} signals (see below) (27). In addition, released ATP enhances the activation processes triggered by GPCR and GPVI via the activation of the ATP gated Ca^{2+} channel P2X₁ (28-30).

All these signaling events converge in the final common step of platelet activation that is the shift of integrin $\alpha\text{IIb}\beta\text{3}$ from a low to a high affinity state, which is a prerequisite for fibrinogen binding and stable thrombus formation (2) (Figure 1). A newly identified platelet receptor involved in thrombus formation is the C-type lectin-like receptor 2 (CLEC-2) (31-32). Studies using a CLEC-2 depleting antibody (31) or genetically modified

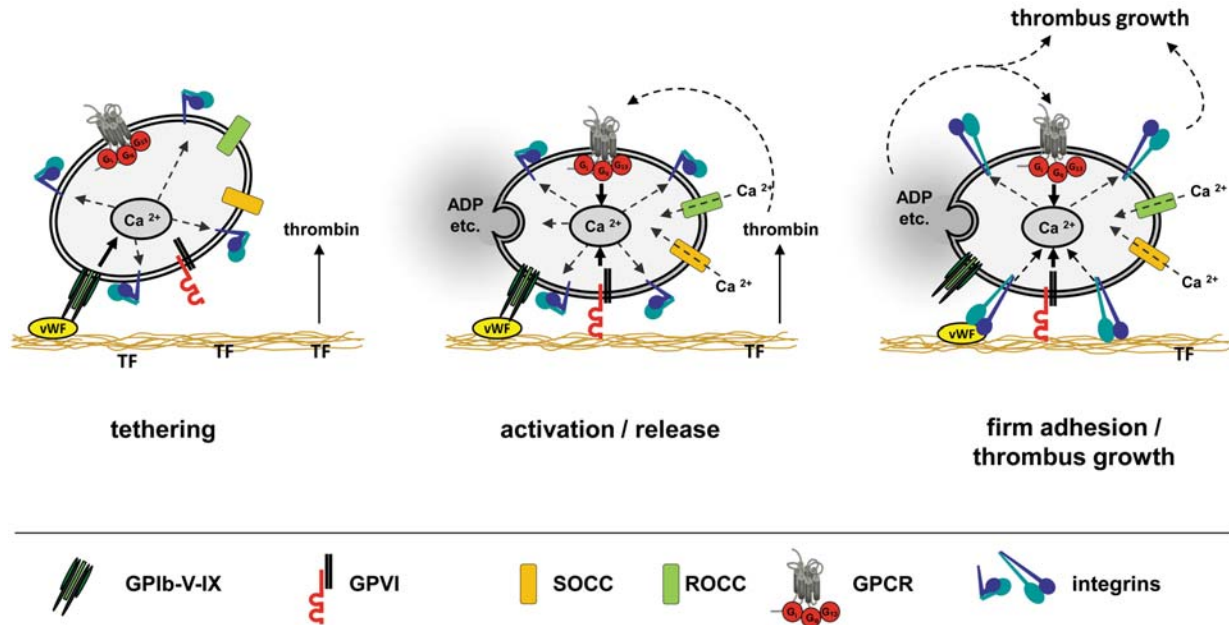


Figure 1. Current model of platelet adhesion and thrombus growth. The initial contact (tethering) of platelets to the ECM is mediated predominantly by GPIb-von Willebrand factor (vWF) interaction. GPIb induces a transient Ca^{2+} release from the intracellular store. The next step is GPVI-collagen interaction which initiates platelet activation by inducing a slow but sustained elevation in cytoplasmic Ca^{2+} concentration by Ca^{2+} release from the intracellular store and activation of store-operated Ca^{2+} channels (SOCC) and receptor-operated Ca^{2+} channels (ROCC). This process leads to integrin activation and the release of second wave mediators, most importantly ADP, ATP, and TxA_2 . In parallel, exposed tissue factor (TF) locally triggers the formation of thrombin on the platelet surface, which in addition to GPVI amplifies Ca^{2+} signaling. On the growing thrombus, activated integrins on the platelet surface mediate firm adhesion by binding to vWF, fibrinogen, and other ligands. Released ATP, ADP, and TxA_2 amplify integrin activation on adherent platelets and mediate thrombus growth by activating additional platelets.

mice (33) have shown that this receptor is of particular importance for the stabilization of growing thrombi. Albeit the physiological ligand of CLEC-2 in this process is not known, stimulation of CLEC-2 with the snake venom toxin rhodocytin revealed a downstream signaling pathway similar to GPVI (32, 34). Therefore, it is likely that, like in GPVI signaling (see below), Ca^{2+} plays also an important role in CLEC-2 signaling. However, this process has not been characterized in detail.

4. Ca^{2+} SENSITIVE PROTEINS INVOLVED IN PLATELET ACTIVATION

In immune cells, Ca^{2+} signals are especially important for the activation of transcription factors, such as nuclear factor of activated T-cells (NFAT), which influence gene expression patterns and thereby shape the outcome of cell activation (6). In sharp contrast, platelets lack a nucleus and, upon vascular injury, have to be activated within seconds. Thus, Ca^{2+} signals have to be rapidly translated into a cellular response. Despite the paramount importance of Ca^{2+} in platelet signaling, not much is known about the molecular identity of the proteins involved in this process.

An essential key regulator in Ca^{2+} -dependent platelet activation is the highly Ca^{2+} -sensitive Ca^{2+} and diacylglycerol regulated guanine nucleotide exchange factor 1 (CalDAG-GEFI) that has been characterized

recently (35-37). In response to Ca^{2+} , CalDAG-GEFI activates Rap1b (35-36), an abundant small GTPase in platelets, required for integrin activation and thus for normal platelet function and hemostasis (38). In consequence, CalDAG-GEFI deficient platelets exhibited marked defects in $\alpha\text{IIb}\beta\text{3}$ -mediated aggregation response to all tested agonists *in vitro*, causing a remarkable bleeding phenotype in tail bleeding assays and protection in different thrombosis models (35, 39). A more detailed analysis of CalDAG-GEFI^{-/-} platelets revealed a critical role of the molecule in Erk-dependent TxA_2 generation, providing a crucial feedback for protein kinase C (PKC) activation and ADP release, thus placing CalDAG-GEFI at the center of Ca^{2+} dependent platelet activation (37).

