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# STIM1 regulates acidic $Ca^{2+}$ store refilling by interaction with SERCA3 in human platelets

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ABSTRACT

$Ca^{2+}$ mobilization regulates a wide variety of cellular functions. Platelets posses agonist-releasable $Ca^{2+}$ stores in acidic organelles where sarcoendoplasmic reticulum $Ca^{2+}$-ATPase-3 (SERCA) pump is involved in store refilling. Stromal interaction molecule 1 (STIM1), which has been presented as a central regulator of platelet function, is a $Ca^{2+}$ sensor of the intracellular $Ca^{2+}$ stores. Here we present that STIM1 is required for acidic store refilling. Electrotransjection of cells with anti-STIM1 ($Y^{231}$–$K^{243}$) antibody, directed towards a cytoplasmic sequence of STIM1, significantly reduced acidic store refilling, which was tested by remobilizing $Ca^{2+}$ from the acidic stores using 2,5-di-(t-butyl)-1,4-hydroquinone (TBHQ) after a brief refilling period that followed thrombin stimulation. Platelet treatment with thrombin or thapsigargin in combination with ionomycin, to induce extensive $Ca^{2+}$ store depletion, resulted in a transient increase in the interaction between STIM1 and SERCA3, reaching a maximum 30 s after stimulation. The coupling between STIM1 and SERCA3 was abolished by electrotransjection with anti-STIM1 antibody. The interaction between STIM1 and SERCA3 induced by thrombin or by treatment with thapsigargin plus ionomycin is reduced in platelets from type 2 diabetic patients, as well as $Ca^{2+}$ reuptake into the acidic $Ca^{2+}$ stores. These findings provide evidence for a role of STIM1 in acidic store refilling in platelets probably acting as a $Ca^{2+}$ sensor and regulating the activity of SERCA3. This action is impaired in platelets from type 2 diabetics, which might lead to the enhanced cytosolic $Ca^{2+}$ concentration observed and, therefore, in platelet hyperactivity.



## 1. Introduction

Cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) is a key regulatory signal involved in the modulation of a wide variety of physiological events [1]. $Ca^{2+}$ mobilization involves mechanisms that rise $[Ca^{2+}]_c$, such as the release of $Ca^{2+}$ from finite intracellular $Ca^{2+}$ stores and $Ca^{2+}$ entry through the plasma membrane, and a finely regulated machinery to remove $Ca^{2+}$ from the cytosol, including $Ca^{2+}$ extrusion by the plasma membrane $Ca^{2+}$-ATPase (PMCA) and/or the $Na^+/Ca^{2+}$ exchanger, and $Ca^{2+}$ sequestration into agonist-releasable organelles by the sarcoendoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) or mitochondria [1–3]. An abnormal platelet $Ca^{2+}$ homeostasis has been reported to be involved in the pathogenesis of a variety of diseases associated to cardiovascular complications, including hypertension and diabetes mellitus [4,5]. The latter is associated to an enhanced $[Ca^{2+}]_c$ and an altered agonist-induced $Ca^{2+}$ mobilization [5].

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SERCA is a membrane protein that pumps cytosolic $Ca^{2+}$ into the intracellular stores against a large concentration gradient. Three different SERCA genes, SERCA1, SERCA2, and SERCA3, encode a variety of SERCA isoforms [6,7]. Human platelets express SERCA2b and six members of the SERCA3 family, named SERCA3a to 3f [8,9]. The SERCA 2b isoform, which shows high sensitivity to thapsigargin (TG), has been identified in the dense tubular system [10], the analog of the endoplasmic reticulum in platelets, while pharmacological studies indicate that SERCA3 isoforms, with low sensitivity to TG but sensitive to 2,5-di-(t-butyl)-1,4-hydroquinone (TBHQ [11], are located in the acidic stores [12,13]).

