Pflugers Arch - Eur J Physiol (2008) 457:377–387
DOI 10.1007/s00424-008-0531-4

Store-operated Ca^{2+} entry in platelets occurs independently of transient receptor potential (TRP) C1

David Varga-Szabo · Kalwant S. Authi · Attila Braun · Markus Bender · Archana Ambily · Sheila R. Hassock · Thomas Gudermann · Alexander Dietrich · Bernhard Nieswandt

Received: 28 April 2008 / Accepted: 9 May 2008 / Published online: 11 June 2008

Abstract Changes in $[Ca^{2+}]_i$ are a central step in platelet activation. In nonexcitable cells, receptor-mediated depletion of intracellular Ca^{2+} stores triggers Ca^{2+} entry through store-operated calcium (SOC) channels. Stromal interaction molecule 1 (STIM1) has been identified as an endoplasmic reticulum (ER)-resident Ca^{2+} sensor that regulates store-operated calcium entry (SOCE), but the identity of the SOC channel in platelets has been controversially debated. Some investigators proposed transient receptor potential (TRP) C1 to fulfil this function based on the observation that antibodies against the channel impaired SOCE in platelets. However, others could not detect TRPC1 in the plasma membrane of platelets and raised doubts about the specificity of the inhibiting anti-TRPC1 antibodies. To address the role of TRPC1 in SOCE in platelets, we analyzed mice lacking TRPC1. Platelets from these mice display fully intact SOCE and also otherwise unaltered calcium homeostasis compared to wild-type. Furthermore, platelet function in vitro and in vivo is not altered in the absence of TRPC1. Finally, studies on human platelets revealed that the presumably inhibitory anti-TRPC1 antibodies have no specific effect on SOCE and fail to bind to the protein. Together, these results provide evidence that SOCE in platelets is mediated by channels other than TRPC1.

Keywords Ca^2 channels · Ca^{2+}-activated channels · Ion channel · Platelets · Thrombin

David Varga-Szabo and Kalwant S. Authi contributed equally to this article.

D. Varga-Szabo · A. Braun · M. Bender · B. Nieswandt (✉)
Rudolf Virchow Center, DFG Research Center for Experimental Biomedicine, University of Würzburg,
Zinklesweg 10,
97078 Würzburg, Germany
e-mail: bernhard.nieswandt@virchow.uni-wuerzburg.de

K. S. Authi · A. Ambily · S. R. Hassock
Cardiovascular Division, King's College London,
Franklin Wilkins Building, Stamford Street,
London SE1 9NH, UK

T. Gudermann · A. Dietrich
Department of Pharmacology and Toxicology,
Philipps-Universität Marburg,
Marburg, Germany

B. Nieswandt
Institute of Clinical Biochemistry and Pathobiochemistry,
University of Würzburg,
Würzburg, Germany

Introduction

Changes in $[Ca^{2+}]_i$ play a central role in signaling processes in virtually all cells. In nonexcitable cells, such as platelets, the major Ca^{2+} entry pathway involves receptor-mediated release of Ca^{2+} from the sarcoplasmic/endoplasmic reticulum (SR/ER) which triggers Ca^{2+} influx through the plasma membrane (PM) by a process referred to as store-operated calcium entry (SOCE) [1]. Stromal interaction molecule (STIM) 1 has recently been identified as an ER-resident calcium sensor that activates store-operated calcium (SOC) channels in T cell lines [2–4] and mast cells [5]. In human T cells, the four transmembrane domain protein Orai1 (CRACM1) appears to be the predominant SOC channel [6, 7], but the C-terminal region of STIM1 may also interact with other SOC channel candidates such as

transient receptor potential channels (TRPCs) 1, 2, and 4 [8]. We have recently shown that an activating EF hand mutant of STIM1 constitutively activates SOC channels in mouse platelets leading to macrothrombocytopenia and a bleeding phenotype [9]. While this indicates that STIM1-dependent SOCE is a major pathway of Ca^{2+} entry in platelets, the identity of the platelet SOC channel remains a matter of considerable debate. TRPC1, which is present in human platelets [10, 11] and murine megakaryocytes (detected on the mRNA level [12]) has been proposed as the major platelet SOC channel based on in vitro studies where a blocking antibody against the protein (obtained from Alomone Labs, Israel) caused impairment of SOCE stimulated by low doses of thrombin and the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) inhibitor thapsigargin (TG) [13]. These investigators reported that store depletion caused the de novo conformational coupling of IP_3R type II in the stores with the TRPC1 channel suggested to be present in the plasma membrane [14, 15]. The same group further suggested that TRPC1 was present in lipid rafts and associated with TRPC4 and TRPC5, as was TRPC3 with TRPC6 associated [16]. Recently, another group also reported the expression of TRPC3 and TRPC4 [17]; however, other investigators were unable to detect TRPC3, TRPC4, or TRPC5 at the protein level, and the low levels of TRPC1 detected were found predominantly in the intracellular stores in contrast to TRPC6 which was strongly detected in the plasma membrane [18]. There is no reported detection of TRPC5 mRNA in platelets and megakaryocytes which questions the expression of this protein [11, 12] and doubts have been raised regarding the antibody used to detect TRPC4 [19]. There are further doubts raised regarding the specificity of the Alomone TRPC1 antibody used by the Rosado group with a reported lack of recognition of overexpressed hTRPC1 in heterologous systems [20] and another report stating that this antibody did not have any significant effect on Ca^{2+} entry induced by either TG or thrombin [21]. Recently, the de novo conformational coupling mechanism itself involving TRPC1 in Ca^{2+} entry has been questioned and a more prominent role for a Na^+/Ca^{2+} exchanger working in reverse mode has been suggested after a possible Na^+ entry event that may be mediated by TRPC1 [22]. In the light of these controversies and to address the role of TRPC1 in platelets directly, we analyzed TRPC1-deficient mice [23, 24]. We show that the lack of TRPC1 has no effect on Ca^{2+} store release and SOCE in platelets and does not influence their function in vitro or in vivo. Our findings on human platelets also indicate the absence of an interaction between TRPC1 and STIM1, suggesting that SOCE in platelets is mediated by channels other than TRPC1.

Materials and methods

Mice Animal studies were approved by the local authorities (Bezirksregierung of Unterfranken). Generation of *TRPC1*$^{-/-}$ mice has been described previously [23].

Chemicals and antibodies Anesthetic drugs: medetomidine (Pfizer, Karlsruhe, Germany), midazolam (Roche Pharma AG, Grenzach-Wyhlen, Germany), and fentanyl (Janssen-Cilag GmbH, Neuss, Germany) and antagonists: atipamezol (Pfizer, Karlsruhe, Germany), flumazenil, and naloxon (both from Delta Select GmbH, Dreieich, Germany) were used according to the regulation of the local authorities. ADP (Sigma, Deisenhofen, Germany), U46619 (Alexis Biochemicals, San Diego, USA), thrombin (Roche Diagnostics, Mannheim, Germany), collagen (Kollagenreagent Horm, Nycomed, Munich, Germany), and TG (Molecular Probes) were purchased. Monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or DyLight-488 were from Emfret Analytics (Würzburg, Germany).

RNA isolation and RT-PCR Platelet mRNA was isolated from 3 ml blood using Trizol reagent (Invitrogen, Karlsruhe, Germany). cDNA was synthesized using 1 μg platelet total mRNA and Super Script Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) as described by the manufacturer. Gradient PCR was performed using TRPC1 and actin-specific primers.

Western blotting TRPC1 was detected after immunoprecipitation of the protein from wild-type and *TRPC1*$^{-/-}$ platelets as described previously [18]. TRPC1 was extracted by immunoprecipitation from platelet lysates using the polyclonal anti-XTRP-1 antibody (a generous gift from G. Barritt, Adelaide, Australia) previously demonstrated to recognize TRPC1 [20]. The immunoprecipitates were Western blotted using the TRPC1 recognizing monoclonal antibody 1F1 (a generous gift from Dr. L. Tsiokas, Oklahoma, USA).

