Antithrombotic Potential of Blockers of Store-Operated Calcium Channels in Platelets

Roger van Kruchten, Attila Braun, Marion A. H. Feijge, Marijke J. E. Kuijpers, Ronny Rivera-Galdos, Peter Kraft, Guido Stoll, Christoph Kleinschnitz, Edouard M. Bevers, Bernhard Nieswandt, Johan W. M. Heemskerk

Objective—Platelet Orai1 channels mediate store-operated Ca2+ entry (SOCE), which is required for procoagulant activity and arterial thrombus formation. Pharmacological blockage of these channels may provide a novel way of antithrombotic therapy. Therefore, the thromboprotective effect of SOCE blockers directed against platelet Orai1 is determined.

Methods and Results—Candidate inhibitors were screened for their effects on SOCE in washed human platelets. Tested antagonists included the known compounds, SKF96365, 2-aminoethyl diphenylborate, and MRS1845 and the novel compounds, Synta66 and GSK-7975A. The potency of SOCE inhibition was in the order of Synta66>2-aminoethyl diphenylborate>GSK-7975A>SKF96365>MRS1845. The specificity of the first 3 compounds was verified with platelets from Orai1-deficient mice. Inhibitory activity on procoagulant activity and high-shear thrombus formation was assessed in plasma and whole blood. In the presence of plasma, all 3 compounds suppressed platelet responses and restrained thrombus formation under flow. Using a murine stroke model, arterial thrombus formation was provoked in vivo by transient middle cerebral artery occlusion. Postoperative administration of 2-aminoethyl diphenylborate markedly diminished brain infarct size.

Conclusion—Plasma-soluble SOCE blockers such as 2-aminoethyl diphenylborate suppress platelet-dependent coagulation and thrombus formation. The platelet Orai1 channel is a novel target for preventing thrombotic events causing brain infarction. (Arterioscler Thromb Vasc Biol. 2012;32:1717–1723.)

Key Words: thrombosis ▪ platelets ▪ calcium channel blockers ▪ stroke ▪ pharmacology

Elevation in cytosolic Ca2+ is fundamental to most platelet responses to physiological agonists, including pseudopod formation, integrin αIIbβ3 activation, secretion, procoagulant activity, and formation of platelet aggregates.1,2 Hence, elevated Ca2+ is a central signaling event in regulating the formation of a multplatelet thrombus after arterial damage. Surprisingly, attempts to pharmacologically attack platelet Ca2+ signaling to suppress thrombus formation and, hence, arterial thrombosis have so far been unsuccessful. This is attributable to a lack of knowledge of the molecular mechanism implicated in platelet Ca2+ signaling and to the absence of suitable Ca2+ signal inhibitors.

Most platelet agonists raise cytosolic Ca2+ via inositol 1,4,5-trisphosphate (InsP3)–mediated mobilization of Ca2+ from internal stores in the endoplasmic reticulum, which is dramatically enhanced by Ca2+ influx from the extracellular medium.1,3 Recent work has shown that, in both human and mouse platelets, the Ca2+ channel Orai1 (also indicated as CRACM1) is responsible for the majority of Ca2+ entry into activated platelets through the pathway of store-operated Ca2+ entry (SOCE).4,6 The permeability of the Orai1 channel appears to be strictly regulated by the interaction with the Ca2+-sensing endoplasmic protein, stromal-interacting molecule 1 (STIM1).7 Platelets also express other channels implicated in Ca2+ influx, particularly isoforms of the transient receptor potential channels, transient receptor potential channel 1 and transient receptor potential channel 6, of which only the latter plays a modest role in platelet activation via the pathway of receptor-operated Ca2+ entry.8,9 In other (nonexcitable) cell types, however, these non-Orai Ca2+ channels can have a more important role.