Two other major Ca^{2+} dependent proteins in platelets are the cysteine proteases calpain-1 and 2 which are involved in the modification of the cytoskeleton, thus affecting cell spreading, aggregation and clot retraction by indirectly reducing the tyrosine phosphorylation status of several proteins involved in integrin signaling (40-41). Ca^{2+} - and integrin-binding protein 1 (CIB1) seems also to be involved in signaling processes downstream of integrins, as its absence has been shown to interfere with the spreading of platelets on collagen (42). Besides this role in integrin signaling, calpains participate in the intracellular cleavage of different platelets receptors, thus

downregulating platelet reactivity (43). The same effect may be achieved by extracellular cleavage of receptors by sheddases. This process is facilitated by the dissociation of the Ca^{2+} sensor calmodulin from the juxtamembrane cytoplasmic sequences of sheddase substrates, including GPVI and GPIb-IX-V and other receptors (44). This occurs upon platelet stimulation (45-46) or treatment with calmodulin inhibitors (47-48). As a consequence, Ca^{2+} positively regulates receptor proteolysis via calpains and calmodulin.

Ca^{2+} is also a potent stimulus for platelet granule release but the molecular basis of this process has not been established. However, calmodulin, calpains, synaptotagmins and other proteins seem to be involved in this process (49).

5: Ca^{2+} HOMEOSTASIS IN PLATELETS

Platelet agonists increase $[\text{Ca}^{2+}]_i$ by two mechanisms: Ca^{2+} release from intracellular stores and Ca^{2+} entry from the extracellular space into the cytosol. The dense tubular system (DTS) - an internal smooth ER membrane system - is the major Ca^{2+} store in platelets. It has a dual function in Ca^{2+} homeostasis: on the one hand it keeps platelets in a resting state by sequestering Ca^{2+} , on the other hand it modulates platelet activation by releasing Ca^{2+} into the cytosol. Physiologically, Ca^{2+} is released from the DTS into the cytosol when activated platelet receptors couple to different isoforms of the phospholipase C (PLC) enzyme and trigger production of the second messenger inositol-1,4,5-trisphosphate (IP_3) by the hydrolysis of phosphatidyl-1,4-bisphosphate (PIP_2). In platelets, IP_3 production is regulated by two different PLC subfamilies, beta and gamma. IP_3 binds and activates IP_3 -receptors (IP_3R) in the DTS membrane and activates Ca^{2+} store release (50). When the Ca^{2+} reservoir of the DTS becomes exhausted, extracellular Ca^{2+} enters into the cytoplasm. During this process, the thin elongated shape of the DTS changes to a rounded vesicular form (51) inducing redistribution of the cortical actin cytoskeleton (52-53).

The other product of PIP_2 hydrolysis, 1,2-diacylglycerol (DAG), induces PKC activation and *receptor-operated Ca^{2+} entry* (ROCE) (50). Platelet ROCE can also occur through an additional pathway, namely the ATP-gated cation channel, P2X_1 (54). This induces transient Ca^{2+} influx through the plasma membrane and contributes to platelet activation by stimulating the PKC/Erk2 pathway leading to shape change and granule release (29, 54-56) (Figure 2).

Platelets possess a Ca^{2+} toolkit (Figure 2) that upon Ca^{2+} influx rapidly removes Ca^{2+} from the cytosol. Ca^{2+} will be either pumped back into the DTS or into other specialized storage compartments like mitochondria and lysosome-like "acidic stores" (57) or it will be pumped out of the cell. Many well known Ca^{2+} pumps are linked to these control mechanisms, including *sarcoplasmic endoplasmic reticulum Ca^{2+} -ATPases* (SERCAs) and *plasma membrane Ca^{2+} -ATPases* (PMCA). However, in platelets these Ca^{2+} store coupled regulatory processes are still poorly understood.

Ca^{2+} store dependent platelet activation is triggered by subendothelial collagens, by TxA_2 and ADP released from activated platelets, and by thrombin generated through the coagulation cascade. These agonists trigger different signaling pathways, all commonly leading to the activation of PLCs (Figure 2). ADP released from dense granules upon platelet activation activates shape change and reversible platelet activation by activating the G-protein coupled P2Y_1 and P2Y_{12} receptors (58). TxA_2 is produced by activated platelets from arachidonic acid and mediates platelet activation through its GPCRs *thromboxane-prostanoid* (TP) α and TP β (59). Thrombin also activates GPCRs, called *protease activated receptors* (PARs). Platelet activation through the Gq coupled PARs, TP and P2Y_1 is very fast as Gq directly binds to the PLC β enzyme (60).

In contrast, GPVI-immunoreceptor *tyrosine-based activation motif* (ITAM) mediated IP_3 production via the PLC γ 2 enzyme is a slower process which is dependent on the subcellular localization, phosphorylation and activation of PLC γ 2 (61-63).

6. STIM PROTEINS REGULATE STORE CONTENT AND SOCE

In the past few years, several models have been proposed for the activation of SOCE. The first model, proposing a diffusible messenger that could link store depletion to the activation of the Ca^{2+} channels in the plasma membrane, was published by Putney and colleagues (64). A second model was based on the conformational coupling of store and plasma membrane resident Ca^{2+} channels. This hypothesis favors the idea that upon store depletion conformational changes of the IP_3R happen and this novel structure of the IP_3R results in its physical attachment to the SOC channel to the plasma membrane (65-66). A third model suggested that upon store depletion, SOC channels are inserted into the plasma membrane through exocytosis of SOC containing vesicles (67). In this case, it has been proposed that in resting cells no or only very few SOC channels are expressed in the plasma membrane. This model was supported by experiments where blocking of vesicle secretion inhibited SOCE (68).

To prove these models experimentally, SOCE was in the focus of platelet research for many years but the molecular identity of the SOC channel and its regulators as well as the physiological function of SOCE *in vivo* have long remained elusive. *Stromal interaction molecules* (STIMs) received much attention during the last few years because a number of results from different mammalian cell types strongly suggest that STIMs are the missing key modulators that link Ca^{2+} store release to Ca^{2+} entry through the plasma membrane and regulate the refilling process of the Ca^{2+} stores (69-71).