Intracellular calcium measurements Mouse platelet intracellular calcium measurements were performed as described [25]. Briefly, platelets isolated from blood were washed, suspended in Tyrode's buffer without calcium, and loaded with Fura-2/AM (5 μM) in the presence of Pluronic F-127 (0.2 μg/ml; Molecular Probes) for 30 min at 37°C. After labeling, platelets were washed once and resuspended in Tyrode's buffer containing 0 or 1 mM Ca^{2+}. Stirred platelets were activated with agonists, and fluorescence was measured with a PerkinElmer LS 55 fluorimeter. Excitation was alternated between 340 and 380 nm, and emission was

measured at 509 nm. Each measurement was calibrated using Triton X-100 and ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). For the experiments with *Stim1*$^{Sax/+}$ mice and the respective controls, intracellular calcium measurements were performed by flow cytometry as described [9].

Platelet aggregation and flow cytometry Washed platelets (200 μl with 0.5×10^6 platelets/μl) were analyzed in the presence of 70 μg/ml human fibrinogen. Transmission was recorded on a Fibrintimer 4 channel aggregometer (APACT Laborgeräte und Analysensysteme, Hamburg, Germany) over 10 min and was expressed in arbitrary units with buffer representing 100% transmission. Flow cytometry: Heparinized whole blood was diluted 1:20 and incubated with appropriate fluorophore-conjugated monoclonal antibodies for 15 min at room temperature and analyzed on a FACScalibur instrument (Becton Dickinson, Heidelberg, Germany).

Adhesion under flow conditions Rectangular coverslips (24×60 mm) were coated with 0.2 mg/ml fibrillar type I collagen (Nycomed, Munich, Germany) for 1 h at 37°C and blocked with 1% bovine serum albumin (BSA). Heparinized whole blood was perfused as described [26]. Image analysis was performed off-line using the Metavue software (Visitron, Munich, Germany). Thrombus formation was expressed as the mean percentage of total area covered by thrombi.

Bleeding time Mice were anesthetized and a 3-mm segment of the tail tip was removed with a scalpel. Tail bleeding was monitored by gently absorbing blood with filter paper at 20 s intervals without making contact with the wound site. When no blood was observed on the paper, bleeding was determined to have ceased. Experiments were stopped after 20 min.

Intravital microscopy of thrombus formation in $FeCl_3$-injured mesenteric arterioles Four-week-old mice were anesthetized, and the mesentery was exteriorized through a midline abdominal incision. Arterioles (35–60 μm diameter) were visualized with a Zeiss Axiovert 200 inverted microscope (×10) equipped with a 100-W HBO fluorescent lamp source, and a CoolSNAP-EZ camera (Visitron, Munich Germany). Digital images were recorded and analyzed off-line using the Metavue software. Injury was induced by topical application of a 3-mm^2 filter paper saturated with $FeCl_3$ (20%) for 10 s. Adhesion and aggregation of fluorescently labeled platelets (Dylight-488 conjugated anti-GPIX Ig derivative) in arterioles was monitored for 40 min or until complete occlusion occurred (blood flow stopped for >1 min).

Human platelet studies Blood from volunteers who had denied taking any medication for 1 week was taken into one tenth volume 3.2% trisodium citrate according to local ethical guidelines and washed platelets prepared according to the methods described in detail previously [18]. Platelets labeled with Fura-2/AM were resuspended at 1.5×10^8 cells/ml and monitored for Ca^{2+} changes using a Cairn spectrofluorimeter (Cairn Research, Faversham, Kent, UK) with excitation at 340 and 380 nm and emission at 510 nm. Reagents were added at the time points described in the legends to the figures. Anti-TRPC1 antibody was from Alomone Labs (Israel) and Ank (raised to the intracellular first ankyrin domain of hTRPC1) was as previously characterized [18]. Where indicated, values represent the means±SEM with numbers of separate experiments indicated by [*n*].

For immunoprecipitation studies, washed platelets were resuspended at 1×10^9 cells/ml in a Tyrode medium containing 10 mM Hepes, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 0.42 mM $NaH_2PO_4{\cdot}H_2O$, 12 mM $NaHCO_3$, 0.2 mM EGTA, 10 μM indomethacin, and 1 U/ml apyrase. Incubations were carried out in aggregation cuvettes at 37°C in the presence of 1 mM Ca^{2+}, and cells were stimulated with either thrombin (1.25 U/ml), collagen-related peptide (CRP, 10 μg/ml), or TG (10 μM). After 1 min stimulation, incubations were stopped with equal volume Triton lysis buffer (containing 2% Triton, 2% sodium deoxycholate, 10 mM EGTA, 150 mM NaCl, 40 mM Tris pH 7.8, 5 μM pepstatin, 1 mM PMSF, 10 μg/ml aprotinin, 0.2 mg/ml soybean trypsin inhibitor, 10 μM E-64, 50 μM leupeptin, 1 mM sodium orthovanadate, 5 mM benzamidine, and 2.5 mM ethylene diamine tetraacetic acid). The lysates were centrifuged at 13,000×*g* for 5 min to remove insoluble material, and after preclearing with protein G sepharose, the STIM1 was immunoprecipitated using polyclonal anti-STIM1 antibody (ProSci, Poway, CA, USA) as described previously [18]. Immunoprecipitated STIM1 was detected by Western blotting using the monoclonal antibody GOK/STIM1 (BD Biosciences, Oxford, UK) and for association to TRPC1 with 1F1.

Results

RT-PCR revealed the presence of wild-type and mutant *TRPC1* mRNA message in control and *TRPC1*$^{-/-}$ platelets, respectively (Fig. 1a). Omission of exon 8 in the mutant mRNA message leads to a frame shift of the original reading frame and results in a STOP codon at positions 2 and 9 of exon 9 coding for the region downstream of the