Recent data using mice deficient in Orai1 or STIM1 have shown that both proteins in platelets play key roles in arterial thrombus formation, as determined in various in vivo thrombosis models.5,11 In addition, deficiency in platelet Orai1 or STIM1 appeared to suppress experimental induction of ischemic brain infarction but did not result in a bleeding phenotype. In agreement with this, deficiency in platelet Orai1...
or STIM1 reduced the buildup of platelet thrombi during high-shear blood flow over a collagen surface. These studies indicated that the Ca\(^{2+}\) entry process via Orai1 and STIM1 enhanced the formation of platelet aggregates, but was essential for platelet procoagulant activity.

Platelet procoagulant activity is induced by strong Ca\(^{2+}\)-mobilizing agonists such as collagen, activating the glycoprotein VI (GPVI) receptor, in combination with thrombin, activating the protease-activated receptors (PAR). This response requires a sustained rise in intracellular Ca\(^{2+}\), surpassing a threshold level of \(\approx 400\) nmol/L. This causes surface exposure of the negatively charged phospholipid, phosphatidylserine (PS), which serves as a binding site for coagulation factors. By providing a key link between platelet and coagulation activation, the platelet procoagulant response was found to regulate arterial thrombus formation in vivo in various experimental mouse models.

Considering the relevant role of Orai1 in arterial thrombosis, we hypothesized that pharmacological blockage of the Orai1 channel may provide a novel way of antithrombotic therapy by suppressing platelet aggregate formation and particularly platelet procoagulant activity. In this article, we first screened established and novel pharmacological inhibitors of SOCE for their suppressive effects on platelet Ca\(^{2+}\) responses and thrombus formation. Using Orai1-deficient platelets, we verified this channel as the target of the most potent inhibitors. We furthermore determined the efficacy of inhibitors to suppress arterial thrombus formation in an established model of ischemic brain infarction.

**Methods**

For extended information, see the online-only Data Supplement.

**Mice**

Experiments with mice were approved by the local animal care and use committees. Wild-type C57BL/6, bone marrow chimeras of C57BL/6 mice with Orai1\(^{-/-}\) or Orai1\(^{+/+}\) platelets have been described before.

**Ca\(^{2+}\) Entry Inhibitors**

SKF96365, MRS1845, and 2-aminoethyl diphenylborinate (2APB) were from Sigma (St. Louis, MO). LOE-908Cl was kindly provided by Boehringer Ingelheim Pharma. The novel inhibitors, Synta66, 3-fluoropyridine-4-carboxylic acid (2',5'-dimethoxybiphenyl-4-yl) amide (compound 66 from patent WO 2005/009954 A2), and GSK-7975A, were from Sigma (St. Louis, MO). LOE-908Cl and the nitrophenyl pyridine, SKF96365, were selected by their ability to suppress Ca\(^{2+}\) mobilization from stores with 10% to 30% (Figure 1A and 1B). In contrast, MRS1845 up to 100 \(\mu\)mol/L did not suppress the Ca\(^{2+}\) entry signal evoked by CaCl\(_2\), whereas they moderately reduced Ca\(^{2+}\) mobilization from stores with 10% to 30% (Figure 1A and 1B). Because platelets lack voltage-dependent Ca\(^{2+}\) channels, specific inhibitors of these were not tested. At maximally effective concentrations, Synta66 and 2APB (10 \(\mu\)mol/L) as well as SKF96365 and GSK-7975A (100 \(\mu\)mol/L) nearly completely blocked the Ca\(^{2+}\) entry signal evoked by CaCl\(_2\) addition, whereas they moderately reduced Ca\(^{2+}\) mobilization from stores with 10% to 30% (Figure 1A and 1B). Because platelets lack voltage-dependent Ca\(^{2+}\) channels, specific inhibitors of these were not tested. At maximally effective concentrations, Synta66 and 2APB (10 \(\mu\)mol/L) as well as SKF96365 and GSK-7975A (100 \(\mu\)mol/L) nearly completely blocked the Ca\(^{2+}\) entry signal evoked by CaCl\(_2\) addition, whereas they moderately reduced Ca\(^{2+}\) mobilization from stores with 10% to 30% (Figure 1A and 1B). Because platelets lack voltage-dependent Ca\(^{2+}\) channels, specific inhibitors of these were not tested. At maximally effective concentrations, Synta66 and 2APB (10 \(\mu\)mol/L) as well as SKF96365 and GSK-7975A (100 \(\mu\)mol/L) nearly completely blocked the Ca\(^{2+}\) entry signal evoked by CaCl\(_2\) addition, whereas they moderately reduced Ca\(^{2+}\) mobilization from stores with 10% to 30% (Figure 1A and 1B).