Although partial $Ca^{2+}$ store refilling can be achieved by reuptake of previously released $Ca^{2+}$, extracellular $Ca^{2+}$ entry is required for full $Ca^{2+}$ store replenishment, and store-operated $Ca^{2+}$ entry (SOCE), a process regulated by the filling state of the intracellular $Ca^{2+}$ stores, is a preeminent mechanism for $Ca^{2+}$ influx in non-electrically excitable cells. The stromal interaction molecule 1 (STIM1), a transmembrane protein located in the $Ca^{2+}$ stores, has recently been identified as the intraluminal $Ca^{2+}$ sensor that communicates the amount of stored $Ca^{2+}$ to plasma membrane channels [14,15]. In human platelets STIM1 interacts with endogenously expressed canonical human transient receptor potential 1 (hTRPC1) protein after store depletion [16]. STIM1 is necessary for the activation of *de novo* conformational coupling between hTRPC1 and the type II $IP_3$ receptor, a mechanism involved in SOCE in platelets [17,18]. Since STIM1 senses the intraluminal $Ca^{2+}$ concentration and has been suggested to regulate $Ca^{2+}$ through store-operated channels we have investigated the possible involvement of STIM1 in the regulation $Ca^{2+}$ store refilling, which might shed new light on the molecular basis underlying cardiovascular pathologies associated to platelet $Ca^{2+}$ homeostasis.

## 2. Materials and methods

### 2.1. Materials

Fura-2/AM was from Molecular Probes (Leiden, The Netherlands). Apyrase (grade VII), aspirin, bovine serum albumin (BSA), sodium dodecyl sulphate (SDS), thapsigargin (TG), and thrombin were from Sigma (Madrid, Spain). Ionomycin (Iono) was from Calbiochem (Nottingham, U.K.). Mouse anti STIM1 antibody ($S^{25}$–$W^{139}$) was from BD Transduction Laboratories (Frankin Lakes, NJ, U.S.A.). Anti STIM1 antibody ($Y^{231}$–$K^{243}$) was from Everest Biotech (Oxford, U.K.). For total SERCA3, the pan-SERCA3 monoclonal antibody (PL/IM430) was used (Chaabane et al. [27]). Horseradish peroxidase-conjugated donkey anti-goat IgG antibody was from Santa Cruz (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated ovine anti-mouse IgG antibody (NA931) and Hyperfilm ECL were from Amersham (Buckinghamshire, U.K.). Protein A-agarose was from Upstate Biotechnology Inc. (Madrid, Spain). Enhanced chemiluminiscence detection reagents were from Pierce (Cheshire, U.K.). All other reagents were of analytical grade.

### 2.2. Platelet preparation

Platelets from type 2 diabetics and healthy volunteers were prepared as described previously [19] in accordance with the Declaration of Helsinki. Blood glucose concentration in diabetic patients was in the range of 180–240 mg/dL. The glycosylated Hb levels (HbA1c) were used as an index of metabolic control. Only blood of diabetic patients with a level of HbA1c >6% was selected for experiments. The control subjects were age- and gender-matched healthy people that had HbA1c levels in the normal range (3.5–5%). Briefly, blood was obtained from drug-free healthy volunteers and mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing (in mM): 85 sodium citrate, 78 citric acid and 111 D-glucose. Platelet-rich plasma was then prepared by centrifugation for 5 min at 700 × *g* and aspirin (100 μM) and apyrase (40 μg/mL) added. Platelets were collected by centrifugation at 350 × *g* for 20 min and resuspended in HEPES-buffered saline (HBS) containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$, pH 7.45 and supplemented with 0.1% (w/v) BSA and 40 μg/mL apyrase.

### 2.3. Measurement of intracellular free calcium concentration ($[Ca^{2+}]_c$)

Human platelets were loaded with fura-2 by incubation with 2 μM fura-2/AM for 45 min at 37 °C. Fluorescence was recorded from 1 mL aliquots of magnetically stirred cellular suspension ($2 \times 10^8$ cells/mL) at 37 °C using a Cary Eclipse Spectrophotometer (Varian Ltd., Madrid, Spain) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_c$ were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to a established method [20]. $Ca^{2+}$ release was estimated using the integral of the rise in $[Ca^{2+}]_c$ for 2 min after the addition of the agents [12], and is expressed as nM s, as previously described [19]. To compare the rate of decay of $[Ca^{2+}]_c$ to basal values after platelet stimulation with thrombin we used the constant of the exponential decay. Traces were fitted to the equation $y = A(1 - e^{K_1 T})e^{-K_2 T}$, where $K_2$ is the constant of the exponential decay.