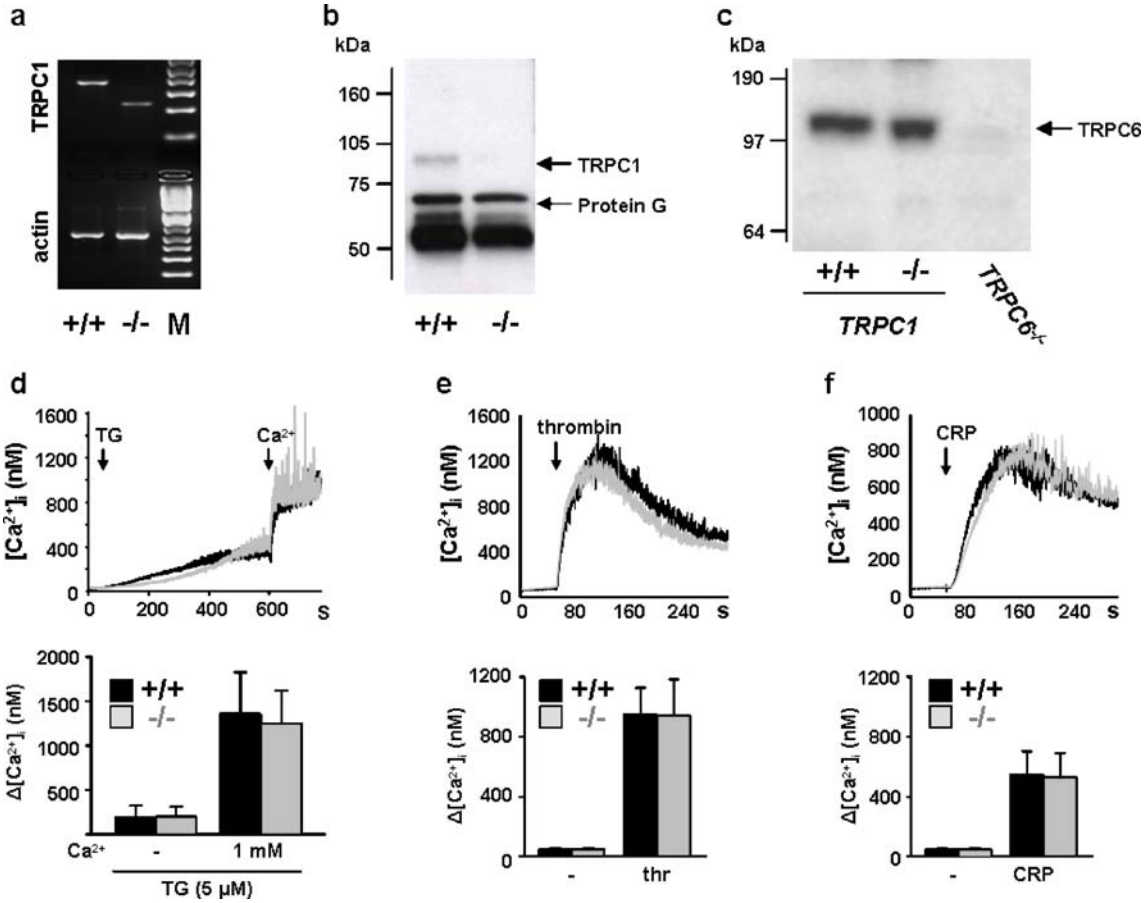

Fig. 1 Ca^{2+} signaling is not altered in *TRPC1*$^{-/-}$ platelets. **a** RT-PCR analysis of platelet *TRPC1* mRNA. In wild-type (+/+) platelets, a band was observed at the expected size of approximately 400 bp, whereas the expected shorter product was obtained from *TRPC1*$^{-/-}$ (−/−) platelets. TRPC1-specific forward (CATGGAGCATCGTATTTCAC) and reverse (GAGTCGAAGGTAACTCAGAA) primers were used. Actin served as control. **b** Immunoprecipitations of lysates from wild-type (+/+) or *TRPC1*$^{-/-}$ (−/−) platelets using XTRP1 were analyzed for TRPC1 using 1F1 antibody. TRPC1 migrates at approximately 84 kDa. Protein G migrating at 65 kDa (*arrowed*) and IgG (50 kDa) are also detected. **c** Western blot analysis of TRPC6 expression in platelets from wild-type, *TRPC1*$^{-/-}$, and *TRPC6*$^{-/-}$ mice. **d–f** Intracellular calcium measurements. Fura-2-loaded wild-type (*black line*) or *TRPC1*$^{-/-}$ (*gray line*) platelets were incubated with 5 μM TG in Tyrode's buffer without Ca^{2+} for 10 min followed by the addition of 1 mM extracellular Ca^{2+} (**d**) or stimulated with thrombin (0.1 U/ml; **e**) or CRP (10 μg/ml; **f**) in the presence of extracellular Ca^{2+} (1 mM), and $[Ca^{2+}]_i$ was monitored. The *upper panels* show representative measurements, and the *lower panels* show maximal changes in intracellular Ca^{2+} concentrations (mean±SD, n=4–6)

ion channel pore [23]. Consequently, the protein was detectable as an approximately 84 kDa band in wild-type but not mutant cells (Fig. 1b). Platelet count and the expression of different platelet surface glycoproteins (GP) were similar in wild-type and *TRPC1*$^{-/-}$ animals (data not shown). Western blot analyses revealed unaltered expression of TRPC6 in the mutant platelets, excluding a compensatory upregulation of this channel (Fig. 1c). Platelets from *TRPC6*$^{-/-}$ mice [27] served as a control. To estimate store content and SOCE in the cells, we performed intracellular Ca^{2+} measurements. Upon Ca^{2+} release from the ER, a conformational coupling between TRPC1 and the type II IP_3 receptor has been proposed to happen, which in turn results in the opening of TRPC1—as SOC channel—and Ca^{2+} entry [14, 15]. To test this directly, we treated platelets with 5 μM TG (a selective inhibitor of the SERCA) in Ca^{2+}-free buffer to empty the intracellular Ca^{2+} stores, and then added 1 mM Ca^{2+} to measure the extent of SOCE. Unexpectedly, neither the store content nor the amplitude of SOCE was different between wild-type and *TRPC1*$^{-/-}$ platelets (Fig. 1d), demonstrating that TRPC1 is not required for TG-induced SOCE in platelets.

Agonist-induced platelet activation triggers the activation of phospholipase (PL) Cβ and/or PLCγ2, which hydrolyze phosphoinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-trisphosphate (IP_3) and diacyl-glycerol (DAG). DAG mediates store-independent Ca^{2+} entry [28], whereas IP_3 releases Ca^{2+} from the ER and in turn leads to SOCE [1]. To test Ca^{2+} responses to physiological agonists, we measured changes in $[Ca^{2+}]_i$ upon platelet activation with thrombin—known to activate PLCβ through G protein-coupled receptors—and with CRP, which activates PLCγ2

through GPVI. Unexpectedly, both agonists evoked comparable Ca^{2+} responses in wild-type and *TRPC1*$^{-/-}$ platelets (Fig. 1e,f).

We further investigated whether the lack of TRPC1 influences agonist-induced platelet activation in vitro. Flow cytometric analysis of integrin αIIbβ3 activation (JON/A-PE) and degranulation, determined as the surface expression of P-selectin, yielded indistinguishable results for *TRPC1*$^{-/-}$ and control platelets in response to all tested agonists and agonist concentrations (Fig. 2a). Similarly, no differences in reactivity were noted between *TRPC1*$^{-/-}$ and control platelets in standard aggregometry (Fig. 2b) and in a flow adhesion assay where whole blood was perfused over a collagen-coated surface at high shear rate (1,000 s^{-1}). Wild-type and *TRPC1*$^{-/-}$ platelets formed stable thrombi to the same extent and with the same kinetics (Fig. 2c).

Next, we analyzed the relevance of TRPC1 deficiency for platelet function in vivo in hemostasis and thrombosis [9]. Bleeding times after amputating the tail tip of wild-type and *TRPC1*$^{-/-}$ mice were comparable (3.1±1.1 min vs. 2.7±1.9 min) (Fig. 3a). Similarly, the application of 20% $FeCl_3$ on the exteriorized mesenteric arteries of *TRPC1*$^{-/-}$ mice resulted in fast platelet adhesion and thrombus growth leading to irreversible vessel occlusion after 13.1±3.0 min, which was comparable to the kinetics of thrombus formation and vessel occlusion observed in the control animals (15.1±3.0 min; Fig. 3b,c).

Very recently, we have shown that the heterozygous expression of an activating EF hand mutant STIM1 (*Stim1*$^{Sax/+}$) constitutively activates SOC channels in murine platelets resulting in elevated basal $[Ca^{2+}]_i$, macrothrombocytopenia, and bleeding [9]. If TRPC1 was the SOC channel on the platelet surface that is regulated by STIM1, lack of the protein should rescue or at least ameliorate the phenotype of the Stim1 mutant platelets. To investigate this, we crossed the two mouse lines and studied their platelets. As shown in Fig. 4, the increase in basal $[Ca^{2+}]_i$ and the resulting macrothrombocytopenia was not altered in *TRPC1*$^{-/-}$/*Stim1*$^{Sax/+}$ compared to *Stim1*$^{Sax/+}$ mice, clearly excluding TRPC1 as the major STIM1-regulated SOC channel on the platelet surface.