Two different STIM isoforms, STIM1 and 2, are expressed in mammals. The STIM1 isoform was originally identified as a potential tumor growth suppressor in rhabdomyosarcoma cell line (72). At that time it was not suspected to be involved in the regulation of SOCE and the

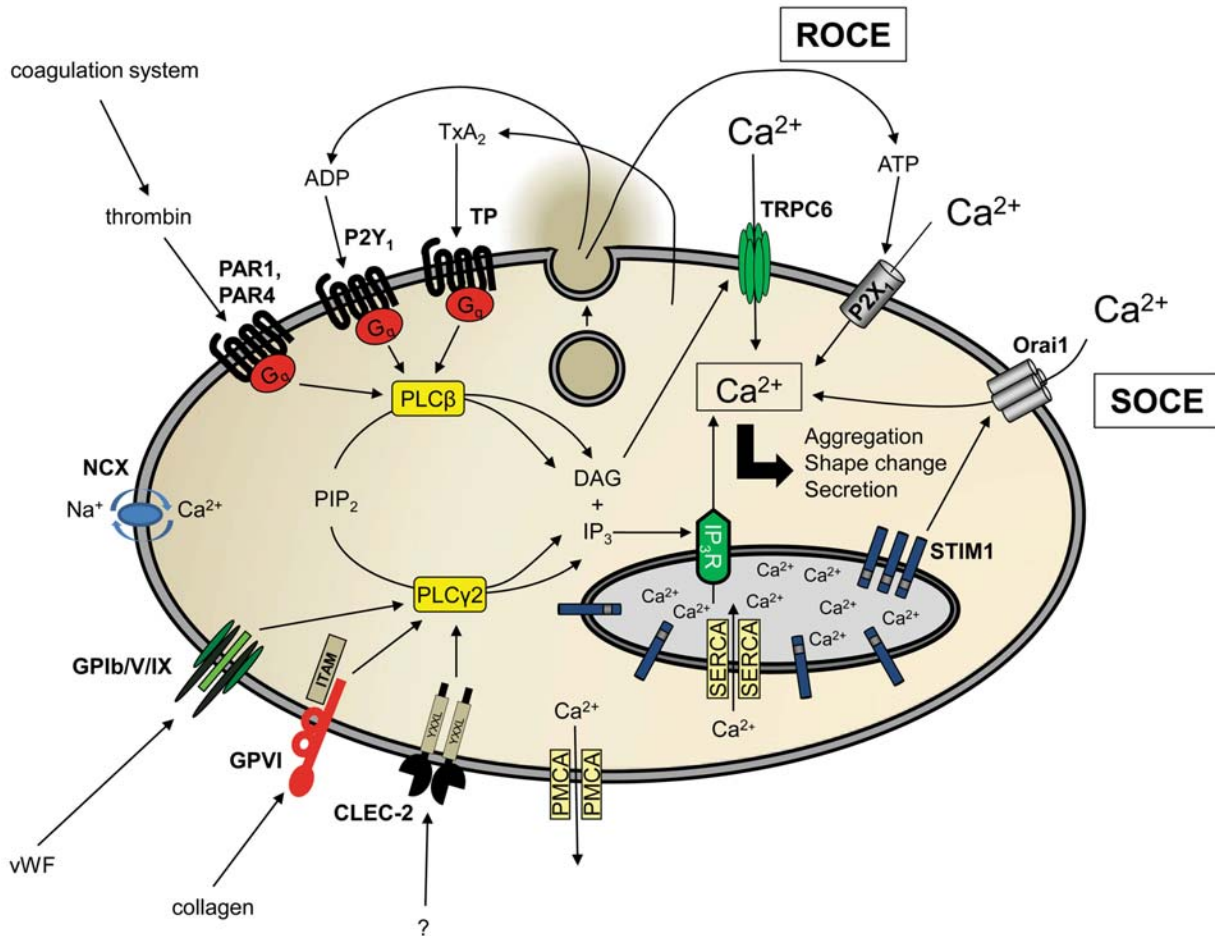


Figure 2. The platelet Ca^{2+} toolkit. Ca^{2+} release is triggered by two major pathways. Collagen activates the GPVI-ITAM pathway leading to $\text{PLC}\gamma 2$ activation, while second wave mediators activate $\text{PLC}\beta$ via binding to GPCRs. PLCs hydrolyze PIP_2 to IP_3 and DAG. IP_3 depletes intracellular Ca^{2+} stores by binding to IP_3R , which in turn activates SOCE via STIM1 and Orai1. DAG binding to transient receptor potential channel TRPC 6 induces ROCE. An additional ROC channel, P2X_1 , contributes to elevation of $[\text{Ca}^{2+}]_i$. The $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) is able to accomplish fast Ca^{2+} extrusion but can also work in reverse mode. PMCA and SERCA are counteracting the elevation of $[\text{Ca}^{2+}]_i$ by pumping Ca^{2+} outside the cell or back into the stores, respectively.

function of the STIM2 protein was entirely unknown. Both STIM isoforms contain a signal peptide, an EF-hand motif, a sterile alpha motif, a transmembrane domain and a long cytoplasmic tail which contains protein interaction sites like a coiled-coil domain and proline rich sequences.

The N-terminus of STIM1 including the EF-hand motif is located in the ER lumen where it binds Ca^{2+} . Upon store depletion, Ca^{2+} dissociation from the EF-hand motif induces conformational changes of STIM1 allowing it to form plasma membrane near “puncta” structures. These “puncta” seem to represent a bridge linking the ER membrane to the plasma membrane, thereby enabling STIM1 to regulate the SOC channel function and the refill of intracellular stores (73). Interestingly, when key residues in the EF-hand - essential for Ca^{2+} binding - were mutated, STIM1 was constitutively active, staying permanently in the “puncta” structure and keeping the SOC channels open without change in the Ca^{2+} store content (70-71).

The *in vivo* biological function of an EF-hand mutant STIM1 was first studied in platelets. Mice homozygously expressing a constitutively active mutant form of STIM1 ($\text{Stim1}^{\text{Sax}}$), due to one single amino acid substitution in the EF-hand motif (D84G), died *in utero* and showed severe diffuse bleedings in different regions of the body, indicating a hemostatic defect. In contrast, mice expressing only one mutant allele ($\text{Stim1}^{\text{Sax/+}}$) were viable but developed severe macrothrombocytopenia associated with megakaryocyte hyperplasia, splenomegaly, bone marrow fibrosis and a bleeding disorder (74). Detailed analysis of megakaryocyte function showed, however, that thrombopoiesis was not impaired in these mice. In contrast, platelets were rapidly cleared from the circulation due to a preactivated state as evidenced by elevated levels of activated integrins on the surface of these cells (74).