### 2.4. Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were performed as previously described [21]. Briefly, aliquots of platelet lysates (1 mL; $2 \times 10^9$ cell/mL) were immunoprecipitated by incubation with 2 μg of mouse anti-STIM1 ($S^{25}$–$W^{139}$) antibody and 25 μL of protein A-agarose overnight at 4 °C on a rocking platform. The immunoprecipitates were resolved by 10% SDS-PAGE and separated proteins were transferred onto nitrocellulose membranes. After blocking residual protein binding sites, immunodetection of SERCA3 or STIM1 was achieved using the anti-pan-SERCA3 (PL/IM430) diluted 1:500 in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1.5 h or the anti-STIM1 ($S^{25}$–$W^{139}$) antibody diluted 1:250 in TBST for 2h. Blots were washed six times for 5 min each with TBST, incubated for 45 min with horseradish peroxidase-conjugated ovine anti-mouse IgG antibody or donkey anti-goat IgG

antibody diluted 1:10,000 in TBST and exposed to enhanced chemiluminescence reagents. Blots were then exposed to photographic films. The data were then analyzed by using NIH ImageJ software.

### 2.5. Reversible electroporation procedure

The platelet suspension was transferred to an electroporation chamber containing 1 μg/mL anti-STIM1 antibody ($Y^{231}$–$K^{243}$), and the antibody was transjected as described previously [16]. Reversible electropermeabilization was performed at 4 kV/cm at a setting of 25 μF capacitance and was achieved by 7 pulses using a Bio-Rad Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA, U.S.A.). Following electroporation, cells were incubated with anti-STIM1 antibody for an additional 60 min at 37 °C, centrifuged and resuspended in HBS prior to the experiments. This procedure did not alter platelet function as previously demonstrated [22].

### 2.6. Statistical analysis

Analysis of statistical significance was performed using Student's unpaired *t*-test for parametric variables. For multiple comparisons, one-way analysis of variance (ANOVA) combined with the Dunnet tests was used. $p < 0.05$ was considered to be significant.

## 3. Results

### 3.1. Electrotransjection with anti-STIM1 antibody inhibits refilling of the acidic stores

We have investigated the role of STIM1 in acidic store refilling by electrotransjection of a specific anti-STIM1 antibody that recognizes the cytosolic amino acid sequence 231–243, which is involved in protein–protein interaction [23]. Several studies have reported that electroporation can be successfully used for transferring antibodies into cells while maintaining the physiological integrity of the cells [24,16]. Human platelets were reversibly electroporated and the presence of this antibody inside platelets was confirmed, in samples from non-electropermeabilized or electropermeabilized cells, both incubated with 1 μg/mL anti-STIM1 antibody, by immunoprecipitation without adding any additional anti-STIM1 antibody and subsequent Western blotting with the anti-STIM1 antibody (Fig. 1C).

We have recently reported that thrombin releases $Ca^{2+}$ from TBHQ-sensitive acidic stores in platelets [12,13]. In order to investigate acidic store refilling, the stores were depleted by treatment with 1 U/mL thrombin and allowed to refill, as previously demonstrated [13]. $Ca^{2+}$ accumulated in the acidic stores was then estimated by treatment with 20 μM TBHQ, which has been shown to induce maximal and selective acidic