The above data in mouse platelets contrast with reports suggesting TRPC1 as the major SOC channel in human platelets on the basis of antibody-induced inhibition of Ca^{2+}

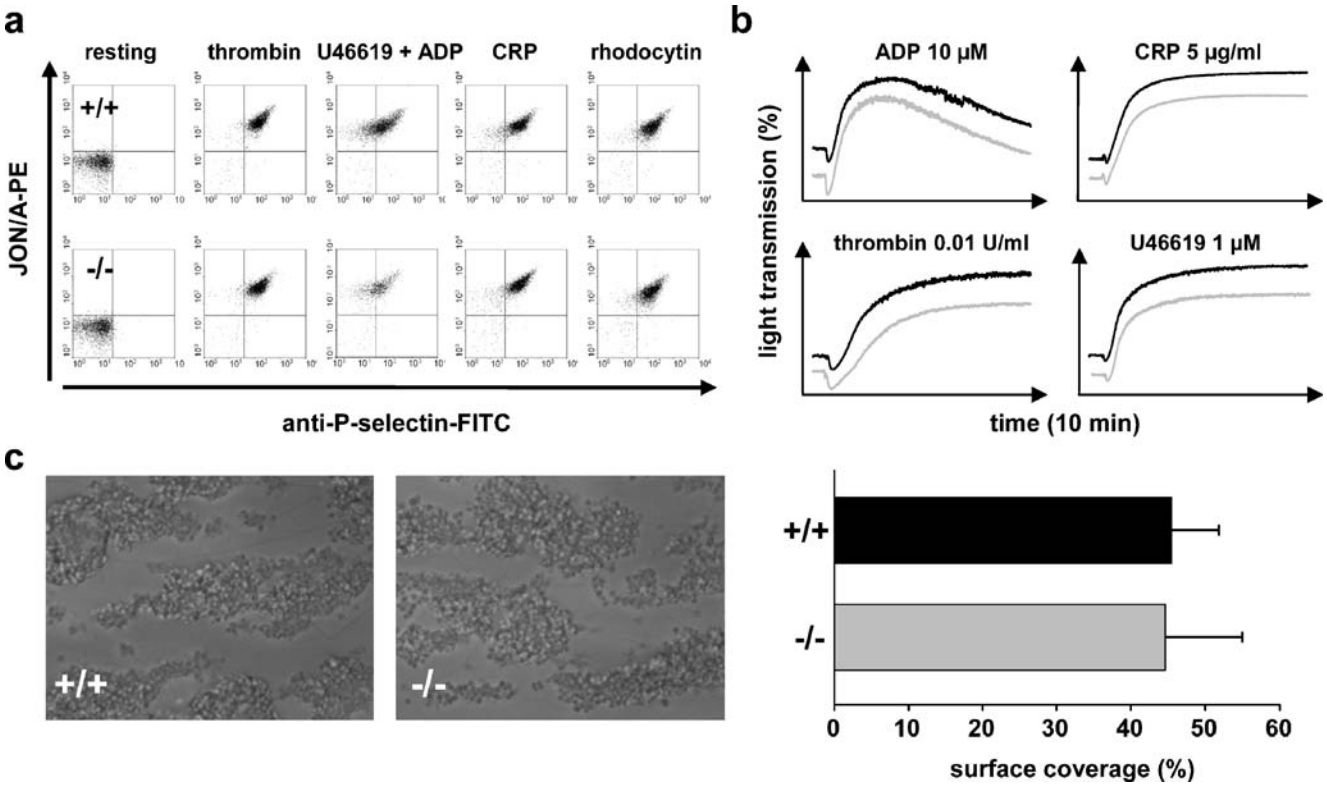

Fig. 2 Normal activation and aggregation of *TRPC1*$^{-/-}$ platelets. **a** Flow cytometric analysis of αIIbβ3 integrin activation and degranulation-dependent P-selectin exposure in response to thrombin (0.1 U/ml), the stable thromboxane A2 analog U46619 (3 µM) and ADP (10 µM), CRP (10 µg/ml) and rhodocytin (1 µg/ml). Representative dot plots of six measurements per group. **b** Normal aggregation of *TRPC1*$^{-/-}$ platelets (*gray lines*) in response to all tested agonists and agonist concentrations. Representative curves of at least four independent measurements. **c** *TRPC1*$^{-/-}$ platelets in whole blood are able to form stable thrombi when perfused over a collagen-coated (0.2 mg/ml) surface at a shear rate of 1,000 s^{-1}. Representative phase contrast images (*left*) and mean surface coverage±SD (n=4, *right*) after 4 min runtime

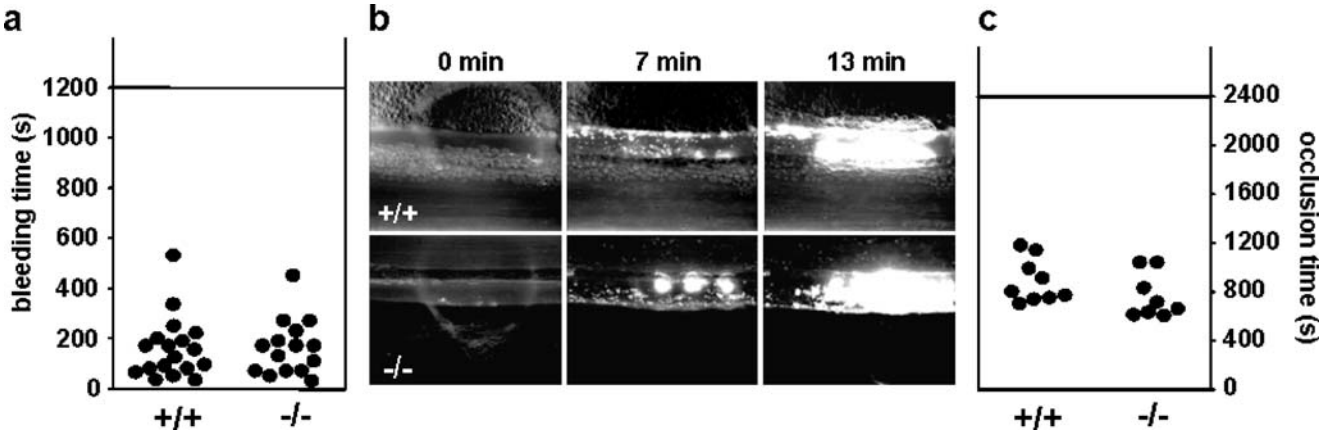

Fig. 3 Unaltered hemostasis and thrombosis in *TRPC1*$^{-/-}$ mice. **a** Tail bleeding times in wild-type and *TRPC1*$^{-/-}$ mice. Each symbol represents one individual. **b** and **c** In vivo microscopy of thrombosis in injured arterioles. Mesenteric arterioles were treated with a 3-mm^2 paper tip saturated by 20% $FeCl_3$ for 10 s, and adhesion and thrombus formation of fluorescently labeled platelets were monitored by in vivo video microscopy. Representative images (**b**) and time to vessel occlusion (**c**) are shown

entry using a commercial antibody raised to a predicted extracellular epitope of TRPC1 [13]. As there are also doubts regarding the specificity of this antibody [20], we decided to reexamine this effect. Human platelets were loaded with Fura-2/AM and $[Ca^{2+}]_i$ measurements performed using 340/380 nm ratio fluorescence as previously described [18]. Figure 5a shows the effects of the incubation of platelets for 10 min with the commercial polyclonal anti-TRPC1 antibody (10 μg/ml final concentration, supplied in a phosphate buffered saline (PBS) vehicle containing 1% BSA and 0.05% sodium azide as preservative) previously reported to inhibit SOCE in human platelets [13]. As controls, water or 10 μg/ml of our previously characterized polyclonal Ank antibody, again in PBS containing 0.05% sodium azide, were used. The Ank antibody has been raised against an intracellular epitope of TRPC1 and does not recognize the protein in intact platelets [18]. Both antibodies showed no effect on Ca^{2+} release from stores, induced by either 0.25 U/ml thrombin or 200 nM TG. It is interesting to note that a similar reduction in the extent of Ca^{2+} entry was seen with both antibodies after the addition of 1 mM Ca^{2+}. With 0.25 U/ml thrombin as the stimulatory agent, the Alomone antibody inhibited the peak height of Ca^{2+} entry by 30.2±3.8% [mean±SEM, n=4], the Ank antibody inhibited the peak height by 31±4.5% [n=3]. Using 200 nM TG as the agonist, the Alomone antibody inhibited the peak height by 17±6.5% [n=3], the Ank antibody inhibited the peak height by 15±5.7% [n=3], suggesting that this effect is not related to antibody recognition of a surface epitope of TRPC1. Furthermore, the inclusion of sodium azide (present in the vehicle of antibodies) for 10 min at 0.0007% final concentration caused no effect on Ca^{2+} release by either agonist but inhibited Ca^{2+} entry to a similar extent as the antibodies (with thrombin, 0.0007% sodium azide inhibited the peak height by 37.2±5% [n=3] and with TG, sodium azide inhibited the peak height by 15±3.7% [n=3]; fluorescence curves not shown for clarity). This suggests that the inhibition observed by the antibodies was due to the presence of sodium azide in the antibody

