Regulation of STIM1/Orai1-dependent Ca\(^{2+}\) signalling in platelets

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Summary
Platelet secretion and aggregation as well as thrombus formation of blood platelets critically depend on increase of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) mainly resulting from intracellular Ca\(^{2+}\) release followed by store operated Ca\(^{2+}\) entry (SOCE) through Ca\(^{2+}\) release activated channels (CRAC). SOCE is in part accomplished by the pore forming unit Orai and its regulator stromal interaction molecule (STIM). Orai1 and STIM1 transcription is stimulated by NF-κB (nuclear factor kappa B). Serum- and glucocorticoid-inducible kinase 1 (SGK1) up-regulates NF-κB-activity in megakaryocytes and thus Orai1-expression and SOCE in platelets. SGK1 is thus a powerful regulator of platelet Ca\(^{2+}\)-signalling and thrombus formation and presumably participates in the regulation of platelet activation by a variety of hormones as well as clinical conditions (e.g. type 2 diabetes or metabolic syndrome) associated with platelet hyperaggregability and increased risk of thromboocclusive events. SOCE in platelets is further regulated by scaffolding protein Homer and chaperone protein cyclolin A (CyPA). Additional potential regulators of Orai1/STIM1 and thus SOCE in platelets include AMP activated kinase (AMPK), protein kinase A (PKA), reactive oxygen species, lipid rafts, pH and mitochondrial Ca\(^{2+}\) buffering. Future studies are required defining the significance of those mechanisms for platelet Orai1 abundance and function, for SOCE into platelets and for platelet function in cardiovascular diseases.

Keywords
Platelet physiology, polymorphisms, kinases, signal transduction, Ca\(^{2+}\) (arterial thrombosis)

Introduction
Platelet adhesion, activation and aggregation are essential for primary haemostasis at sites of vascular injury but by the same token account for the development of acute thrombotic occlusion at regions of atherosclerotic plaque rupture leading to ischaemic diseases such as myocardial infarction or ischaemic stroke (1, 2). Moreover, platelets play a decisive role in the pathogenesis of several inflammatory diseases, particularly in vascular inflammation and atherogenesis (2).

Activation of platelets by several agonists such as subendothelial collagen, adenosine diphosphate (ADP), thrombin and collagen-related peptide (CRP) is followed by platelet granule release, integrin αIIbβ3 activation, aggregation and thrombus formation (3). All those functions are triggered by an increase of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) (3, 4). In platelets, both, Ca\(^{2+}\) and diacylglycerol-regulated guanine nucleotide exchange factor I (Caldag-GEFI) and protein kinase C (PKC) have been shown to be critical elements linking increased cytosolic Ca\(^{2+}\) concentration to platelet secretion and integrin activation (inside-out signalling) (3, 5, 6).

A strong and sustained rise in [Ca\(^{2+}\)]\(_{i}\), further triggers phosphatidylserine (PS) exposure (7) and cell shrinkage due to efflux of K\(^{+}\) ions through Ca\(^{2+}\)-activated K\(^{+}\)-channels (8). Activation-dependent exposure of phosphatidylserines is required for the procoagulant platelet response involving assembly of tenase and prothrombinase complexes which results in formation of factor Xa and thrombin, respectively (9).

STIM1/Orai1 in platelet Ca\(^{2+}\) signalling
In platelets, Ca\(^{2+}\) release from intracellular stores is triggered by inositol 1,4,5-trisphosphate (IP\(_{3}\)) via activation of IP\(_{3}\) receptors (10-12). IP\(_{3}\) production occurs due to agonist-mediated activation of phospholipase C by thrombin (via activation of G protein-coupled receptors), downstream of G\(_{\text{q}}\) protein coupled phosphoinositide 3-kinase (PI3K) signalling or after stimulation of the tyrosine kinase linked collagen receptor glycoprotein VI (GPVI) by collagen or CRP (13).

Depletion of the intracellular Ca\(^{2+}\) stores in the endoplasmic reticulum triggers store operated Ca\(^{2+}\) entry (SOCE), which is ac-
completed in part by the pore forming Ca\(^{2+}\) release-activated channel (CRAC) moiety (CRACM) Orai (3, 14, 15). Following store depletion Orai is activated by the Ca\(^{2+}\) sensing stromal interaction molecule (STIM), which is primarily located in the intracellular Ca\(^{2+}\) stores (16). STIM protrudes into the intracellular store lumen and senses the Ca\(^{2+}\) content with its Ca\(^{2+}\) binding EF-hand domain (17).

