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 Ca\textsuperscript{2+} 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;32:0–0.)

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

Elevation in cytosolic Ca\textsuperscript{2+} is fundamental to most platelet responses to physiological agonists, including pseudopod formation, integrin activation, secretion, procoagulant activity, and formation of platelet aggregates.\textsuperscript{1,2} Hence, elevated Ca\textsuperscript{2+} is a central signaling event in regulating the formation of a multipotent thrombus after arterial damage. Surprisingly, attempts to pharmacologically attack platelet Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+} signaling and to the absence of suitable Ca\textsuperscript{2+} signal inhibitors.

Most platelet agonists raise cytosolic Ca\textsuperscript{2+} via inositol 1,4,5-trisphosphate (InsP\textsubscript{3})–mediated mobilization of Ca\textsuperscript{2+} from internal stores in the endoplasmic reticulum, which is dramatically enhanced by Ca\textsuperscript{2+} influx from the extracellular medium.\textsuperscript{3,4} Recent work has shown that, in both human and mouse platelets, the Ca\textsuperscript{2+} channel Orai1 (also indicated as CRACM1) is responsible for the majority of Ca\textsuperscript{2+} entry into activated platelets through the pathway of store-operated Ca\textsuperscript{2+} entry (SOCE).\textsuperscript{5–6} The permeability of the Orai1 channel appears to be strictly regulated by the interaction with the Ca\textsuperscript{2+}-sensing endoplasmic protein, stromal-interacting molecule I (STIM1). Platelets also express other channels implicated in Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+} entry.\textsuperscript{8,9} In other (nonexcitable) cell types, however, these non-Orai Ca\textsuperscript{2+} 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.\textsuperscript{10,11} 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.5,6,10 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.6,10

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 \(\sim 400\) nM.\(^{3,11}\) This causes surface exposure of the negatively charged phospholipid, phosphatidylerine (PS), which serves as a binding site for coagulation factors.12–14 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.\(^{5,10}\)

Considering the relevant role of Orai1 in arterial thrombosis, we hypothesized that pharmacological blockage of the Orai1 channels 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.

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

### Methods

#### 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.\(^{10}\)

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

SKF96365, MRS1845, and 2-aminoethyl diphenylborinate (2APB) were from Sigma. LOE-908Cl was kindly provided by Boehringer Ingelheim Pharma. The novel inhibitors, Synta66, 3-fluoropyridine-4-carboxylic acid (\(2',5',\)dimethoxyphenyl-4-yl)amide (compound 66 from patent \(\text{WO 2005/009954 A2}\), and GSK-7975A, 2,6-difluoro-\(\text{N}-(1-(4-hydroxy-2-(trifluoromethyl)phenyl)methyl)\)1H-pyrrol-3-yl)benzamide (compound 36 from patent \(\text{WO 2010/122089 A1}\)) were kindly provided by GlaxoSmithKline (UK). The latter 2 compounds were selected by their ability to suppress \(L_{\text{SOCE}}\) in Jurkat or basophilic leukemia cells (see patents). Synta66 and GSK-7975A were \(>99\%\) pure, as determined by proton nuclear magnetic resonance spectroscopy, liquid chromatography-mass spectrometry, and high-performance liquid chromatography.

#### Platelet Responses in Suspension

Details on platelet responses in suspension are given in the online-only Data Supplement.

#### Thrombus Formation and Platelet Ca\(^{2+}\) Responses Under Flow

Glass coverslips were coated with 10 \(\mu\)L of 50 \(\mu\)g/mL Horm collagen (1 cm\(^2\)) for 1 hour in a hummid box, and subsequently blocked with 1% BSA in HEPES buffer, pH 7.45. Collagen-coated coverslips were perfused with whole blood that was anticoagulated with PPACK/fragmin (human)\(^{17}\) or PPACK/heparin (mouse).\(^{11,18}\) Platelets in thrombi were postlabeled with fluorescein isothiocyanate-annexin A5. To measure single-cell Ca\(^{2+}\) responses under flow, platelets were loaded with fluo-4 acetoxyethyl ester and re-added to autologous blood at an amount of 10% fluo-4–loaded platelets.\(^{19}\) Inhibitor or vehicle was added 10 minutes before perfusion.

#### In Vivo Thrombosis

Wild-type or chimeric Orai1\(^{-/-}\) mice were injected with vehicle solution or 2APB (3 mg/kg) as indicated. In blood samples isolated 60 minutes after injection, collagen-induced thrombus formation was measured, as described above. To induce focal cerebral ischemia in mice, the middle cerebral artery (MCA) was transiently occluded for 60 minutes using an intraluminal filament as described elsewhere (transient MCA occlusion model).\(^{20}\) Immediately after reperfusion of the MCA territory, vehicle solution or 2APB (3 mg/kg) was injected postoperatively. Animals were euthanized on day 1 after transient MCA occlusion, and brain sections were stained with 2% 2,3,5-triphenyltetrazolium chloride to quantify the ischemic brain volume (corrected for edema).\(^{20}\)

### 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.\(^{21}\) Platelet stimulation in the presence of low EGTA (baseline Ca\(^{2+}\) \(\approx 15\) nM) resulted in full mobilization of Ca\(^{2+}\) from intracellular stores, which was detected as a transient Ca\(^{2+}\) rise peaking at 400 nM/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 nM/L. Candidate compounds tested included the known SOCE inhibitor, bimethoxyphenyl imidazole, SKF96365;\(^{22}\) the less well-studied lipophilic biaromate, 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.\(^{24,25}\) Other tested compounds were LOE-908CI and the nitrophenyl pyridine, MRS1845, both of which block Ca\(^{2+}\) entry in HL-60 cells.\(^{26,27}\) 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, 2APB, or GSK-7975A (100 \mu mol/L) was without further effect on PS exposure and aggregation (Figure 2A and 2B). Subsequent experiments with fura-2–loaded platelets indicated that, at the same flow conditions, all 3 SOCE inhibitors did reduce the Ca\(^{2+}\) rises of collagen-adenhred platelets by 25\% (Figure 3).