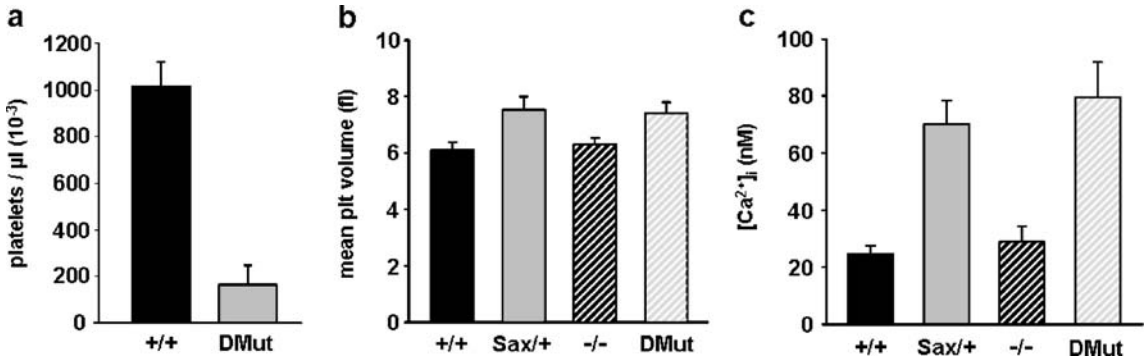

Fig. 4 TRPC1 deficiency does not rescue the *Stim1*$^{Sax/+}$ phenotype in platelets. **a** Platelet count was measured by flow cytometry in *TRPC1*$^{-/-Sax/+}$ (*DMut*) and control mice and are expressed as mean±SD (n=6). **b** Platelet size was determined in wild-type, *Stim1*$^{Sax/+}$, *TRPC1*$^{-/-}$, and *TRPC1*$^{-/-Sax/+}$ (*DMut*) mice by an automated hematology analyzer (Sysmex Deutschland, Norderstedt, Germany). Mean platelet size±SD (n=4). **c** Flow cytometric analysis of basal $[Ca^{2+}]_i$ in wild-type, *Stim1*$^{Sax/+}$, *TRPC1*$^{-/-}$, and *TRPC1*$^{-/-Sax/+}$ (*DMut*) mice. Mean basal $[Ca^{2+}]_i$±SD (n=4–6)

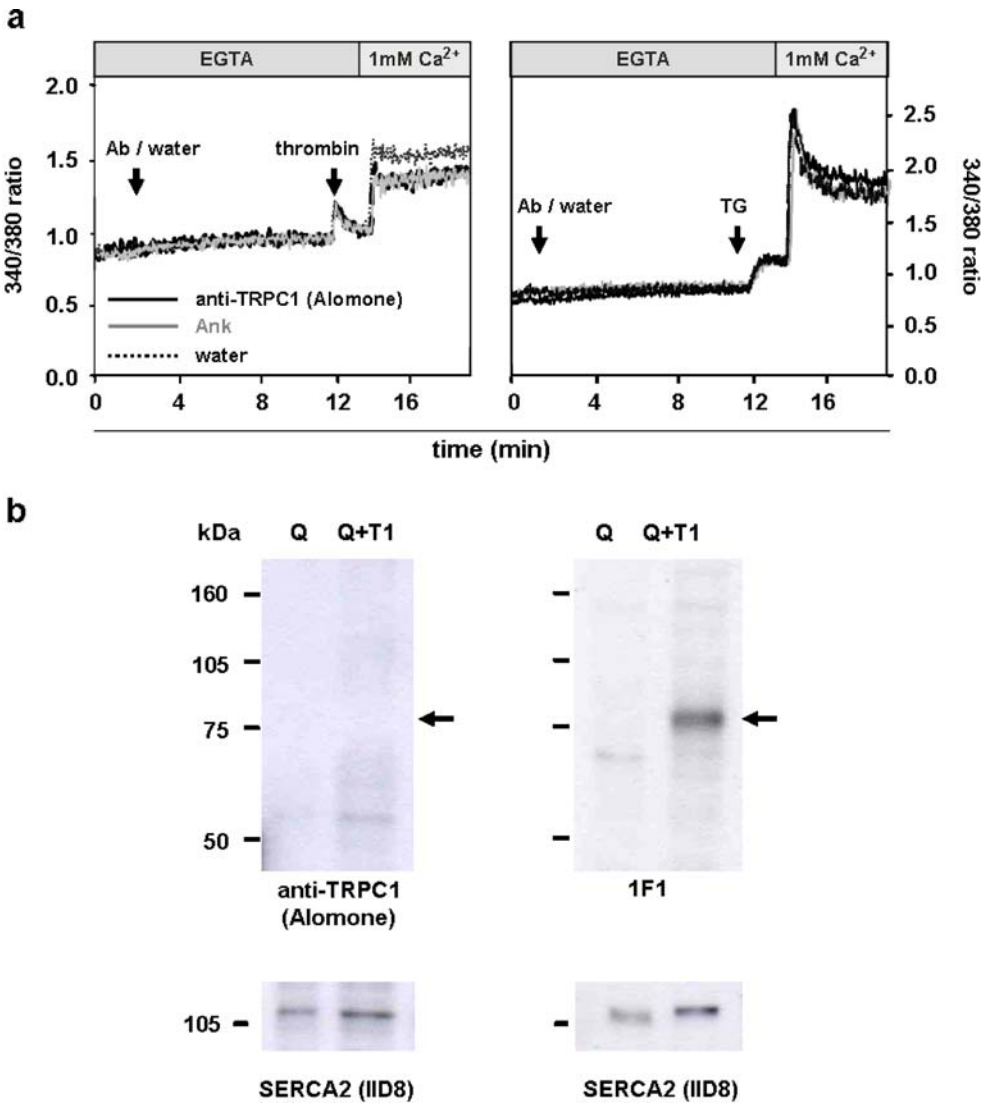

Fig. 5 No specific effect of anti-TRPC1 (Alomone Labs) on SOCE in human platelets. **a** Anti-TRPC1 antibody or Ank does not affect Ca^{2+} elevation in human platelets induced by thrombin or *TG*. Fura-2-loaded human platelets were incubated in the presence of EGTA (0.1 mM) and anti-TRPC1 (Alomone Labs, *continuous black lines*) or Ank (*gray lines*) (both at a final concentration of 10 μg/ml), or water (*dashed black lines*) added for 10 min followed by 0.25 U/ml thrombin (*left panel*) or 200 nM TG (*right panel*). One millimolar Ca^{2+} was added at times indicated and 340/380 nm ratio fluorescence was recorded. Traces are representative of three separate experiments. **b** Alomone anti-TRPC1 antibody fails to recognize hTRPC1 overexpressed in QBI-293 cells under conditions where 1F1 yields a strong specific signal. *Q* QBI-293 cells, *Q+T1* QBI-293 cells overexpressing hTRPC1. Each lane contains 50 μg protein. SERCA2 served as control. *Arrows* indicate the position of overexpressed hTRPC1. *Numbers on the left* indicate the position of molecular size markers. Blot is representative of at least six experiments carried out between 2005 and 2007

preparations. Next, we performed Western blot analyses to test the specificity of the TRPC1 antibody, as other investigators have raised doubts about it [20]. In agreement with these reports, the Alomone anti-TRPC1 antibody failed to detect hTRPC1 overexpressed in QBI-293 cells, whereas a strong signal was obtained with the 1F1 antibody (Fig. 5b). This negative result was consistently obtained with at least three separate purchases of the Alomone antibody over a 3-year period.