**Results**

**Pharmacological Inhibition of Platelet Ca\(^{2+}\) and Procoagulant Responses**

Several compounds known to affect agonist-evoked influx of extracellular Ca\(^{2+}\) were screened for their potency to block SOCE and exposure of procoagulant PS in washed human platelets. The platelets were stimulated with a combination of strong agonists, convulxin (activating GPVI) and thrombin (activating protease-activated receptors), that is, a condition known to maximally trigger Ca\(^{2+}\) signaling. Platelet stimulation in the presence of low EGTA (baseline Ca\(^{2+}\) = 15 nmol/L) resulted in full mobilization of Ca\(^{2+}\) from intracellular stores, which was detected as a transient Ca\(^{2+}\) rise peaking at 400 nmol/L (Figure 1A). Subsequent addition of a surplus of CaCl\(_2\) induced massive Ca\(^{2+}\) influx attributable to SOCE, resulting in Ca\(^{2+}\) peak levels up to 1300 nmol/L. Candidate compounds tested included the known SOCE inhibitor, bimethoxyphenyl imidazole, SKF96365; the less well-studied lipophilic biaromatics, Synta66 and GSK-7975A (Figure 1 in the online-only Data Supplement); and furthermore the diphenylborate 2APB, initially used as Ins\(_3\) receptor inhibitor at high concentrations but now recognized as a more potent antagonist of Ca\(^{2+}\) entry. Other tested compounds were LOE-908Cl and the nitrophenyl pyridine, MRS1845, both of which block Ca\(^{2+}\) entry in HL-60 cells. Because platelets lack voltage-dependent Ca\(^{2+}\) channels, specific inhibitors of these were not tested. At maximally effective concentrations, Synta66 and 2APB (10 \(\mu\)mol/L) as well as SKF96365 and GSK-7975A (100 \(\mu\)mol/L) nearly completely blocked the Ca\(^{2+}\) entry signal evoked by CaCl\(_2\) addition, whereas they moderately reduced Ca\(^{2+}\) mobilization from stores with 10% to 30% (Figure 1A and 1B). In contrast, MRS1845 up to 100 \(\mu\)mol/L did not suppress the Ca\(^{2+}\) entry. The compound LOE-908Cl strongly interfered with the fura-2 fluorescence signal and, hence, was not used for experiments. Dose–response curves for all inhibitors showed that the IC\(_{50}\) concentration for SOCE in washed platelets increased in the order of Synta66<2APB<GSK-7975A<SKF96365<MRS1845 (Figure 1C).
Platelet procoagulant activity resulting from surface exposure of PS is known to be greatly impaired by deficiency or mutations of the SOCE channel, Orai1. Using fluorescent-labeled annexin A5, we investigated the ability of all inhibitors to affect PS exposure in platelets stimulated with convulxin and thrombin. Flow cytometric analysis showed that Synta66 (10 μmol/L), 2APB (10 μmol/L), GSK-7975A (30 μmol/L), and SKF96365 (100 μmol/L) each potently suppressed the fraction of PS-exposing platelets (Figure II in the online-only Data Supplement). Quantitative analysis indicated that among these, at a maximally effective concentration, 2APB was the most potent inhibitor. These results thus showed that all tested SOCE inhibitors restrain platelet procoagulant activity.