Ca^{2+} signaling was investigated in $\text{Stim1}^{\text{Sax/+}}$ platelets to better understand this complicated *in vivo*

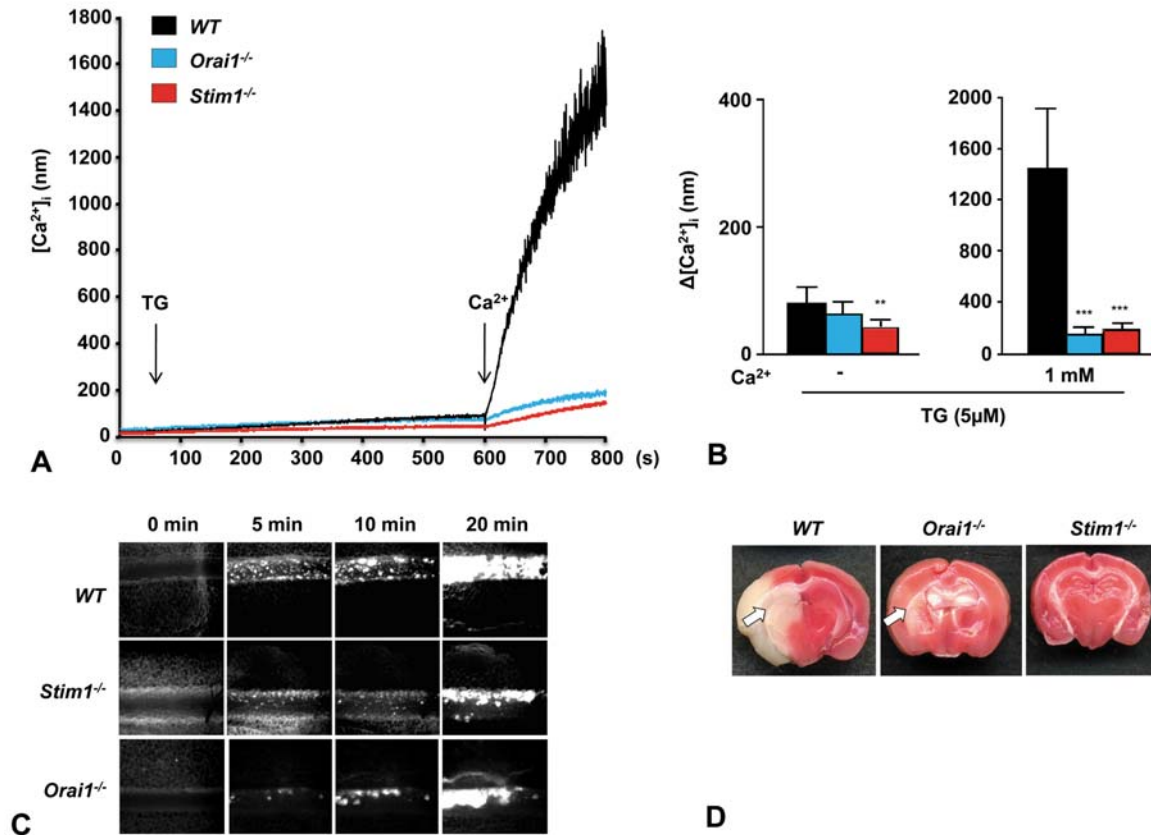


Figure 3. Orai1-STIM1 signalosome regulates SOCE in platelets and this process is essential for thrombus formation *in vivo*. (A) SOCE was studied in wild-type (WT), *Orai1*^{-/-} and *Stim1*^{-/-} platelets using Fura-2 loaded platelets and fluorimetric Ca^{2+} measurements. SERCA pump inhibitor thapsigargin (TG) was used to determine store content and Ca^{2+} release from the DTS. After 10 minutes of store depletion SOCE was measured by the addition of extracellular Ca^{2+} into the buffer. Representative curves (A) and mean changes in the intracellular Ca^{2+} concentration ($\Delta[Ca^{2+}]_i$) (B) are presented. (C) $FeCl_3$ -induced chemical injury model was used to study thrombus formation in small mesenteric arteries *in vivo*. Impaired thrombus formation was only observed in *Stim1*^{-/-} mice. However, both knockout mice were protected in the *in vivo* model of ischemic stroke (D).

phenotype. Increased basal Ca^{2+} levels were found in the cytosol of unstimulated *Stim1*^{Sax/+} platelets that were based on permanently opened SOC channels in the plasma membrane. SOCE through the two major signaling pathways – namely the Gq-PLC β and GPVI-ITAM-PLC γ 2 pathways (Figure 2) – was also investigated. Using different agonists, interestingly only the GPVI-ITAM-PLC γ 2 dependent signaling was defective in *Stim1*^{Sax/+} platelets both *in vitro* and *in vivo* (74). These very first experiments already indicated that STIM1 might play a pivotal role in the regulation of platelet SOCE, and suggested that SOCE is not equally important for all signaling pathways, at least in platelets.

Shortly after, mice lacking STIM1 were generated and platelet function was studied (75). Since *Stim1*^{-/-} mice show marked growth retardation and die around 4 weeks after birth, these studies were performed in irradiated wild-type mice transplanted with *Stim1*^{-/-} bone marrow. In these mice, injected *Stim1*^{-/-} bone marrow stem cells repopulated all lineages of blood cells, including megakaryocytes, and mutant platelets were analyzed.

In *Stim1*^{-/-} platelets a severely defective Ca^{2+} response to all major agonists was found, clearly establishing SOCE as the major way of Ca^{2+} entry in platelets. Like for *Stim1*^{Sax/+} platelets, flow-cytometric measurements of integrin activation and degranulation revealed a selective signaling defect in the GPVI-ITAM-PLC γ 2 pathway in STIM1 deficient platelets. This was also observed in aggregation studies, where the response of STIM1 deficient platelets towards GPVI-agonists was completely abolished at low agonist concentrations and strongly reduced at higher concentrations, whereas it was normal in response to GPCR-agonists at all concentrations.

Despite the severe reduction in Ca^{2+} entry, a residual Ca^{2+} influx was still detected in *Stim1*^{-/-} platelets (Figure 3). This indicates that other molecules can also regulate Ca^{2+} influx in platelets, but only to a minor extent. One possible candidate molecule is STIM2 that has been shown to activate SOC channels in eukaryotic cells and is also expressed in platelets. Alternatively, the residual Ca^{2+} entry could be mediated by ROCE, independent of SOCE.

