Study of calcium transport processes in blood platelets using knock out mouse models. Regulation and role of *"store-operated calcium entry"* (SOCE)

Ph.D. Thesis

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Introduction

Platelet activation and aggregation at sites of vascular injury are essential processes to arrest bleeding; however, on the other hand the same processes contribute also to pathological thrombus formation in diseased vessels, such as found in atherosclerosis. For this reason, platelet activation must be tightly regulated in order to be sufficient to provide a platelet plug big enough to seal the wounded artery, but also controlled to avoid undesired vessel occlusion.

A wide variety of extracellular matrix proteins – most notably collagen and von Willebrand factor – and soluble agonists, released from platelet granules or produced locally, can elicit platelet activation, thereby supporting firm platelet adhesion and the recruitment of further platelets from the circulation. These agonists activate different signaling pathways, in most of which a common final step is the rise in intracellular calcium concentration ($[Ca^{2+}]_i$).

Calcium is an essential second messenger in virtually all cells, regulating fundamental cellular processes from cell differentiation, through cell division to gene transcription and many others. An elevation in $[Ca^{2+}]_i$ is also a prerequisite of platelet activation, since the blockade of this elevation – by the

use of extra- and intracellular calcium scavengers – has been shown to impair the process. Despite its importance, not much is known about the exact regulation of calcium homeostasis in platelets.

There are two major sources of cytosolic calcium elevation: the release of compartmentalized free Ca^{2+} and the entry of extracellular free Ca^{2+} through the plasma membrane.

The mechanism of calcium release from the intracellular stores is a rather well established process. Agonist binding of platelet surface receptors leads to the activation of phospholipase (PL) C isoforms, which hydrolyze phosphoinositide-4,5-bisphosphate (PIP_2) to inositol-1,4,5-trisphosphate (IP_3) and diacyl-glycerol (DAG). IP_3 in turn releases the calcium content of intracellular stores while DAG is involved in calcium entry from the extracellular compartment.

In contrast, less well-known is how calcium entry through the plasma membrane happens. In non-excitable cells, such as platelets, the major way of calcium entry involves IP_3 mediated Ca^{2+} release from the intracellular stores followed by calcium influx from the extracellular space; a process referred to as *store-operated calcium entry* (SOCE). Despite intensive efforts, the molecular components and the functional relevance of SOCE in platelets remained elusive.

The so called *de novo* conformational coupling model has been the leading hypothesis regarding the molecular mechanism of SOCE in blood platelets. This model suggests canonical transient receptor potential channel 1 (TRPC1) – a six transmembrane domain protein – to be the major SOC channel in these cells and emphasizes a *de novo* coupling between TRPC1 and the IP_3-receptor (IP_3-R) type 2 in the endoplasmic reticulum (ER) membrane upon store release as the mechanism leading to channel opening.

There is, however, considerable debate in the literature regarding the correctness of this hypothesis. Already the localization of TRPC1 is unclear. It is supposed to be present in the plasma membrane in order to fulfill the SOC channel function, however, there are reports showing it to be expressed mainly in internal membranes. Furthermore, the *de novo* coupling model is based largely on *in vitro* studies where an antibody against TRPC1 resulted in reduced SOCE in platelets, although the same antibody was unable the detect TRPC1 in other studies. Finally, there are reports stating no effects of anti-TRPC1 treatment on platelet SOCE.

In 2005 and 2006 stromal interaction molecule 1 (STIM1) and Orai1, respectively, have been identified as conserved components of SOCE in *Drosophila* S2 cells and T cell lines.

STIM1, a single transmembrane protein, has been shown to be a calcium sensor in the ER membrane of these cells that upon calcium release moves to plasma membrane near puncta and regulates SOCE. Orai1 on the other hand has been identified as a calcium channel bearing all the characteristics of SOC channels.

Aim of the study

As already mentioned, calcium entry is generally believed to be of pivotal importance for platelet activation; however, definitive evidence of this is completely missing. The above mentioned conflicting data regarding the *de novo* conformational coupling hypothesis and the role of TRPC1 in platelet SOCE and the description of novel proteins in the regulation of calcium entry inspired me to investigate the process in more details.

During my PhD work, I aimed to study the possible role of STIM1, TRPC1 and Orai1 in platelet calcium homeostasis and signaling. Furthermore, I investigated the functional relevance of these molecules and store-operated calcium entry in *in vitro* platelet function and thrombus formation under flow conditions.