All three Orai isoforms and both STIM isoforms are expressed in human platelets, but Orai1 appears to be mainly involved in SOCE (9, 18). Orai1 is considered particularly important for platelet function as Orai1 deficiency renders mice resistant to pulmonary thromboembolism, arterial thrombosis, and ischaemic brain infarction (14). Megakaryopoiesis, platelet number and platelet size are, however, normal in Orai1 and STIM1 knockout mice (14, 16). Humans expressing mutant forms of Orai1 apparently do not suffer from major functional defects of platelets (15, 19). Expression of the inactive mutant Orai1<sup>R93W</sup> reduced SOCE, impaired agonist-induced increases in [Ca\(^{2+}\)], blunted integrin activation, impaired degranulation at low agonist concentrations and counteracted cell membrane scrambling with phosphatidylserine exposure, but did not significantly affect the ability of platelets to aggregate or to adhere to collagen under arterial flow conditions ex vivo (12). STIM1/Orai1 is critical for the procoagulant activity but not for the proadhesive function of platelets (20). At the site of vascular injury, STIM1 and CalDAG-GEFI are critical for the first wave of thrombin generation mediated by procoagulant platelets (20).

Pharmacological blockade of Orai1-dependent SOCE in human platelets has a thromboprotective potential by suppressing thrombus formation and reducing platelet procoagulant activity. Using Orai1 knockout murine platelets as an excellent control, 2-APB was shown to be the most specific SOCE inhibitor of Orai1 (21). Platelet activation and thus platelet-dependent coagulation and thrombus formation are blunted by Orai inhibitors (22), by antibodies directed against Orai1 or STIM1 (23–25) and by non-steroidal oestrogens (26). In human platelets, Ca\(^{2+}\) entry is a function of Orai1 protein abundance in the cell membrane (27).

In addition to SOCE, platelets express non-store-operated Ca\(^{2+}\) (non-SOC) and receptor operated Ca\(^{2+}\) (ROC) channels (28). Ca\(^{2+}\) entry into platelets further involves the mammalian homologue of Drosophila transient receptor potential channel (TRP) channels TRPC1 and TRPC6 (29-32). Upon store depletion, TRPC1 and TRPC6 associate to a complex with STIM and Orai proteins (24, 29-32). Upon store depletion Orai is activated by the Ca\(^{2+}\) sensing stromal interaction molecule (STIM), which is primarily located in the intracellular Ca\(^{2+}\) stores (16). STIM protrudes into the intracellular store lumen and senses the Ca\(^{2+}\) content with its Ca\(^{2+}\) binding EF-hand domain (17).

Regulation of STIM1/Orai1

NF-\(\kappa\)B (nuclear factor kappa B)

The transcription factor NF-\(\kappa\)B is critically important for inflammatory signalling in several metabolic disorders (35), such as diabetes mellitus or metabolic syndrome, clinical conditions typically associated with platelet hyperaggregability and atherothrombotic complications including myocardial infarction or ischaemic stroke (36). The majority of NF-\(\kappa\)B family members, including the regulatory proteins IkB \(\alpha\) and IkB kinase (IKK), are expressed in both megakaryocytic MEG-01 cells and megakaryocytes (37). Together with other well described megakaryocytic transcription factors involved in megakaryocyte maturation, such as erythroid transcription factor GATA-binding factor 1 (GATA-1) or nuclear factor erythroid-derived 2 (NF-E2), NF-\(\kappa\)B regulates megakaryocytic differentiation and plays a decisive role in the regulation of megakaryocytic gene expression and platelet function (38, 39). Genes regulated by NF-\(\kappa\)B in megakaryocytes (4) and other cell types (40) include Orai1 and STIM1.

SGK1 (serum- and glucocorticoid-inducible kinase 1)

Platelet activation critically depends on stimulation of PI3K-dependent signalling (41). Interference with PI3K signalling has previously been shown to compromise Ca\(^{2+}\) influx into platelets (42, 43). Signalling molecules regulated by PI3K signalling include AGC kinases, such as Akt (protein kinase B, PKB) or protein kinase C (PKC); which have been shown to be decisive players in platelet secretion, aggregation and thrombus formation (44-46). A further PI3K-sensitive AGC kinase is SGK1, a kinase regulated by a variety of triggers, including thrombin, insulin-like growth factor (IGF)-1, transforming growth factor (TGF)-\(\beta\), oxidative stress and ischaemia (47).