SOCE Blockers Suppress Human Platelet Activation in Plasma and Whole-Blood Thrombus Formation

In plasma or whole blood systems, lipophilic inhibitors often need to be added at 10× to 50× higher concentrations than in nonplasma-based buffer systems to affect platelet function. This also appeared to be the case for the SOCE inhibitors. When added to platelet-rich plasma, concentrations of 100 μmol/L Synta66, 2APB, or GSK-7975A were required for inhibition of convulxin-induced Ca2+ rises and PS exposure with 41% to 49% (data not shown). To verify that these inhibitors influenced platelet procoagulant activity, the effects of Synta66, 2APB, or GSK-7975A (100 μmol/L) in platelet-rich plasma were measured on thrombin generation. Upon triggering with 1 μmol/L tissue factor, peak heights of thrombin generation were reduced with Synta66, 2APB, and GSK-7975A to 29±2%, 58±2%, and 28±2% of control, respectively. SKF96365 (100 μmol/L) was inactive in platelet-rich plasma (not shown).

Because Synta66, 2APB, or GSK-7975A retained their inhibitory activity in plasma, the compounds were tested in whole-blood thrombus formation. Therefore, phenylanalyl-prolyl-arginine choromethyl ketone-anticoagulated human blood was flowed at high shear rate over collagen (ie, a condition where the thrombus-forming process is regulated via Orai1-mediated platelet activation). In this flow perfusion assay, collagen-adherent platelets are activated in a GPVI-dependent way by elevating intracellular Ca2+ and aggregating and exposing PS. With all 3 inhibitors, the deposition of platelets on the collagen surface was markedly reduced in comparison with the vehicle control, as was the number of platelets exposing PS (Figure 2A and 2B). Subsequent experiments with fluo-4–loaded platelets indicated that, at the same flow conditions, all 3 SOCE inhibitors did reduce the Ca2+ rises of collagen-adhered platelets by 25% (Figure 3).

In control flow experiments, the effect of chelation of extracellular Ca2+ by EGTA was examined. This resulted in the formation of small aggregates on the collagen surface, while PS exposure (measured with fluorescein isothiocyanate–labeled lactadherin) was also reduced by >90%. With EGTA present, the extra addition of 2APB, Synta66, or GSK-7975A (100 μmol/L) was without further effect on PS exposure and platelet deposition (surface area coverage: vehicle 7.8%; 2APB 7.8%; Synta66 8.3%; GSK-7975A 7.5%). Together, these data demonstrate that the tested compounds reduce Ca2+ signaling and markedly suppress collagen-dependent thrombus formation of flowing human blood.

SOCE Blockers Suppress Murine Platelet Responses and Thrombus Formation

Considering that Orai1 forms the main SOCE channel in mouse platelets, we used platelets from wild-type and Orai1-deficient mice to verify the selectivity of the inhibitors. In fura-2–loaded platelets from wild-type mice, the GPVI agonist CRP caused a prominent Ca2+ rise in the presence of CaCl2, attributable to SOCE (Figure III in the online-only Data Supplement). This Ca2+ rise with CRP was inhibited by >50% with Synta66 (10 μmol/L), 2APB (100 μmol/L), or GSK-7975A (100 μmol/L). In fura-2–loaded Orai1−/− platelets, CRP evoked a much lower Ca2+ response because of the absence of SOCE, which is in confirmation with earlier results. The Ca2+ signal in knockout platelets was not affected by Synta66 or 2APB, whereas GSK-7975A was slightly inhibitory (Figure III in the online-only Data Supplement). In wild-type platelets, all 3 inhibitors markedly suppressed thrombin-evoked Ca2+ rises,
whereas Synta66 and GSK-7975A but not 2APB had a limited reducing effect on the already impaired thrombin-evoked Ca\(^{2+}\) rise in Orai\(^{1-}\) platelets. Together, these data point to 2APB as a most specific inhibitor of murine Orai1.