Our studies using the $TRPC1^{-/-}/Stim1^{Sax/+}$ mutation in mouse platelets (Fig. 4) imply that STIM1 does not interact with TRPC1 in a functional capacity in mouse platelets. A recent report, again using the Alomone TRPC1 antibody, suggested that STIM1 interacts with TRPC1 in resting platelets and increases modestly upon activation of platelets with TG plus ionomycin [29]. We, therefore, investigated a possible interaction between STIM1 and TRPC1 in human platelets using our currently described antibodies. Washed platelets at 1×10^9 cells/ml were incubated with 1 mM Ca^{2+} in aggregometer cuvettes in the presence of thrombin (1.25 U/ml), CRP (10 μg/ml), or TG (10 μM) for 1 min followed by immunoprecipitation of STIM1 using a polyclonal antibody to STIM1 (ProSci, USA). The extractions were probed on Western blots for the presence of STIM1 (using the monoclonal GOK/STIM1 antibody) and for TRPC1 (using 1F1). Figure 6 shows a typical experiment repeated three times on separate occasions where STIM1 extraction is apparent in all incubations but there is no association to TRPC1 in either resting or stimulated platelets.

Discussion

STIM1 has been identified as the ER-resident Ca^{2+} sensor that activates SOC channels in T cells [2–4, 30], mast cells [5], and platelets [9]. However, while the identity of the SOC channel in human T cells [6, 7, 31] and mast cells [32]

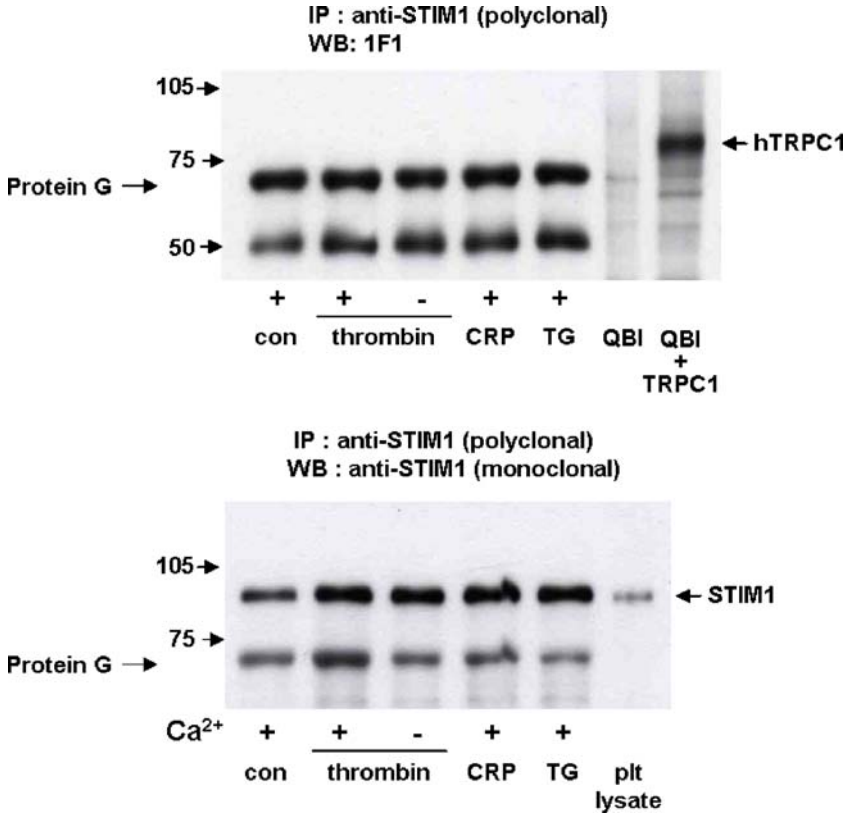

Fig. 6 STIM1 does not associate with TRPC1 in human platelets under resting or stimulated conditions. Platelets were incubated with indicated agonists for 1 min in the presence of 1 mM Ca^{2+} (an additional incubation of thrombin in the absence of Ca^{2+} is also included). After cell lysis, STIM1 was immunoprecipitated with polyclonal anti-STIM1 antibody and analyzed by Western blotting using a monoclonal anti-GOK/STIM1 antibody for STIM1 and 1F1 for TRPC1. Also detected are protein G (approximately 65 kDa) and IgG at 50 kDa. Platelet lysate is used as a positive control for STIM1 and overexpressed hTRPC1 for 1F1, respectively (*Con* control platelets). The blots shown are representative of three separate determinations each

has been established as Orai1 (CRACM-1), the proposed function of TRPC1 as a major platelet SOC channel has been considerably debated [21, 33, 34]. TRPC1, which was the first mammalian TRPC channel to be identified, has probably been the most intensively studied and has had many functions ascribed to it including a role in SOCE or being a mechanosensitive channel (for review, see Beech [35]). However, with the exception of a role in salivary glands where TRPC1 is well-expressed [24], its physiological role in other tissues has remained elusive. In the current study, we demonstrate unaltered Ca^{2+} homeostasis and cellular activation in *TRPC1*$^{-/-}$ platelets, clearly excluding a major role of this channel in SOCE in mouse platelets. Furthermore, we provide experimental evidence that some of the published data that had previously lead to the proposal of TRPC1 as a major SOC channel in human platelets may have been misleading due to the lack of specificity of reagents used.

Changes in $[Ca^{2+}]_i$ are essential during cellular activation and are, therefore, tightly regulated. As platelets have to respond to vascular injury very rapidly, their signal transduction machinery is optimized for maximal activation within seconds including the rapid occurrence of maximal cytosolic Ca^{2+} levels upon agonist-induced stimulation [36–38]. This is perhaps best exemplified by the steep slope of Ca^{2+} entry after TG treatment of mouse or human platelets (Fig. 1d and Fig. 5a, respectively) which suggests it to be mediated by the activation of a highly abundant SOC channel. In agreement with this notion, STIM1, the principal regulator of SOCE, is highly expressed in platelets, even at higher relative density than in T cells [9]. In contrast, TRPC1 is expressed in both mouse (Fig. 1b) and human (not shown) platelets at extremely low levels, making it necessary to enrich the protein by immunoprecipitation for detection. Furthermore, it has previously been reported that most of the TRPC1 detected is not present in the plasma membrane of (human) platelets but is rather localized in intracellular stores, indicating it to perform functions other than SOCE [18]. In support of the latter, other investigators have also reported a widespread intracellular location for hTRPC1 overexpressed in HEK-293 cells [39]. In addition, others have suggested that TRPC1 alone is unable to increase whole cell currents in resting and carbachol-stimulated cells, suggesting that it may not fulfil a channel function but that it can form heteromers with TRPC4 and TRPC5 that are activated by Gq-coupled receptors rather than as SOC channels [40]. Furthermore, analysis of SOCs in freshly isolated smooth muscle cells from cerebral arteries or from thoracic aortas from wild-type and *TRPC1*$^{-/-}$ mice revealed no difference, again suggesting an absence of a major role in SOCE [23]. In agreement with this, we found that TRPC1 deficiency does not have any effect on the kinetics and extent of TG-induced store depletion or subsequent SOCE or agonist-

induced Ca^{2+} mobilization and entry in mouse platelets (Fig. 1d–f). Its absence in platelets has no measurable effect on platelet aggregation or secretion induced by any of the agonists examined (Fig. 2) nor on thrombus formation on a collagen-coated surface (Fig. 2c) or in a mesenteric arteriolar injury model utilizing ferric chloride where vessel occlusion is largely driven by thrombin stimulation of platelets and fibrin clot formation (Fig. 3). This data further suggests that the SOC channel in platelets comprises subunits other than TRPC1.