SGK1 is highly expressed in megakaryocytes, acts as an important transcriptional regulator of NF-\(\kappa\)B-dependent Orai1 expression in megakaryocytes and thus influences Ca\(^{2+}\) response, activation and thrombus formation of released platelets (4, 48). SGK1 phosphorylates IkB kinase alpha/beta (IKKa/b), which in turn leads to phosphorylation of the NF-\(\kappa\)B inhibitory protein IkB with subsequent degradation of IkB and thus translocation of NF-\(\kappa\)B subunit p65 to the nucleus (49) (Figure 1).

SGK1 has previously been reported to regulate a wide variety of further ion channels including TRPV4-6 or Kv1.3 (50), carriers including NHE1 and several glucose and amino acid transporters (51) as well as the Na\(^{+}/K\(^{-}\)-ATPase (51). Several SGK1 sensitive channels, carriers, enzymes and transcription factors are expressed in platelets and/or megakaryocytes. To which extent their regulation by SGK1 influences platelet function, remains to be shown.

SGK1 presumably participates in the influence of several hormones and mediators on platelet function (4). SGK1 expression is stimulated by a variety of triggers, including thrombin, gluco- and mineralocorticoids, TGF\(\beta\), interleukin (IL) 6, endothelin and advanced glycation end products (AGE) (47). Moreover, SGK1 expression is up-regulated by excessive glucose concentrations, metabolic acidosis, ischaemia, heat shock and oxidative stress (47).
SGK1 is activated by growth factors via PI3K and the 3-phosphoinositide dependent kinase 1 (PDK1), by mammalian target of rapamycin mTOR complex-2 (mTORC2) and by WNK1 (with no lysine kinase 1) (47). Much is yet to be learned about the role of SGK1 in the regulation of platelet function by those hormones and cell stressors.

A gain-of-function SGK1 gene variant (~3-5% prevalence in Caucasians) is associated with hypertension, stroke, obesity and type 2 diabetes (47). The same gene variant is associated with ischaemic stroke (52). The association is only in part explained by higher blood pressure of the gene variant carriers (52). Additional mechanisms involved may include platelet hyperaggregability.

Complete SGK1 deficiency does not fully abrogate store operated Ca\(^{2+}\) entry (SOCE) into platelets and Ca\(^{2+}\)-sensitive platelet function but decreases the sensitivity of the platelets to stimulators of Ca\(^{2+}\) entry (4). In theory, the function of SGK1 could in the absence of SGK1 be taken over by similar signalling molecules, such as the SGK3 isoform (53) and the related kinase Akt/PKB (54), as shown in dendritic cells (53) and vascular smooth muscle cells (54), respectively.

ERK1/2 (extracellular-signal-regulated kinases 1 and 2) and CyPA (cyclophilin A)

STIM1 is phosphorylated by ERK1/2 leading to enhanced SOCE (55, 56). Disruption of the phosphorylation site does not affect the association of STIM1 with Orai1, but decreases SOCE (55, 56). Phosphorylation of STIM1 and activation-induced Ca\(^{2+}\) mobilisation from intracellular stores and SOCE in platelets are fostered by the chaperone protein CyPA (55). Phosphorylation of STIM1 is abrogated, and Ca\(^{2+}\) mobilisation from intracellular stores as well as Ca\(^{2+}\) influx from extracellular space are impaired by CyPA deficiency (55). Along those lines, CyPA-deficient mice are protected against arterial thrombosis while bleeding time is not appreciably affected in those mice (55). The lack of STIM1 phosphorylation in CyPA-deficient platelets does, however, not prevent the association of STIM1 and Orai1 following platelet activation with thrombin (55). In unstimulated platelets CyPA colocalises with (55) and participates in the regulation of (57) the sarco/endoplasmatic reticulum Ca\(^{2+}\) ATPase SERCA2b. Thrombin stimulation attenuates the colocalisation of CyPA and SERCA2b.

Further regulators of STIM1/Orai1

STIM1 and Orai1 associate with Homer, a cytoplasmic scaffolding protein participating in the signalling of G-protein-coupled receptors, which was shown to support the agonist-induced Ca\(^{2+}\) entry into thrombin-stimulated platelets (58). The significance of Homer in the regulation of platelet function remains, however, to be shown.