We then examined the efficacy of the compounds to suppress murine thrombus formation in flow experiments over collagen with isolated wild-type blood. In comparison with the vehicle condition, all 3 compounds (100 μmol/L) significantly lowered platelet deposition by ≈30% (leaving small aggregates) and PS exposure by 50% (Figure 4A and 4B). This was again accompanied by a marked reduction in Ca\(^{2+}\) responses of the collagen-adherent platelets (Figure 4C). The relatively high reduction in PS exposure in comparison with platelet aggregate formation is compatible with the results of flow studies using Orai1\(^{-}\) blood. Previous data have indicated that platelet adhesion and aggregation are less dependent on the Ca\(^{2+}\) signal than PS exposure, which requires a prolonged elevation in cytosolic Ca\(^{2+}\).

Flow experiments were also performed with Orai1\(^{-}\) blood to check for potential non-Orai effects of 2APB, Synta66, and GSK-7975A (Figure IV in the online-only Data Supplement). However, in the absence of Orai1 none of these compounds (100 μmol/L) caused a further reduction in platelet deposition or PS exposure. Together, this points to Orai1 as principal target of these inhibitors in whole-blood thrombus formation.

Given the marked effects of 2APB in flow experiments, this compound was used to examine the consequences of SOCE inhibition on arterial thrombus formation in vivo. Wild-type mice were injected with a bolus of 2APB (3 mg/kg) or vehicle solution, and blood was taken after 60 minutes to assess collagen-dependent thrombus formation under flow. The in vivo 2APB treatment resulted in the formation of smaller platelet aggregates and in a marked decrease in platelet deposition by ≈35% (Figure 5A and 5B). This resembled the diminished thrombus formation after in vitro addition of 2APB (Figure 4).

Because platelet activation via glycoprotein Ib, GPVI, and Orai1 significantly contributes to the development of brain infarction after thrombus formation in the cerebral arteries, we tested the efficacy of 2APB on this process using an established experimental stroke model. Herein, ischemic stroke is evoked by transient MCA occlusion, and the brain infarct volume is assessed after 1 day. Administration of 2APB to wild-type mice significantly reduced the infarction volume by ≈30% compared with the vehicle control (Figure 5C).
Orai\(^{1/−}\) mice, transient MCA occlusion caused a smaller infarction volume, which is in agreement with previous results.\(^5\) Importantly, administration of 2APB to Orai\(^{1/−}\) mice did not result in a further decrease in infarction size (Figure 5C and 5D). Altogether, these results indicate that the blockade of platelet SOCE via Orai1 has a thromboprotective effect.

**Discussion**

In the present study, we show for the first time that pharmacological blockage of platelet SOCE channels has a thromboprotective potential by suppressing thrombus formation and reducing platelet procoagulant activity. In both human and mouse platelets, the novel compounds Synta66, GSK-7975A, and 2APB, at concentrations that inhibited SOCE, effectively antagonized whole-blood thrombus formation and PS exposure. These results are in agreement with the earlier reported impaired thrombus formation and platelet procoagulant activity in mice with Orai1-deficient platelets\(^5,10\) and in human platelets with a dysfunctional Orai1R91W mutation.\(^6\) In contrast, we found that the compound SKF96365, earlier identified as blocker of \(\text{Ca}^{2+}\) influx in platelets, was ineffective in the presence of blood plasma.

We find that in suspensions of washed human platelets, 2APB, Synta66, and GSK-7975A effectively suppressed agonist-induced \(\text{Ca}^{2+}\) influx via SOCE (>85%), at concentrations where \(\text{Ca}^{2+}\) mobilization from stores was only moderately affected (10%–25%). The mechanism of this moderate reduction of \(\text{Ca}^{2+}\) mobilization is unclear. Similar to 2ABP, both inhibitors may modulate InsP\(_3\) receptor function (eg, by affecting \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release). Alternatively, they may interfere in a proposed interaction of InsP\(_3\) receptors with Orai1 channels.\(^{29}\)