Platelets have been reported to express other TRPC isoforms and members of the TRPM subfamily [12]. Thus, a possibility exists that the function of TRPC1 may be compensated for by another TRP channel member. However, we consider this unlikely for the following reasons. Previous analysis of the *TRPC1*$^{-/-}$ mouse vasculature suggested that there is no upregulation or downregulation of other TRPC channels [23], unlike the situation in *TRPC6*$^{-/-}$ mice where TRPC3 levels are elevated [27]. We have found that levels of TRPC6 in wild-type versus *TRPC1*$^{-/-}$ platelets are unchanged (Fig. 1c). Other members of the TRPC family (TRPC2, TRPC3, TRPC4, TRPC5, and TRPC7) have previously not been detected in mouse megakaryocytes from which platelets are produced [12] and are, therefore, unlikely to compensate for the absence of TRPC1 in this model system. Furthermore, with the exception of TRPC4 shown in endothelial cells [41], there is little evidence that any other TRPC channel is able to fulfil a SOCE role. In human platelets, TRPC4 expression has been detected and has been suggested to allow SOCE to be sensitive to alkalosis [17]. Although it is able to form heteromers with TRPC1 [39], its exact role in SOCE remains to be determined. Thus, there is little indication that another TRP channel compensates for the loss of the putative SOC activity of TRPC1 in the knockout platelets. We are convinced that, if a function for TRPC1 in platelet Ca^{2+} homeostasis was important, it would have been evident in at least one of the assays described in this study.

Although our results with mouse platelets do not fully exclude the possibility that species-specific differences might exist in the function of TRPC1 in platelets, we consider this to be unlikely for the following reasons. The proposal of TRPC1 as the major SOC channel in human platelets was largely based on the observation that a commercially available anti-TRPC1 antibody partially inhibited Ca^{2+} entry stimulated by low-dose thrombin and TG in those cells [14, 15]. However, in this study, we show that they have no specific effect on SOCE in human platelets and that the inhibition seen is not related to an antibody-recognized surface epitope but due to the presence of sodium azide in the preparations. In agreement with the studies of Ong et al. [20], we found that the Alomone TRPC1 antibody does not bind to hTRPC1 in a specific manner. Currently, we cannot rule out batch differences in the antibody preparations obtained from the manufacturer, but different purchases (different lot numbers) consistently yielded negative results over the last 3 years.

Our studies using the *TRPC1*$^{-/-Sax/+}$ mutation (Fig. 4) also imply that STIM1 does not interact with TRPC1 in a functional capacity in mouse platelets. The Sax mutation represents a constitutively active protein that is unable to sense the Ca^{2+} in intracellular stores and leads to the activation of the SOC channel resulting in increased basal cytosolic Ca^{2+} levels [9]. The observations in the *Stim1*$^{Sax/+}$ mouse model supports earlier studies in heterologous expression systems suggesting mutations in the EF hand domain to lead to constitutive activation of SOC channels [3]. If TRPC1 was the important entry channel or an important part of the channel complex linked to STIM1, then crossing the two mouse lines would have resulted in rescue of the *Stim1*$^{Sax/+}$ phenotype. That the *TRPC1*$^{-/-}$/*Stim1*$^{Sax/+}$ showed no difference compared to *Stim1*$^{Sax/+}$ mice confirms without doubt that TRPC1 plays no major role in SOCE in platelets. Using the antibodies described in our study, we failed to observe STIM1–TRPC1 interaction in human platelets at rest or upon stimulation with thrombin, CRP, or TG (Fig. 6) further supporting our other data showing a lack of effect of TRPC1 deficiency on platelet responses and suggesting it to be the same in human platelets. A recent study, again using the Alomone TRPC1 antibody, came to the conclusion that STIM1 interacts with TRPC1 in resting platelets and that a modest increase occurs upon stimulation with TG [29]. It was further reported that "electrotransjection" (electroporation at 4 kV/cm electric field) of a STIM1 antibody (GOK/STIM1) reduced SOCE, interaction of STIM1–TRPC1, and interaction of IP_3R type II with TRPC1.

While it is very difficult to know all the reasons for the differences, use of different TRPC1 reagents may be partly responsible, and for reasons already described, we are not confident of their detection of TRPC1. The reported electroporation experiments [29] are also surprising. This technique was widely used in the 1980s by ourselves (K.S. A.) and other investigators to permeabilize the plasma membrane to allow free diffusion of small molecular size molecules (less than 1,000 Da) such as Ca^{2+}, ATP, inositol, etc., but this technique did not allow diffusion of proteins [42–44]. It is, therefore, surprising that antibodies, which represent near globular structures of 150,000 Da, are reported to pass through the plasma membrane of electroporated platelets. If such a possibility exists, this would be accompanied by loss of cytosolic proteins and, with it, platelet integrity. Furthermore, the GOK/STIM1 antibody is known to recognize the N-terminal portion of STIM1 (amino acids 25–139) that houses the EF hand domain which is predicted to point into the lumen of the ER where

Ca^{2+} binding occurs or to the outside if the STIM1 is plasma membrane-associated. If the electroporated GOK/STIM1 antibody is to have an effect, the antibody needs to penetrate into the lumen of the ER. With electroporation techniques, this is considered unlikely (see also Hughes and Crawford [43] and Authi et al. [44]).

Taken together, our results provide compelling evidence that TRPC1 is not a major SOC channel in platelets and further studies will be required to identify this channel in platelets. This will be of considerable importance as SOC channels in general and the platelet SOC channel in particular have been recognized as very attractive potential targets for pharmacological intervention.

Acknowledgement We would like to thank Sylvia Hengst for the excellent technical support. This work was supported by grant Ni556/7-1 and the SFB 688 from the Deutsche Forschungsgemeinschaft (DFG) (to B.N.). AA is supported by the Medical Research Council (UK), and KSA is supported by the British Heart Foundation.