Ca\(^{2+}\) entry into platelets is further regulated by AMP activated kinase (AMPK) (59), a kinase activated by cellular energy depletion (60). Both the a1 and a2 AMPK isoforms are expressed by human and murine platelets (61). Pharmacological inhibition of AMPK blunts the thrombin-induced platelet aggregation and clot retraction without affecting the initial increase in cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_{cyt}\)) (61). Clot retraction is also impaired in platelets from mice lacking AMPKα2 but not in platelets from mice lacking AMPKα1 (61). Whether or not AMPK influences Orai1/STIM1 in platelets is unknown. In other cell types (62), AMPK has been shown to downregulate Orai1. AMPK is activated by increase of [Ca\(^{2+}\)], through Calcium/calcmodulin-dependent protein kinase kinase β (CaMKKβ) and CaMKKβ-dependent phosphorylation (63). STIM1 silencing blunts the increase of [Ca\(^{2+}\)], and subsequent AMPK phosphorylation following hypoxia (63). AMPK is thus part of a negative feedback loop limiting Ca\(^{2+}\) entry following energy depletion and/or increase of cytosolic Ca\(^{2+}\). Energy depletion compromises the function of the sarco/endoplasmatic reticulum Ca\(^{2+}\) ATPase SERCA and thus fosters the depletion of intracellular Ca\(^{2+}\) stores. Without the inhibition of Orai1 by AMPK the store depletion would lead to activation of Orai1 by STIM1.

The translocation of STIM1 to the junctions between ER and the plasma membrane and thus binding of STIM1 to Orai1 is stimulated by cAMP and protein kinase A (64). STIM1 is further regulated by tyrosine phosphorylation, the kinases involved remaining, however, elusive (65).

Orai1 has been shown to be regulated by reactive oxygen species (ROS) (66) and lipid rafts (67). In platelets, lipid rafts are particularly required for the inactivation of SOCE by extracellular Ca\(^{2+}\) (68). Furthermore, extracellular pH as well as intracellular

<table>
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<th>Regulator</th>
<th>Direction</th>
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<td>(4)</td>
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<td>Homer</td>
<td>upregulation</td>
<td>platelets</td>
<td>(58)</td>
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<td>ERK1/2</td>
<td>upregulation</td>
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<td>(55)</td>
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<td>upregulation</td>
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<td>tyrosine kinase</td>
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Table 1: Regulators of STIM1 and Orai1.

<table>
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<td>RACK1</td>
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acidic Ca\(^{2+}\) stores are important regulators of platelet SOCE (69). Acidosis (pH 6.9) inhibits while alkalosis (pH 7.9) augments platelet SOCE and platelet aggregation (69). Recently, it has been shown that depletion of platelet acidic Ca\(^{2+}\) stores, sensed by STIM1 and STIM2 located in lysosome-related organelles and dense granules, is critically involved in SOCE activation after store depletion (70, 71).

The association of STIM1 and Orai1 is supported by mitochondria, which buffer the Ca\(^{2+}\) release from the Ca\(^{2+}\) stores and thus foster emptying of the Ca\(^{2+}\) stores and subsequent activation of Orai1 (72). Mitochondrial Ca\(^{2+}\) buffering lowers cytosolic Ca\(^{2+}\) activity in the proximity of the channels and thus blunts Ca\(^{2+}\) dependent slow inactivation of the channels (72, 73). Along those lines mitochondrial depolarisation reduces movement of STIM1 across the ER membrane (72, 73).

Regulators of Orai1 in other cell types further include the ubiquitin ligase Nedd4-2 (neuronal precursor cells expressed developmentally downregulated), an enzyme ubiquitinating Orai1 thus preparing the channel protein for proteasomal degradation (74). Nedd4-2 appears, however, not to play a major role in the regulation of Orai1 in platelets (4). Orai1 is further regulated by RACK1 (receptor for activated protein kinase C-1) (75). Whether or not RACK1 plays a role in the regulation of Orai1 in platelets, has, however, not been reported.

**Role of STIM1/Orai1 in thromboocclusive cardiovascular disease: Pathophysiological and potential therapeutic implications**

Platelet hyperaggregability is one of the key mechanisms involved in the atherothrombotic complications associated with type 2 diabetes mellitus, such as myocardial infarction or ischemic stroke (36). Platelets isolated from patients with type 2 diabetes show increased P-selectin expression, adhesion, and aggregation (36). The mechanisms responsible for enhanced platelet activity in type 2 diabetes include enhanced TRPC3, Orai1 and STIM1 expression (76) with subsequent increase of SOCE, cytosolic Ca\(^{2+}\) concentration and Ca\(^{2+}\) entry in response to agonists (77, 78). On the other hand, the thapsigargin (TG) or thrombin induced interaction of STIM1 with Orai1, hTRPC1 and hTRPC6 is blunted in platelets from diabetic patients (79).