Both 2APB and Synta66 abrogated GPVI-induced \(\text{Ca}^{2+}\) signaling in wild-type but not in Orai1\(^{1/−}\) mouse platelets, whereas GSK-7975A caused limited additional inhibition in Orai1\(^{1/−}\) platelets. Furthermore, 2APB did not affect thrombin-induced \(\text{Ca}^{2+}\) signaling in Orai1\(^{1/−}\) mouse platelets, thus pointing to this compound as a more selective inhibitor of the Orai1 channels. Synta66 has been shown to block I\(_{\text{CRAC}}\) currents in...
mast cells, whereas the structural analog GSK-7975A has not been tested before. The compound 2APB was previously considered to be an antagonist of InsP$_4$ receptors but later identified as a much more potent Ca$^{2+}$ influx blocker, which is confirmed by the current data. Other tested compounds were much less effective in inhibiting SOCE and could not be used in the presence of blood plasma (ie, MRS1845 and SKF96365, the first described antagonist of Ca$^{2+}$ influx in isolated platelets and leukocytes). $^{22}$

In GPVI-stimulated platelets, Snyt66, GSK-7975A, and 2APB markedly inhibited the procoagulant response (ie, PS exposure) at concentrations that also affected Ca$^{2+}$ responses. This inhibition was observed in washed platelets with thrombin as coagulant and in collagen-adhered platelets after blood perfusion. Previous studies suggested a role of Ca$^{2+}$ influx in PS exposure of human platelets, $^{31,32}$ and the present data are the first to demonstrate that pharmacological blockade of SOCE suppresses this process in whole blood. However, the moderate reduction in Ca$^{2+}$ responses in collagen-adhered platelets points to partial inhibition of SOCE in whole blood despite the presence of high concentrations (10$^{-4}$ mol/L) of blockers, which is compatible with their incomplete inhibition of PS exposure. Given the limited water solubility of all tested compounds, next generation Orai1 antagonists preferably combine reduced hydrophobicity with increased affinity to the Ca$^{2+}$ channel.

Injection of a single bolus of 2APB into wild-type mice effectively reduced whole-blood thrombus formation ex vivo in mouse and significantly diminished brain infarct volume after middle cerebral artery occlusion (ie, an arterial thrombosis model known to rely on platelet Orai1 activity). $^{5}$ Given the fact that this model of brain infarction relies on glycoprotein Ib- and GPVI-dependent platelet activation and on factor Xa, thrombin, and fibrin(ogen) on thrombi at venous shear.

In conclusion, we have shown that SOCE inhibitors including 2APB, directed against Orai1, have a high potential in reducing platelet-dependent coagulation and thrombus formation. The relatively high doses needed for the presently available SOCE (Orai1) inhibitors, however, urge for a search to higher affinity channel blockers before starting preclinical studies. Given the reported immune deficiency of patients with missense Orai1 mutations, total or long-term blockade of SOCE may not be desirable in the treatment of patients with thrombosis. Hence, possible toxic side effects after administration of Orai1 channel blockers need to be monitored thoroughly. With such compounds available, our data indicate that the platelet Orai1 channel is a novel target for attacking arterial thrombosis to brain infarction.

Acknowledgments

We thank Drs M. Begg and D. House (GlaxoSmithKline, Stevenage, UK) for their stimulating discussions.

Sources of Funding

We acknowledge support from the Cardiovascular Centre (HVC), Maastricht; the Center for Translational Molecular Medicine (CTMM) INCOAG; and the Deutsche Forschungsgemeinschaft (DFG), SFB 688 (TP A13 to C.K.).

Disclosures

None.

References

Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. *EMBO J*. 2001;20:2120–2130.


Supplemental Material

The antithrombotic potential of blockers of store-operated calcium channels in platelets

Roger van Kruchten¹, Attila Braun², Marion A. H. Feijge¹, Marijke J. E. Kuijpers¹, Ronny Rivera-Galdos², Peter Kraft³, Guido Stoll¹, Christoph Kleinschnitz³, Edouard M. Bevers¹, Bernhard Nieswandt², Johan W. M. Heemskerk¹

¹Department of Biochemistry and Cardiovascular Centre, Cardiovascular Research Institute Maastricht (CARIM), University of Maastricht, The Netherlands; ²Chair of Vascular Medicine, University Clinic, and Rudolf Virchow Center, DFG Research, Center for Experimental Biomedicine, University of Würzburg; ³Department of Neurology, University Hospital, Würzburg, Germany