DAG could be one of the metabolites which induces ROCE by direct activation of *transient receptor potential* (TRP) channels. TRPC6 is a non-selective cation channel that is activated directly by DAG in a membrane-delimited fashion, independently of PKC (76). In line with this, TRPC6 has indeed been proposed as a candidate mediating store-independent Ca^{2+} entry in human platelets (77). In addition, the ATP-gated Ca^{2+} channel P2X_1 must be also considered to contribute to the residual Ca^{2+} influx in *Stim1*^{-/-} platelets.

Besides the severely impaired SOCE, a reduced Ca^{2+} release from intracellular stores upon agonist-induced platelet activation was seen in *Stim1*^{-/-} platelets (75). Similar observations have also been made in *Stim1*^{-/-} mast cells (78) indicating that STIM1 could be involved in the refilling process of the intracellular Ca^{2+} store.

Although STIM2 is expressed in platelets, no obvious abnormalities in Ca^{2+} homeostasis of *Stim2*^{-/-} platelets could be shown so far (79). The normal Ca^{2+} store release and SOCE of these cells indicate that STIM2 has a minor role in platelet Ca^{2+} homeostasis or its function can be fully compensated by STIM1.

7. IDENTIFICATION OF THE MAJOR SOC CHANNEL IN PLATELETS

Studies on SOCE in platelets have long been hampered by the lack of identified SOC channels. The first potential candidate SOC channel, a TRP Ca^{2+} channel, was discovered in *Drosophila* (80). This finding accelerated the efforts to identify more members of the TRP gene family. Searching genome-project databases, three *Drosophila* TRP genes and at least 33 TRP genes in mammalian systems were identified (81). Based on sequence identities, the TRP protein family has been divided into seven sub-families; especially the TRPC subfamily received growing attention in platelet research (82). The subunit structure of all TRPCs has been preserved during evolution with six putative transmembrane helices and unique protein binding motifs in the amino- (ankyrin repeat) and carboxyl terminal regions (Dystrophin-homologous sequence).

Two members of the TRPC family are expressed in platelets: TRPC1 and TRPC6. The already mentioned TRPC6 is highly abundant in the platelet plasma membrane and mediates nonselective-cation influx in response to activation with DAG (77). For over a decade, TRPC1 was suggested to be the major SOC channel in platelets. The *de novo* conformational coupling model proposed TRPC1 to mediate SOCE by a coupling-dependent activation through interaction of the protein with the type II IP_3R upon intracellular store depletion (83-85). Other investigators, however, suggested TRPC1 to be located rather in intracellular membranes of human platelets, indicating a role for TRPC1 other than SOCE (77).

Mice lacking TRPC1 display fully intact SOCE and generally unaltered Ca^{2+} homeostasis in platelets (86). In addition, platelet activation *in vitro* and *in vivo* is not altered in the absence of TRPC1. Furthermore, studies on

human platelets revealed that the presumably inhibitory anti-TRPC1 antibodies used in previous studies to demonstrate the function of the channel, had no specific effect on SOCE and failed to bind to the protein (86). Together, these results provided evidence that SOCE in platelets is mediated by Ca^{2+} channels other than TRPC1 or that the lack of TRPC1 function could be fully compensated in platelets. The discovery of a novel putative SOC channel, Orai1, led to the long awaited breakthrough in this field.

Orai1 was originally identified in a *Drosophila* cell line by three independent groups (87-89). It is a 33-kDa plasma membrane protein with four transmembrane domains. Glycosylation of Orai1 in human platelets increases its apparent molecular weight to 50 kDa (90). It appears that tetramer of Orai1 proteins assemble to form a functional SOC unit (91). *In vitro* studies have shown that upon store depletion Orai1 co-localizes and directly binds to STIM1 and allows extracellular Ca^{2+} to enter into the cytosol (92). The physiological role of Orai1, as a potential mediator of SOCE, was first studied in two human patients suffering from severe combined immunodeficiency (SCID) syndrome, caused by a point mutation in the Orai1 gene (93). Shortly after this discovery, a role for Orai1 in platelet SOCE was proposed (94), based mainly on the finding, that quantitative RT-PCR analysis of candidate SOC channels in human platelets showed a >30-fold higher expression of Orai1 when compared to TRPC1 or other TRPC channels.

The function of this novel Ca^{2+} channel in platelets was first studied in mice lacking Orai1 (90). Orai1 deficiency in platelets resulted in severely reduced SOCE (Figure 3) in response to all major physiological agonists, but in contrast to STIM1 deficiency it had no effect on the store Ca^{2+} content. These data suggest that functional SOCE is not a prerequisite for proper store refilling and indicates that STIM1 presumably plays a direct, yet unidentified, role in this process. Further, lack of Orai1 resulted in strongly reduced, but not completely abolished SOCE, indicating that other channels might mediate this residual Ca^{2+} influx. Alternatively, the residual Ca^{2+} entry could be mediated by DAG induced ROCE, similar to *Stim1*^{-/-} platelets. *In vitro*, the functional consequences of reduced SOCE were rather mild. Orai1 deficient platelets aggregated normally in response to GPCR agonists, like ADP, thrombin or the TxA_2 analog U46619. Aggregation towards stimulation with GPVI agonists was diminished, but, in contrast to *Stim1*^{-/-} platelets, these defects were completely overcome at higher agonist concentrations. This selective impairment of GPVI-ITAM signaling was also observed in flow-cytometric measurements of integrin activation and degranulation.

At the same time, Bergmeier *et al.* generated Orai1 knock-in mice expressing the Orai1^{R93W} mutant protein (95), which is the mouse equivalent to the Orai1^{R91W} mutation that abrogates SOCE in T cells of human SCID patients (87). Mutant platelets were characterized by markedly reduced SOCE and impaired agonist-induced increase in $[\text{Ca}^{2+}]_i$. Further, Orai1^{R93W} platelets showed reduced integrin activation and impaired

degranulation when stimulated with low agonist concentrations under static conditions. This defect did, however, not significantly affect the ability of *Orai1*^{R93W} platelets to aggregate or to adhere to collagen under arterial flow conditions *ex vivo* (see below).

8. STIM1 AND ORAI1 IN THROMBUS FORMATION

Because of the pivotal importance of Ca^{2+} signaling for platelet activation, molecules involved in this process may serve as target structures for antithrombotic therapy. In different genetic mouse models, it has been demonstrated that attenuation of intracellular Ca^{2+} signals or the deletion of a Ca^{2+} responsive protein leads to impaired thrombus formation. For instance, the genetic ablation of the ATP sensitive Ca^{2+} channel P2X_1 or the Ca^{2+} sensor CalDAG-GEFI both resulted in a reduced aggregation response towards collagen *in vitro* and resistance against lethal collagen/epinephrine induced pulmonary thromboembolism *in vivo* (29, 35). Conversely, the overexpression of human P2X_1 in the megakaryocytic cell lineage produced a prothrombotic phenotype (30). Furthermore, enhanced SOCE has been suggested to contribute to the hypercoagulable state of platelets from patients suffering from Diabetes mellitus type 2 (96).