In control flow experiments, the effect of chelation of extracellular Ca\(^{2+}\) by EGTA was examined. This resulted in the formation of small aggregates on the collagen surface, while PS exposure (measured with fluoresein isothiocyanate–labeled lactadherin) was also reduced by >90\%. With EGTA present, the extra addition of 2APB, Synta66, or GSK-7975A (100 \mu mol/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 Ca\(^{2+}\) 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 inhibitory compounds for this Ca\(^{2+}\) channel. In fura-2–loaded platelets from wild-type mice, the GPVI agonist CRP caused a prominent Ca\(^{2+}\) rise in the presence of CaCl\(_2\), attributable to SOCE (Figure III 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 show 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\times to 50\times 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 PRP, concentrations of 100 \mu mol/L Synta66, 2APB, or GSK-7975A were required for inhibition of convulxin-induced Ca\(^{2+}\) 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 \mu mol/L) in PRP were measured on thrombin generation. Upon triggering with 1 pmol/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 \mu mol/L) was inactive in PRP (not shown).

Because Synta66, 2APB, or GSK-7975A retained its inhibitory activity in plasma, the compounds were tested in whole-blood thrombus formation. Therefore, PPACK-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-adenhred platelets are activated in a GPVI-dependent way by elevating intracellular Ca\(^{2+}\) 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).
whereas Synta66 and GSK-7975A but not 2APB had a limited reducing effect on the already impaired thrombin-evoked Ca\textsuperscript{2+} rise in Orai\textsuperscript{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 \textmu mol/L) significantly lowered platelet deposition by \(\approx 30\%\) (leaving small aggregates) and PS exposure by 50\% (Figure 4A and 4B). This was again accompanied by a marked reduction in Ca\textsuperscript{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 Orai\textsuperscript{1−/−} blood.\textsuperscript{5,10} Earlier data have indicated that platelet adhesion and aggregation are less dependent on the Ca\textsuperscript{2+} signal than PS exposure, which requires a prolonged elevation in cytosolic Ca\textsuperscript{2+}.\textsuperscript{1}

Flow experiments were also performed with Orai\textsuperscript{1−/−} 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 \textmu mol/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 \(\approx 35\%\) (Figure 5A and 5B). This resembled the diminished thrombus formation after in vitro addition of 2APB (Figure 4).

Because platelet activation via GPIb, GPVI, and Orai1 significantly contributes to the development of brain infarction after thrombus formation in the cerebral arteries,\textsuperscript{5,20} 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 \(\approx 30\%\) compared with the vehicle control (Figure 5C).
In Orai$^{-/-}$ mice, transient MCA occlusion caused a smaller infarction volume, which is in agreement with previous results. Administration of 2APB to Orai$^{-/-}$ 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 blockade 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 and in human platelets with a dysfunctional Orai1 R91W mutation.

In contrast, we found that the compound SKF96365, earlier identified as blocker of 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 Ca$^{2+}$ influx via SOCE ($\approx$85%), at concentrations where Ca$^{2+}$ mobilization from stores was only moderately affected (10%–25%). The mechanism of this moderate reduction of Ca$^{2+}$ mobilization is unclear. Similar to 2ABP, both inhibitors may modulate InsP$_3$ receptor function (eg, by affecting Ca$^{2+}$-induced Ca$^{2+}$ release). Alternatively, they may interfere in a proposed interaction of InsP$_3$ receptors with Orai1 channels.

Both 2APB and Synta66 abrogated GPVI-induced Ca$^{2+}$ signaling in wild-type but not in Orai1$^{-/-}$ mouse platelets, whereas GSK-7975A caused limited additional inhibition in Orai1$^{-/-}$ platelets. Furthermore, 2APB did not affect thrombin-induced Ca$^{2+}$ signaling in Orai1$^{-/-}$ mouse platelets, thus pointing to this compound as a more selective inhibitor of the Orai1 channels. Synta66 has been shown to block I$_{CRAC}$ 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$_3$ receptors but later identified as a much more potent Ca$^{2+}$ influx blocker, which was 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).

In GPVI-stimulated platelets, Synta66, 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 coagonist and in collagen-adhered platelets after blood perfusion. Previous studies suggested a role of Ca$^{2+}$ influx in PS exposure of human platelets, 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 SOCE (Orai1) inhibitors, however, urge for a search for new SOCE (Orai1) 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.

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

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**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 platelets. 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. 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.

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. 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) or PPACK/heparin (mouse), 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$^{2+}$ 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 figures

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) Orai1\(^{-/-}\) mouse platelets. (C-F) Quantitative effect of inhibitors on maximal Ca\(^{2+}\) rises in wildtype (C, E) or Orai1\(^{-/-}\) 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 Orai1\(^{-/-}\) 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.