Corresponding author: Johan W. M. Heemskerk PhD, Dept. of Biochemistry (CARIM), Maastricht University, Universiteitszidingel 50, Maastricht. PO Box 616, 6200 MD Maastricht, The Netherlands. Tel: +31-43-3881671, fax: +31-43-3884159; e-mail: jwm.heemskerk@maastrichtuniversity.nl
Supplemental Methods

Mice- Bone-marrow chimeras of mice with Orai1\(^{+/+}\) or Orai1\(^{+/-}\) platelets at C57BL/6 background were generated as described \(^1,2\). Deficiency in Orai1 was confirmed by reverse transcription PCR analysis. Wildtype mice were at C57BL/6 genetic background.

Materials- H-Phe-Pro-Arg chloromethyl ketone (PPACK) was obtained from Calbiochem; fragmin from Pfizer; annexin A5 labeled with fluorescein isothiocyanate (FITC) from PharmaTarget; fibrillar type I collagen (Horm) from Nycomed. Fura-2, and Fluo-4 acetoxymethyl esters, Alexa Fluor-647 annexin A5, and pluronic F-127 were all from Invitrogen. Apyrase (grade V), bovine serum albumin (BSA), heparin, 2,3,5-triphenyltetrazolium chloride (TTC) and thrombin were from Sigma. Convulxin was purified as described \(^3\).

Blood collection and platelet preparation- Human blood was obtained from healthy volunteers after full informed consent. For perfusion studies, the blood was collected into 40 µmol/L PPACK and 40 U/mL fragmin (f.c.). For the preparation of washed platelets, blood was collected into acid citrate dextrose (ACD) anticoagulant \(^4\). Platelets were resuspended in Hepes buffer pH 7.45 (136 mmol/L NaCl, 10 mmol/L Hepes, 2.7 mmol/L KCl, 2 mmol/L MgCl\(_2\), 0.1% glucose and 0.1% BSA) \(^5\). For the preparation of platelet-poor plasma, blood was collected in 1/10 volume 129 mmol/L trisodium citrate. Cells were counted with a Coulter counter.

Mouse blood was collected via retro-orbital puncture. For perfusion experiments, blood was collected into 40 µM PPACK and 5 U/mL low molecular weight heparin (f.c). Washed mouse platelets were prepared as described \(^1\).

Ca\(^{2+}\) measurements of platelets in suspension- Human \(^6\) and mouse \(^1\) platelets were loaded with Fura-2 acetoxymethyl ester and washed, as described. Calcium responses in the loaded platelets
(1×10⁸/mL) were measured by ratio fluorometry under stirring at 37°C. Calibration of Fura-2 fluorescence ratios to nanomolar levels of Ca²⁺ was performed by lysis with Triton X-100 and subsequent application of a surplus of EGTA in Tris buffer pH 8, as reported for human and mouse platelets¹. Cells were pretreated with vehicle (DMSO) or inhibitor during 10 min.

Flow cytometry- Surface exposed PS as a measure of procoagulant activity of platelets in suspension was determined by flow cytometry, as described ⁷. Briefly, cells (1×10⁸/mL) were preincubated with vehicle or inhibitor for 10 min, activated with the indicated agonist in the presence of 2 mmol/L CaCl₂, and stained with appropriate label, e.g. with FITC-annexin A5 to determine PS exposure. For measurements in plasma environment, washed platelets were added to plasma that was recalcified in the presence of 40 µmol/L PPACK and 40 U/mL fragmin, as described ⁸.

Thrombin generation- PRP and platelet-free plasma collected on citrate were used to measure thrombin generation under standard conditions with the calibrated automated thrombogram method ⁵. Thrombin generation was initiated by 1 pmol/L tissue factor, and first-derivative curves of fluorescence generation from cleaved Z-GGR-AMC were made using Thrombogram software. Thrombin peak heights were corrected for contribution of microparticles.