In line with this, the strongly decreased Ca^{2+} influx in response to all major agonists found in *Stim1*^{-/-} and *Orai1*^{-/-} platelets resulted in dramatically impaired thrombus formation when blood of these mice was perfused over a collagen-coated surface *ex vivo* (97). At high shear rates, resembling arterial flow conditions, wild-type platelets adhere to collagen fibers and build aggregates that grow into large thrombi during the perfusion period. In the case of *Stim1*^{-/-} and *Orai1*^{-/-} platelets, the surface area covered by thrombi was reduced by 42% and 60%, respectively, and the total thrombus volume was reduced by even more than 80% (75, 90). Given that under static *in vitro* conditions integrin activation, granule release and aggregation of *Stim1*^{-/-} and *Orai1*^{-/-} platelets was not affected in response to GPCR agonists and only partially defective towards GPVI agonists, these results indicate that STIM1 and Orai1 mediated SOCE is especially important under flow conditions where agonist potency becomes limiting because of rapid dilution. In contrast to *Orai1*^{-/-} platelets, *Orai1*^{R93W} platelets exhibited normal aggregation to fibrillar collagen in a flow adhesion assay and the surface area covered by platelets was not significantly reduced compared to wild-type (95). These differences may be explained by different experimental setups; e.g. the lower flow rate used (1,700s⁻¹ vs 1,000s⁻¹ in (90) and (95), respectively), or, as the authors assume, the *Orai1*^{R93W} mutation may not completely abolish Orai1 function in murine platelets.

In line with the flow adhesion results, *Stim1*^{-/-} and *Orai1*^{-/-} bone marrow chimeric (BMc) mice were protected in different *in vivo* models of thrombus formation (75, 90). This effect was most profound in a mechanical injury model of the abdominal aorta, where thrombus formation is triggered predominantly by collagens and thus occurs in a highly GPVI/PLCgamma2-dependent manner

(98). Likewise *Orai1*^{-/-} BMC mice were protected in a model of otherwise lethal pulmonary thromboembolism, triggered by intravenous injection of collagen and epinephrine (90). These results are in accordance with the dramatically reduced SOCE and the selectively impaired aggregation response of STIM1 and Orai1 deficient platelets in response to the GPVI-specific agonist collagen related peptide or collagen *in vitro*.

On the contrary, in a chemical injury model, where vessel injury is induced by topical application of FeCl_3 on mesenteric arterioles, thrombus formation is more dependent on soluble mediators such as thrombin (99), and thus on GPCR signaling. In this model, only *Stim1*^{-/-} BMC mice displayed impaired thrombus formation. The appearance of first aggregates was delayed in 50% of these chimeras and formation of stable thrombi was almost completely abrogated (75). In contrast, normal thrombus formation was observed in *Orai1*^{-/-} BMC mice in this model (90) which is most likely attributed to the slightly higher $[\text{Ca}^{2+}]_i$ achieved after stimulation compared to *Stim1*^{-/-} platelets (Fig 3). The mechanistic explanation for this observation is the decreased store content found in *Stim1*^{-/-} but not in *Orai1*^{-/-} platelets. This indicates that already minor alterations in $[\text{Ca}^{2+}]_i$ can make the difference between stable and unstable thrombi.

In conclusion, these studies showed that SOCE is of particular importance for thrombus growth under conditions of high shear flow, which is mainly driven by the GPIb-GPVI-ITAM axis. The protective effect of STIM1, but not of Orai1 deficiency in the more thrombin-driven chemical injury model is likely to arise from the role of STIM1 in refilling intracellular stores and appears therefore not to be attributable to the defect in SOCE.

The hemostatic and thrombotic function of platelets is not only dependent on their ability to adhere and aggregate at sites of vascular injury, but also on their ability to support blood coagulation. Procoagulant conversion of platelets is associated with specific biochemical and morphological changes, including calpain (and Ca^{2+})-dependent proteolytic cleavage of actin cytoskeleton proteins, activation of caspases, blebbing, membrane contraction and microvesiculation. In the process of procoagulant conversion, the redistribution of PS on the platelet surface is one of the early characteristics. This allows binding and assembly of enzyme complexes of the coagulation pathways and accelerates the clotting process. In non-stimulated platelets, PS is mostly sequestered in the inner layer of the plasma membrane (100). Upon platelet activation, platelets lose plasma membrane phospholipid asymmetry and PS is rapidly exposed on the surface. Although an increase in $[\text{Ca}^{2+}]_i$ is essential to trigger the redistribution of PS, the Ca^{2+} dependent signaling mechanism underlying this externalization process is not fully understood. It has been demonstrated that PS exposure is dependent on store release and extracellular Ca^{2+} influx since a continuously elevated $[\text{Ca}^{2+}]_i$ is obligate to maintain the externalization process (101). Ca^{2+} mobilizing agents - ionophores, thapsigargin (an inhibitor of SERCA) or thrombin - induce PS exposure and the formation of microvesicles in platelets (102).

Platelet stimulation with single G-protein coupled agonists, like thrombin or ADP, results in limited PS exposure (103-104) whereas GPVI activation causes appreciable procoagulant activity (105-106). The basis of this difference might be that thrombin or ADP induced activation of Gq-PLC β is a rather rapid but transient process, whereas GPVI-ITAM stimulation causes more sustained activation of PLC γ 2 dependent signaling (107). Combined stimulation of the GPVI-ITAM-PLC γ 2 and the Gq-PLC β pathway results in high PS exposure (11). Accordingly, the moderate procoagulant response caused by thrombin is strongly amplified in the presence of thapsigargin in platelets, emphasizing the important role of store release and sustained high level of $[Ca^{2+}]_i$ in this process. However, Ca^{2+} release from the stores alone is insufficient to explain such an effect (108) and SOCE has also been proposed to regulate, at least in part, the transmembrane movements of PS. Inhibition of SOCE with extracellular Ca^{2+} chelators reduces PS externalization, thereby supporting this notion (109).