The enhanced Orai1 expression, SOCE and agonist sensitivity of platelets in type 2 diabetes could well result from enhanced SGK1 expression (47). Diabetes mellitus is characterised by hyperglycaemia and increased generation of advanced glycation end products (AGEs) which are strongly associated with platelet activation (76). Excessive glucose concentrations and advanced glycation end products (AGEs) are powerful stimulators of SGK1 expression (51) suggesting that increased stimulation of SGK1 in these patients could contribute to megakaryocytic NF-kB induction and stimulation of Orai1 expression resulting in enhanced SOCE and increased activation-dependent platelet responsiveness.

Excessive SGK1, Orai1 and STIM1 expression may be expected in hyperaldosteronism and glucocorticoid excess (47). Cytosolic Ca\(^{2+}\) activity is enhanced in platelets from patients with chronic renal failure (80, 81). At least in theory, the increased Ca\(^{2+}\) activity could result from hyperaldosteronism with subsequent stimulation of SGK1 expression (47). Moreover, SGK1 expression is further up-regulated by TGFβ1 (47) and presumably participates in alterations of platelet function during inflammation.

Considering their impact on platelet activation and thrombotic occlusion, STIM1, Orai1 and their regulators, such as SGK1, may be considered promising targets for the development of anti-thrombotic drugs. To date, several antiplatelet inhibitors (targeting cyclooxygenase-1, purinergic receptor P2Y12 and activated integrin αIIbβ3) are available, but all antiplatelet therapies still have limitations including lack of efficacy (non-responder) or serious bleeding complications. Thus, novel antiplatelet strategies are warranted to control undesired platelet activation without disrupting haemostasis and risking life-threatening bleeding complications (82). Since mice lacking STIM1 and Orai1 or its regulator SGK1, display markedly reduced arterial occlusive thrombus formation, but only mildly prolonged (STIM1/Orai1) or even normal (SGK1) bleeding times (4, 11), these molecules are potential targets for anti-thrombotic therapy. As SGK1 participates in the regulation not only of Orai1/STIM1 but of a wide variety of functions (47), treatment with SGK1 inhibitors may be limited by multiple side effects. However, the phenotype of SGK1 knockout mice is mild, indicating that SGK1 is not critically important for house keeping func-
tions (83). Instead, SGK1 is relevant primarily following excessive SGK1 expression leading to or supporting several disorders including platelet hyperaggregability (83).

Conclusions

Recent observations shed new light on the regulation of the pore forming Orai1 and its regulator STIM1, which accomplish a large part of store-operated Ca\(^{2+}\) entry (SOCE) or Ca\(^{2+}\) release activated channels (CRAC) and are thus critically important for platelet activation. A powerful regulator of Orai1 and STIM1 is SGK1, which is in turn under control of cell stress, such as ischaemia, energy depletion and oxidation. Moreover, SGK1 is regulated by a large variety of hormones and mediators, including aldosterone and glucocorticoids as well as IGF-1, TGF-β or thrombin. SGK1 regulates Orai1/STIM1 by stimulating transcription through up-regulation of transcription factor NF-κB in megakaryocytes. It is tempting to speculate that SGK1-sensitive Orai1 expression participates in platelet hyperaggregability of disorders associated with enhanced SGK1 expression, such as glucocorticoid excess. SGK1 is up-regulated in and contributes to the various clinical features of metabolic syndrome. Diseases expected to up-regulate SGK1 and thus Orai1/STIM1 activity include type 2 diabetes, metabolic syndrome and inflammation and are associated with a significantly increased risk for thromboocclusive cardiovascular events. Orai1/STIM1 and thus SOCE are further modulated by AMP activated kinase (AMPK), receptor for activated C kinase 1 (RACK1), Ca\(^{2+}\) sensitive protein kinase A, tyrosine phosphorylation, the chaperone protein cyclophilin A (CyPA), ROS, lipid rafts, pH as well as mitochondrial Ca\(^{2+}\) buffering (Table 1). There is little doubt that future studies will reveal further signalling molecules regulating platelet Orai1/STIM1 protein abundance and function. Moreover, Orai1/STIM1 and its regulators may be considered attractive targets for the treatment of arterial thrombosis underlying myocardial infarction or ischaemic stroke.

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Conflicts of interest

None declared.

References

34. Redondo PC, et al. Intracellular Ca2+ store depletion induces the formation of macromolecular complexes involving hTRPC1, hTRPC6, the type II IP3 receptor and SERCA3 in human platelets. Biochim Biophys Acta 2008; 1783: 1163-1176.
50. Lang F, Shumilina E. Regulation of ion channels by the serum- and glucocorticoid-inducible kinase SGK1. FASEB J 2013; 27: 3-12.
60. Steinberg GR, Kemp BE. AMPK in Health and Disease. Physiol Rev 2009; 89: 1025-1078.