Thrombus formation and platelet Ca²⁺ responses under flow- Collagen type I coated coverslips, placed in a transparent parallel-plate flow chamber, were perfused with whole blood anticoagulated with PPACK/fragmin (human) ⁹ or PPACK/heparin (mouse) ²-¹⁰, thus containing physiologically high levels of free Ca²⁺ and Mg²⁺. Pretreatment with vehicle or inhibitors was for 10 min. After 4 min perfusion at arterial shear rate of 1000 s⁻¹, platelet thrombi formed on the collagen surface were post-labeled with FITC-annexin A5 (0.25 µg/mL) in Hepes buffer pH 7.45 containing 2 mmol/L CaCl₂. Phase contrast and fluorescent images were recorded by a Visitech
digital imaging system, equipped with two intensified, charge-coupled device cameras. Images were analyzed off-line by Metamorph software.

To measure single-cell Ca\textsuperscript{2+} responses under flow, human or mouse platelets were loaded with Fluo-4 acetoxymethyl ester, and re-added to the autologous blood at an amount of 10% Fluo-4-loaded platelets. During blood perfusion over collagen, Fluo-4 fluorescence images were recorded at high frequency (1 Hz) from the optical plane of the collagen coating. Changes in fluorescence were analyzed off-line by selecting regions of interest, representing single adhered platelets. Traces of fluorescence intensity (F) per platelet were converted into pseudo-ratio $F/F_0$ values.

**Statistics**- Significance of differences was determined with the paired t-test or the independent samples t test, as appropriate, using the statistical package for social sciences (SPSS 11.0).
References


Supplemental Fig. I. Chemical structure of (A) Synta 66, 3-fluoropyridine-4-carboxylic acid [2',5'-dimethoxybiphenyl-4-yl)amide, and (B) GSK-7975A, 2,6-difluoro-N-(1-[[4-hydroxy-2-(trifluoromethyl)phenyl)methyl]-1H-pyrazol-3-yl)benzamide.
Supplemental Fig. II. SOCE inhibitors suppress platelet procoagulant reactivity. Human platelets in Hepes buffer plus 2 mmol/L CaCl₂ were 10 min preincubated with vehicle (control), Synta66 (10 µmol/L), SKF96365 (100 µmol/L), 2APB (10 µmol/L) or GSK-7975A (30 µmol/L), and stimulated with convulxin (50 ng/mL) plus thrombin (4 nmol/L) during 15 min. Using FITC-annexin A5, PS-exposing platelets (marker, M1) were detected by flow cytometry. (A) Representative histograms. (B) Effects of various compounds on PS exposure. Mean ± SEM (n=4-7), *p<0.05.
Supplemental. Fig. III. SOCE blockers primarily block Orai1 signaling. Rises in Ca\(^{2+}\) were measured in wildtype and Orai1\(^{-/-}\) mouse platelets, loaded with Fura-2. Stimulation was with collagen-related peptide (CRP, 10 µg/mL) or thrombin (0.9 nM) in CaCl\(_2\)-containing Hepes buffer; preincubation was with vehicle (DMSO), Synta66 (10 µmol/L), 2APB (100 µmol/L) or
GSK-7975A (100 µmol/L). (A, B) Representative traces of Ca\(^{2+}\) rises in the presence of 2APB or vehicle for (A) wildtype and (B) Orai\(^1^-\) mouse platelets. (C-F) Quantitative effect of inhibitors on maximal Ca\(^{2+}\) rises in wildtype (C, E) or Orai\(^1^-\) platelets (D, F) after activation by CRP (C, D) or thrombin respectively (E, F). Mean ± SEM (n=3-5), *p<0.05 vs vehicle control.

**Supplemental Fig IV.** PPACK-anticoagulated wildtype and Orai\(^1^-\) mouse blood was perfused 4 min over collagen at 1000 s\(^{-1}\). Samples were preincubated with vehicle (control), Synta66, 2APB or GSK-7975A (100 µmol/L). Surface area coverage (SAC) of total and Annexin A5-binding platelets. Mean ± SEM (n=3), *p<0.05.