Analysis of STIM1-, STIM2- and Orai1-deficient platelets has shown that the Stim1-Orai1 complex also contributes to PS exposure (79). Platelets from *Stim1*^{-/-} and *Orai1*^{-/-} BMc mice formed only small aggregates on collagen under flow *in vitro*, and PS exposure was almost completely absent. The procoagulant index, calculated as the ratio of the surface coverage of PS-exposing platelets to the surface coverage of all platelets, was also greatly reduced in both knockout mice. Quantitative analysis of Ca^{2+} rises in single platelets demonstrated a nearly complete ablation of the average Ca^{2+} signal in *Stim1*^{-/-} and a greatly reduced signal in *Orai1*^{-/-} platelets. These data pointed out the importance of Stim1-Orai1 dependent Ca^{2+} entry in PS exposure under conditions of anticoagulation (thrombin inhibition). In line with these results, Bergmeier *et al.* also found that *Orai1*^{R93W} platelets are defective in surface PS exposure, suggesting that Orai1 mediated SOCE is crucial for the procoagulant response of the platelet rather than for other Ca^{2+} -dependent cellular responses (95). However, deficiency in STIM1 and Orai1 did not abolish PS exposure and thrombus formation under non-anticoagulation conditions *in vitro*. In the presence of thrombin, GPVI dependent PS exposure and procoagulant activity were only partly affected (79). *In situ* thrombin generation seemed to be sufficient for high PS exposure even in the absence of SOCE. This result suggested the presence of a compensating Ca^{2+} entry mechanism, which could be triggered either by STIM2 or by ROCE.

In contrast, normal Ca^{2+} store release and influx in response to all agonists were found in *Stim2*^{-/-} platelets (79). Furthermore, normal SOCE, thrombus formation and PS exposure were observed both under coagulation and non-coagulation conditions (79). The role of ROCE in PS exposure in the absence of SOCE was also investigated by using SKF96365, a selective Ca^{2+} entry blocker. Strikingly, in both, *Stim1*^{-/-} and *Orai1*^{-/-} platelets SKF96365 further suppressed the GPVI-ITAM-PLC γ 2 and the Gq-PLC β induced Ca^{2+} influx, suggesting ROCE as an alternative Ca^{2+} entry pathway in platelets which independently regulates PS exposure (79).

9. INHIBITION OF STIM1 AND ORAI1 FUNCTION: A THERAPEUTICAL OPTION FOR STROKE?

Despite the marked protection of *Stim1*^{-/-} and *Orai1*^{-/-} BMc mice in collagen-dependent models of arterial thrombosis, these and also the *Orai1*^{R93W} mice, exhibited only mildly prolonged tail bleeding times, indicating that SOCE is more important for arterial thrombus formation than for primary hemostasis. This makes STIM1 and Orai1 potential targets for antiplatelet therapy. However, problems may arise from the widespread expression of STIM1 and Orai1 as well as from differences in STIM1/Orai1 expression and function between mice and humans (93).

The most prominent defect in *Stim1*^{-/-} and *Orai1*^{-/-} mice is a pronounced perinatal lethality. Surviving mice display strongly reduced body size and weight and have a dramatically shortened life time (75, 78, 90, 110-113). The exact cause of death is not known, but myopathy, due to defects in skeletal muscle (112-113) and (cardio) pulmonary defects (75, 78) have been observed. Within the hematopoietic system, defects in mast cells (78, 113), macrophages (114) and B cells (110) have been found, while *Stim1*^{-/-} T cells, despite exhibiting a pronounced hyperproliferative phenotype (111, 115), were still able to fulfill some effector functions (115). These impairments within the hematopoietic system are, however, not life limiting, as *Stim1*^{-/-} and *Orai1*^{-/-} BMc mice were viable and did not show obvious signs of disease (75, 90, 110). It is important to note, however, that laboratory mice grow up and are kept in a pathogen free environment and the ability of *Stim1*^{-/-} and *Orai1*^{-/-} BMc mice or conditional knock-out mice to cope with complex infections has not been tested.

In contrast to mice, in humans lacking either functional STIM1 or ORAI1, a defect within the immune system is the life limiting factor (93, 116-117). The few patients described all suffered from combined immunodeficiency due to impaired lymphocyte activation resulting in recurrent and often lethal infections. The most apparent non-immune phenotypes observed in STIM1 and ORAI1 deficient patients are congenital myopathy and defects in dental enamel calcification (116-117). Importantly, patients deficient in either STIM1 or ORAI1 function did not exhibit an overt bleeding diathesis. These results correlate well with the largely intact primary hemostasis in *Stim1*^{-/-} and *Orai1*^{-/-} BMc mice. The thrombocytopenia observed in all three STIM1 and in one ORAI1 deficient patient was caused by autoantibodies against platelet surface antigens and not by developmental defects (93). Of note, ORAI1 seems to have a non-redundant function in human platelets, as Ca^{2+} influx after treatment with thapsigargin in platelets of a patient with a loss of function mutation in ORAI1 was profoundly impaired (116, 118).

In conclusion, inhibition of STIM1 or Orai1 function may be a suitable approach for antithrombotic therapy in human, however, not for long-term therapy, due to the expected side effect of immunosuppression. However, it may be useful in some clinical settings where a

short term inhibition of platelet function is sufficient to achieve the therapeutic goal, e.g. in the treatment of acute ischemic stroke (119).

Stroke is the second leading cause of mortality worldwide (3). In most cases, stroke is caused by occlusion of a major or multiple smaller intracerebral arteries leading to focal cerebral ischemia and subsequent neuronal cell death in the center of the ischemic territory. At present, early thrombolysis of vessel occluding thrombi is the only established therapeutic option (120-121). This recanalization of major intracerebral arteries is a prerequisite for salvage of tissue; however, sufficient reflux does not guarantee the prevention of infarct growth, as secondary thrombus formation in the microvasculature at distal sites of the infarct area may occur (122). Platelets have a multifaceted role in ischemic stroke, as they are critically involved in its pathogenesis but are also required for prevention of intracranial bleeding (121).