Methods

In order to accomplish these plans, genetically modified knock out mice – and where necessary bone marrow chimeras – were generated in our laboratory and *in vitro* and *in vivo* experiments were carried out to study the platelets of these animals.

In vitro experiments:

Using flow cytometry and aggregometry, I investigated platelet morphology, the expression of different surface receptors and the capability of knock out platelets to change their shape, activate integrins, release their granules and form stable aggregates. I performed intracellular calcium measurements to study possible differences in the calcium store content and calcium entry in knock out platelets as compared to wild-type controls. Finally, using a flow chamber system I examined the capability of platelets to form stable adhesions and aggregates under high shear conditions.

In vivo experiments:

Using two different arterial thrombosis models – one chemical injury model of small mesenteric arteries and one mechanical injury model of the abdominal aorta – and a collagen/epinephrine induced pulmonary embolization model, I investigated how STIM1, TRPC1 and Orai1 influence platelet function and thrombus formation *in vivo*. Furthermore, in collaboration with the Neurology Department of the University of Würzburg the impact of platelet store-operated calcium entry on stroke development was studied in a transient middle cerebral artery occlusion model.

Results

In my PhD work, I show that

1. however, mice lacking STIM1 or Orai1 born severely ill, megakaryopoiesis and platelet production is unaffected in these animals.
2. lack of STIM1 and Orai1 both cause dramatically reduced SOCE and calcium response to all major agonists in platelets.
3. furthermore, STIM1-deficiency not only reduces platelet SOCE, but also results in a lower filling state of the intracellular calcium stores.

4. *Stim1*$^{-/-}$ and *Orai1*$^{-/-}$ platelets have a selective activation defect through PLCγ2, whereas activation through the G-protein coupled receptor (GPCR) / PLCβ pathway is unaffected.
5. lack of STIM1 or Orai1 barely influences aggregate formation in the absence of flow, whereas thrombus formation is severely impaired under flow conditions.
6. the formation of stable thrombi *in vivo* is defective in the absence of STIM1 or Orai1, however, this defect is more pronounced in the absence of STIM1.
7. *Stim1*$^{-/-}$ and *Orai1*$^{-/-}$ bone marrow chimeras are protected against ischemic stroke in a transient middle cerebral artery occlusion model without suffering from bleeding time prolongation.
8. TRPC1 plays no significant role in platelet SOCE.

Conclusion

The existence of store-operated calcium entry in platelets has been known for over a decade, but the underlying mechanisms and its relevance in platelet physiology remained elusive. My results establish STIM1 and Orai1 as essential components of platelet SOCE, where STIM1 is a calcium sensor in the ER

membrane that regulates Orai1 as the major SOC channel in the plasma membrane.

The severely reduced calcium response to all major platelet activating agonists shows that store-operated calcium entry is the major source of calcium increase in platelets. Whereas this process seems to be dispensable for megakaryopoiesis and platelet production, it is necessary for complete platelet activation through PLCγ2. Why functional SOCE is obligatory for the PLCγ2 activation pathway and not for the PLCß pathway is unknown at present. The slower kinetic of PLCγ2 activation, as compared to PLCβ activation, could be responsible for the observed differences; however, further investigation of this is needed.

In my PhD thesis I show that despite the relatively normal platelet activation observed *in vitro*, platelet SOCE is of major importance for aggregate formation under flow conditions and thrombus formation *in vivo*. The defect in the formation of stable thrombi was more pronounced in *Stim1*$^{-/-}$ mice than in *Orai1*$^{-/-}$ animals most probably due to the fact that *Stim1*$^{-/-}$ platelets not only display impaired SOCE but also have reduced calcium content in the intracellular stores. This strongly suggests that besides mediating calcium entry, STIM1

also regulates the filling of cytoplasmic calcium stores through a mechanism independent of SOCE.

Finally, our stroke studies indicate that platelet SOCE is of huge relevance in a clinically important disease model. The fact that lack of STIM1 or Orai1 protects bone marrow chimeras from ischemic cerebrovascular events with no or only very mild bleeding prolongation, makes these molecules – or alternatively the whole platelet SOCE process – promising candidates in the prevention and treatment of ischemic cardio- and cerebrovascular events.

Publication list

Publications involved in the thesis:

Braun A, **Varga-Szabo D (joint first)**, Kleinschnitz C, Pleines I, Bender M, Austinat M, Bosl M, Stoll G, Nieswandt B. Orai1 (CRACM1) is the platelet SOC channel and essential for pathological thrombus formation. *Blood* Prepublished online Oct 2, 2008; DOI:10.1182/blood-2008-07-171611

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