References

1. Berridge MJ, Bootman MD, Roderick HL (2003) Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol 4:517–529
2. Liou J, Kim ML, Heo WD, Jones JT, Myers JW, Ferrell JE Jr, Meyer T (2005) STIM is a Ca^{2+} sensor essential for Ca^{2+}-store-depletion-triggered Ca^{2+}influx. Curr Biol 15:1235–1241
3. Zhang SL, Yu Y, Roos J, Kozak JA, Deerinck TJ, Ellisman MH, Stauderman KA, Cahalan MD (2005) STIM1 is a Ca^{2+} sensor that activates CRAC channels and migrates from the Ca^{2+} store to the plasma membrane. Nature 437:902–905
4. Roos J, DiGregorio PJ, Yeromin AV, Ohlsen K, Lioudyno M, Zhang S, Safrina O, Kozak JA, Wagner SL, Cahalan MD, Velicelebi G, Stauderman KA (2005) STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. J Cell Biol 169:435–445
5. Baba Y, Nishida K, Fujii Y, Hirano T, Hikida M, Kurosaki T (2008) Essential function for the calcium sensor STIM1 in mast cell activation and anaphylactic responses. Nat Immunol 9:81–88
6. Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, Hogan PG, Lewis RS, Daly M, Rao A (2006) A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. Nature 441:179–185
7. Vig M, Peinelt C, Beck A, Koomoa DL, Rabah D, Koblan-Huberson M, Kraft S, Turner H, Fleig A, Penner R, Kinet JP (2006) CRACM1 is a plasma membrane protein essential for store-operated Ca^{2+} entry. Science 312:1220–1223
8. Huang GN, Zeng W, Kim JY, Yuan JP, Han L, Muallem S, Worley PF (2006) STIM1 carboxyl-terminus activates native SOC, I(crac) and TRPC1 channels. Nat Cell Biol 8:1003–1010
9. Grosse J, Braun A, Varga-Szabo D, Beyersdorf N, Schneider B, Zeitlmann L, Hanke P, Schropp P, Muhlstedt S, Zorn C, Huber M, Schmittwolf C, Jagla W, Yu P, Kerkau T, Schulze H, Nehls M, Nieswandt B (2007) An EF hand mutation in Stim1 causes premature platelet activation and bleeding in mice. J Clin Invest 117:3540–3550
10. Berg LP, Shamsher MK, El-Daher SS, Kakkar VV, Authi KS (1997) Expression of human TRPC genes in the megakaryocytic cell lines MEG01, DAMI and HEL. FEBS Lett 403:83–86
11. den DE, Molin DG, Breikers G, van OR, Akkerman JW, van Eys GJ, Heemskerk JW (2001) Expression of transient receptor potential mRNA isoforms and Ca(2+) influx in differentiating human stem cells and platelets. Biochim Biophys Acta 1539:243–255
12. Carter RN, Tolhurst G, Walmsley G, Vizuete-Forster M, Miller N, Mahaut-Smith MP (2006) Molecular and electrophysiological characterization of transient receptor potential ion channels in the primary murine megakaryocyte. J Physiol 576:151–162
13. Rosado JA, Brownlow SL, Sage SO (2002) Endogenously expressed Trp1 is involved in store-mediated Ca^{2+} entry by conformational coupling in human platelets. J Biol Chem 277:42157–42163
14. Rosado JA, Sage SO (2001) Activation of store-mediated calcium entry by secretion-like coupling between the inositol 1,4,5-trisphosphate receptor type II and human transient receptor potential (hTrp1) channels in human platelets. Biochem J 356:191–198
15. Rosado JA, Sage SO (2000) Coupling between inositol 1,4,5-trisphosphate receptors and human transient receptor potential channel 1 when intracellular Ca^{2+} stores are depleted. Biochem J 350(Pt 3):631–635
16. Brownlow SL, Sage SO (2005) Transient receptor potential protein subunit assembly and membrane distribution in human platelets. Thromb Haemost 94:839–845
17. Wakabayashi I, Marumo M, Graziani A, Poteser M, Groschner K (2006) TRPC4 expression determines sensitivity of the platelet-type capacitative Ca^{2+} entry channel to intracellular alkalosis. Platelets 17:454–461
18. Hassock SR, Zhu MX, Trost C, Flockerzi V, Authi KS (2002) Expression and role of TRPC proteins in human platelets: evidence that TRPC6 forms the store-independent calcium entry channel. Blood 100:2801–2811
19. Flockerzi V, Jung C, Aberle T, Meissner M, Freichel M, Philipp SE, Nastainczyk W, Maurer P, Zimmermann R (2005) Specific detection and semi-quantitative analysis of TRPC4 protein expression by antibodies. Pflugers Arch 451:81–86
20. Ong HL, Chen J, Chataway T, Brereton H, Zhang L, Downs T, Tsiokas L, Barritt G (2002) Specific detection of the endogenous transient receptor potential (TRP)-1 protein in liver and airway smooth muscle cells using immunoprecipitation and Western-blot analysis. Biochem J 364:641–648
21. Authi KS (2007) TRP channels in platelet function. Handb Exp Pharmacol(179):425–443
22. Harper AG, Sage SO (2007) A key role for reverse Na+/Ca^{2+} exchange influenced by the actin cytoskeleton in store-operated Ca^{2+} entry in human platelets: evidence against the de novo conformational coupling hypothesis. Cell Calcium 42:606–617
23. Dietrich A, Kalwa H, Storch U, Mederos YS, Salanova B, Pinkenburg O, Dubrovska G, Essin K, Gollasch M, Birnbaumer L, Gudermann T (2007) Pressure-induced and store-operated cation influx in vascular smooth muscle cells is independent of TRPC1. Pflugers Arch 455:465–477
24. Liu X, Cheng KT, Bandyopadhyay BC, Pani B, Dietrich A, Paria BC, Swaim WD, Beech D, Yildrim E, Singh BB, Birnbaumer L, Ambudkar IS (2007) Attenuation of store-operated Ca^{2+} current impairs salivary gland fluid secretion in TRPC1(−/−) mice. Proc Natl Acad Sci U S A 104:17542–17547
25. Heemskerk JW, Feijge MA, Rietman E, Hornstra G (1991) Rat platelets are deficient in internal Ca^{2+} release and require influx of extracellular Ca^{2+} for activation. FEBS Lett 284:223–226
26. Nieswandt B, Brakebusch C, Bergmeier W, Schulte V, Bouvard D, Mokhtari-Nejad R, Lindhout T, Heemskerk JW, Zirngibl H, Fassler R (2001) Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. EMBO J 20:2120–2130

27. Dietrich A, Mederos YS, Gollasch M, Gross V, Storch U, Dubrovska G, Obst M, Yildirim E, Salanova B, Kalwa H, Essin K, Pinkenburg O, Luft FC, Gudermann T, Birnbaumer L (2005) Increased vascular smooth muscle contractility in TRPC6$^{-/-}$ mice. Mol Cell Biol 25:6980–6989
28. Bird GS, Aziz O, Lievremont JP, Wedel BJ, Trebak M, Vazquez G, Putney JW Jr (2004) Mechanisms of phospholipase C-regulated calcium entry. Curr Mol Med 4:291–301
29. Lopez JJ, Salido GM, Pariente JA, Rosado JA (2006) Interaction of STIM1 with endogenously expressed human canonical TRP1 upon depletion of intracellular Ca^{2+} stores. J Biol Chem 281:28254–28264
30. Oh-Hora M, Yamashita M, Hogan PG, Sharma S, Lamperti E, Chung W, Prakriya M, Feske S, Rao A (2008) Dual functions for the endoplasmic reticulum calcium sensors STIM1 and STIM2 in T cell activation and tolerance. Nat Immunol 9:432–443
31. Peinelt C, Vig M, Koomoa DL, Beck A, Nadler MJ, Koblan-Huberson M, Lis A, Fleig A, Penner R, Kinet JP (2006) Amplification of CRAC current by STIM1 and CRACM1 (Orai1). Nat Cell Biol 8:771–773
32. Vig M, DeHaven WI, Bird GS, Billingsley JM, Wang H, Rao PE, Hutchings AB, Jouvin MH, Putney JW, Kinet JP (2008) Defective mast cell effector functions in mice lacking the CRACM1 pore subunit of store-operated calcium release-activated calcium channels. Nat Immunol 9:89–96
33. Sage SO, Brownlow SL, Rosado JA (2002) TRP channels and calcium entry in human platelets. Blood 100:4245–4246
34. Authi KS, Hassock S, Zhu MX, Flockerzi V, Trost C (2002) TRPC channels in calcium entry in human platelets. Blood 100:4246–4247
35. Beech DJ (2005) TRPC1: store-operated channel and more. Pflugers Arch 451:53–60
36. Ruggeri ZM (2002) Platelets in atherothrombosis. Nat Med 8:1227–1234
37. Offermanns S (2006) Activation of platelet function through G protein-coupled receptors. Circ Res 99:1293–1304
38. Nieswandt B, Watson SP (2003) Platelet-collagen interaction: is GPVI the central receptor. Blood 102:449–461
39. Hofmann T, Schaefer M, Schultz G, Gudermann T (2002) Subunit composition of mammalian transient receptor potential channels in living cells. Proc Natl Acad Sci U S A 99:7461–7466
40. Strubing C, Krapivinsky G, Krapivinsky L, Clapham DE (2001) TRPC1 and TRPC5 form a novel cation channel in mammalian brain. Neuron 29:645–655
41. Freichel M, Suh SH, Pfeifer A, Schweig U, Trost C, Weissgerber P, Biel M, Philipp S, Freise D, Droogmans G, Hofmann F, Flockerzi V, Nilius B (2001) Lack of an endothelial store-operated Ca^{2+} current impairs agonist-dependent vasorelaxation in TRP4$^{-/-}$ mice. Nat Cell Biol 3:121–127
42. Knight DE, Scrutton MC (1984) Cyclic nucleotides control a system which regulates Ca^{2+} sensitivity of platelet secretion. Nature 309:66–68
43. Hughes K, Crawford N (1989) Reversible electropermeabilisation of human and rat blood platelets: evaluation of morphological and functional integrity ‘in vitro’ and ‘in vivo’. Biochim Biophys Acta 981:277–287
44. Authi KS, Hughes K, Crawford N (1989) High incorporation of [3H]inositol into phosphoinositides of human platelets during reversible electropermeabilisation. FEBS Lett 254: 52–58