To test the functional relevance of STIM1 and Orai1 mediated SOCE in stroke, *Stim1*^{-/-} and *Orai1*^{-/-} BMC mice have been subjected to transient middle cerebral arterial occlusion (tMCAO) (119, 123). In this animal model of ischemic stroke, a thread is advanced through the carotid artery into the middle cerebral artery, reducing blood flow by about 95% (119). After 1 hour, the thread is withdrawn, allowing reperfusion of the ischemic area, thus mimicking the clinical situation with vessel occlusion, followed by thrombolysis and reperfusion. Remarkably, in both *Stim1*^{-/-} and *Orai1*^{-/-} BMC mice infarct volumes 24 hours after induction of tMCAO were reduced to ~30% of those observed in control BMC mice (75, 90) (Figure 3). Magnetic resonance imaging, performed in living animals, further showed that this protection was sustained, since no secondary infarct growth was observed during the entire observation of 7 or 5 days after infarct induction, respectively. Moreover, no intracranial hemorrhage was detected in T2-weighted gradient echo images, demonstrating that the protective effect did not occur at the expense of intracranial bleeding. Importantly, the reduced infarct sizes were of functional relevance, as both, *Stim1*^{-/-} and *Orai1*^{-/-} BMC mice reached significantly better scores than control mice in tests assessing the motoric or global neurological function (75, 90). Of note, STIM2 deficient mice have also been shown to be protected in the tMCAO model, but this phenotype could be linked to alterations within the neuronal system and was not due to altered platelet function (124).

Taken together, these results revealed STIM1 and Orai1 as essential mediators of ischemic brain infarction and established these two proteins and SOCE as potential targets for the prevention and treatment of ischemic stroke. This is of particular relevance because the utility of current antithrombotic or anticoagulant therapeutics is limited by their inherent effect on primary hemostasis and thus the risk of intracranial bleeding complications. Recent experiments have provided evidence that administration of platelet GPIIb inhibiting antibodies after the induction of stroke can be as beneficial as prophylactic treatment, indicating that platelet inhibition

can be effective even at later stages of disease progression (119). Thus, a single bolus of a STIM1 and Orai1 inhibitor, applied to a patient with acute ischemic stroke, might be sufficient to improve the clinical outcome, while avoiding side effects on other cell types which would likely arise from long term application (see above). It is important to note, however, that data obtained in mice cannot directly be extrapolated to the human situation as differences in the pathomechanisms of ischemic tissue injury may exist. However, in the case of integrin α IIb β 3 blockade the results obtained in mice (119) were predictive for the effect observed in humans (125).

Orai1 seems to be a good target protein for the development of SOCE inhibiting drugs, because it is located in the plasma membrane and therefore easily accessible. STIM1 has also been reported to be located to some extent in the plasma membrane in some cell types, including human platelets (71, 126). However, the suitability of plasma membrane STIM1 as a therapeutic target appears questionable, as a recent study raised the interesting possibility that plasma membrane STIM1 may participate in the inhibition of SOCE (126). Different pharmaceutical companies are currently working on Orai1 inhibitors, mainly with the purpose to develop novel immunosuppressive drugs (127). However, those drugs should also be effective in the inhibition of platelet SOCE given the dominant role of Orai1 in this process (94, 118).

10. CONCLUSION AND OPEN QUESTIONS

The identification of STIM and Orai as the key molecules mediating SOCE was a milestone in cell biology, and has important implications for platelet biology. Analysis of genetically modified mice clearly established STIM1 and Orai1 as the main store Ca^{2+} sensor and SOC channel in platelets, respectively. Considering the well-known importance of Ca^{2+} signals in platelet physiology and the reported defective T-cell function in the absence of SOCE, it was quite surprising that platelets in the absence of SOCE still developed normally and were able to fulfill their role in primary hemostasis. The activation of SOCE involves receptor stimulation, signaling from the membrane to the ER, store depletion, clustering of STIM1 and finally activation of SOC channels. The time required for this process, may be too long for platelets as they have to react very rapidly to activating stimuli. Thus, store release of Ca^{2+} appears to be sufficient to trigger the main responses of initial platelet activation, like integrin activation and granule release. In contrast, SOCE seems to be especially important for subsequent events, like growth and stabilization of thrombi (75, 90) and coagulant activity (79, 95).

Primary hemostasis and thrombus formation have long been considered to be intrinsically tied together. STIM1/Orai1-mediated SOCE, however, seems to be of greater relevance for pathological processes than for primary hemostasis, given the protective effect of STIM1- and Orai1 deficiency in different thrombosis models and the absence of spontaneous hemorrhages and excessive bleeding after injury. Thus, selective targeting of

pathological thrombus formation without or only mildly affecting the cell's ability to seal wounds at sites of injury, may be possible. Future developments will have to show if these observations can be transferred to the human system and if novel powerful, yet safe, antithrombotics can be developed.

These recent advances, however, did not only answer many questions but also raised new ones. One first question – not only for platelet biology – is how STIM1 regulates store content and which other proteins or signaling pathways are involved in this process. Also, it is not known why defective SOCE impairs GPVI-ITAM coupled receptor signaling while GPCR signaling remains largely intact. Further open questions are: What is the function of STIM2 in platelets? Is Orai1 the only SOC channel in platelets? Is there a pathway that negatively regulates the STIM1-Orai1 interaction? New insights into the regulation of Ca^{2+} signaling and SOCE will be of pivotal importance to understand the complexity of platelet function.

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Abbreviations: ECM: extracellular matrix; ADP: adenosine diphosphate; ATP: adenosine triphosphate; TxA₂: thromboxane A₂; Ca²⁺: calcium; [Ca²⁺]_i: intracellular free Ca²⁺ levels; SOCE: store-operated Ca²⁺ entry; ER: endoplasmic reticulum; STIM1: stromal interaction molecule 1, vWF: von Willebrand factor; GP: glycoprotein; PS phosphatidylserine; GPCR: G-protein coupled receptor; CLEC-2: C-type lectin-like receptor 2; CalDAG-GEFI: Ca²⁺ and diacylglycerol regulated guanine nucleotide exchange factor I; PKC: protein kinase C; CIB1: Ca²⁺- and integrin-binding protein 1; DTS: dense tubular system; PLC: phospholipase C; IP₃: inositol-1,4,5-trisphosphate; IP₃R: IP₃-receptor; PIP₂: phosphatidyl-1,4-bisphosphate; DAG: 1,2-diacyl-glycerol, ROCE: receptor operated Ca²⁺ entry; SERCA: sarcoplasmic endoplasmic reticulum Ca²⁺-ATPases; PMCA: plasma membrane Ca²⁺-ATPase; TP: thromboxane-prostanoid; PAR: protease activated receptor; ITAM: immunoreceptor tyrosine-based activation motif; TRP: transient receptor potential I; SCID: severe combined immunodeficiency, tMCAO: transient middle cerebral arterial occlusion

Key Words: Platelets, Thrombus Formation, Hemostasis, Review

STIM and Orai in hemostasis and thrombosis

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