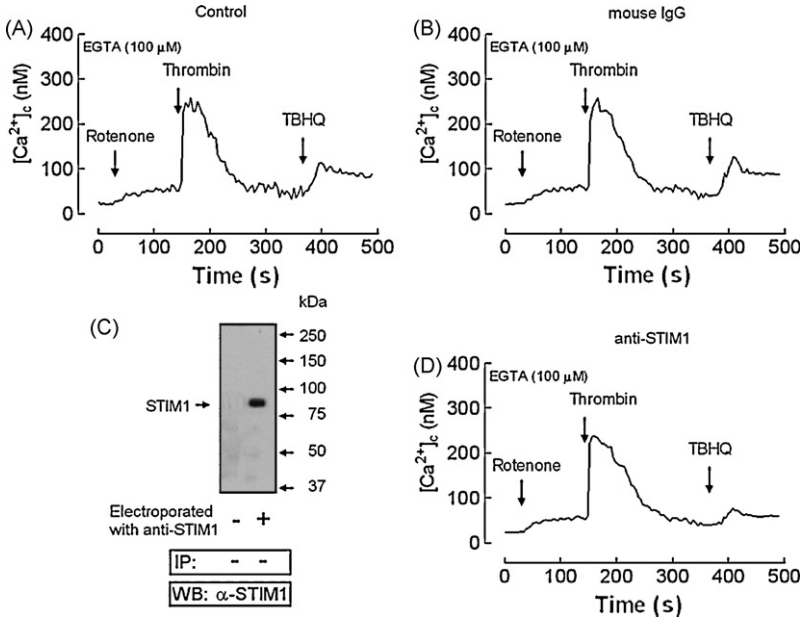


**Fig. 1 – Inhibition of acidic store refilling by electrotransjection with anti-STIM1 antibody. Human platelets were electropermeabilized in a Gene Pulser and incubated in the absence of antibodies (Control; A) or in the presence of 1 μg/mL mouse IgG (B) or anti-STIM1 antibody ($Y^{231}$–$K^{243}$) (C and D) for 60 min, as indicated. At the time of experiment 100 μM EGTA was added. Cells were treated with 10 μM rotenone and 2 min latter stimulated with 1 U/mL thrombin followed by addition of 20 μM TBHQ 3.5 min later. Changes in fura-2 fluorescence were monitored using the 340/380-nm ratio and calibrated in terms of $[Ca^{2+}]_c$. Traces are representative of six to seven independent experiments. C, resting platelets (lane 1) or platelets electropermeabilized in a Gene Pulser as described in Section 2 (lane 2) were incubated in the presence of 1 μg/mL anti-STIM1 antibody (α-STIM1) for 60 min as indicated, and then lysed. Whole cell lysates were immunoprecipitated in the absence of antibodies but adding protein A-agarose, and immunoprecipitated proteins were analyzed by Western blotting using anti-STIM1 antibody (α-STIM1). These results are representative of three independent experiments.**

store depletion [25]. In cells electroporated in the absence of antibodies and in the absence of external $Ca^{2+}$ (100 μM EGTA added) thrombin induced a transient increase in $[Ca^{2+}]_c$ (the integral of the rise in $[Ca^{2+}]_c$ above basal for 2 min after the addition of thrombin, taking data every 1 s, was 7064 ± 145 nM s; mean ± S.E.M.; Fig. 1A; $n = 6$). Subsequent addition of 20 μM TBHQ to the platelet suspension resulted in a rise in $[Ca^{2+}]_c$ indicative of the amount of $Ca^{2+}$ accumulated in the acidic compartments (the integral of the rise in $[Ca^{2+}]_c$ above basal after the addition of TBHQ was 3249 ± 602 nM s; Fig. 1A). During the performance of the experiments, rotenone (10 μM), an inhibitor of complex I of the respiratory chain that dissipates the mitochondrial membrane potential, was added to the platelet suspension to release mitochondrial $Ca^{2+}$ and avoid interference with this organelle.

In cells electrotransjected with 1 μg/mL anti-STIM1 antibody (Fig. 1D) or 1 μg/mL mouse IgG of the same nature of the anti-STIM1 antibody (Fig. 1B) thrombin-induced $Ca^{2+}$ release was not significantly modified (the integral of the rise in $[Ca^{2+}]_c$ above basal for 2 min after the addition of thrombin in cells electrotransjected with anti-STIM1 antibody or mouse IgG was 6806 ± 556 and 6767 ± 351 nM s, respectively; $n = 6$). In contrast, electrotransjection with anti-STIM1 antibody significantly reduced the rate of decay of the $[Ca^{2+}]_c$ to basal levels after stimulation with thrombin (the decay constants were 0.0094 ± 0.0004 and 0.0078 ± 0.0004 in electroporated cells

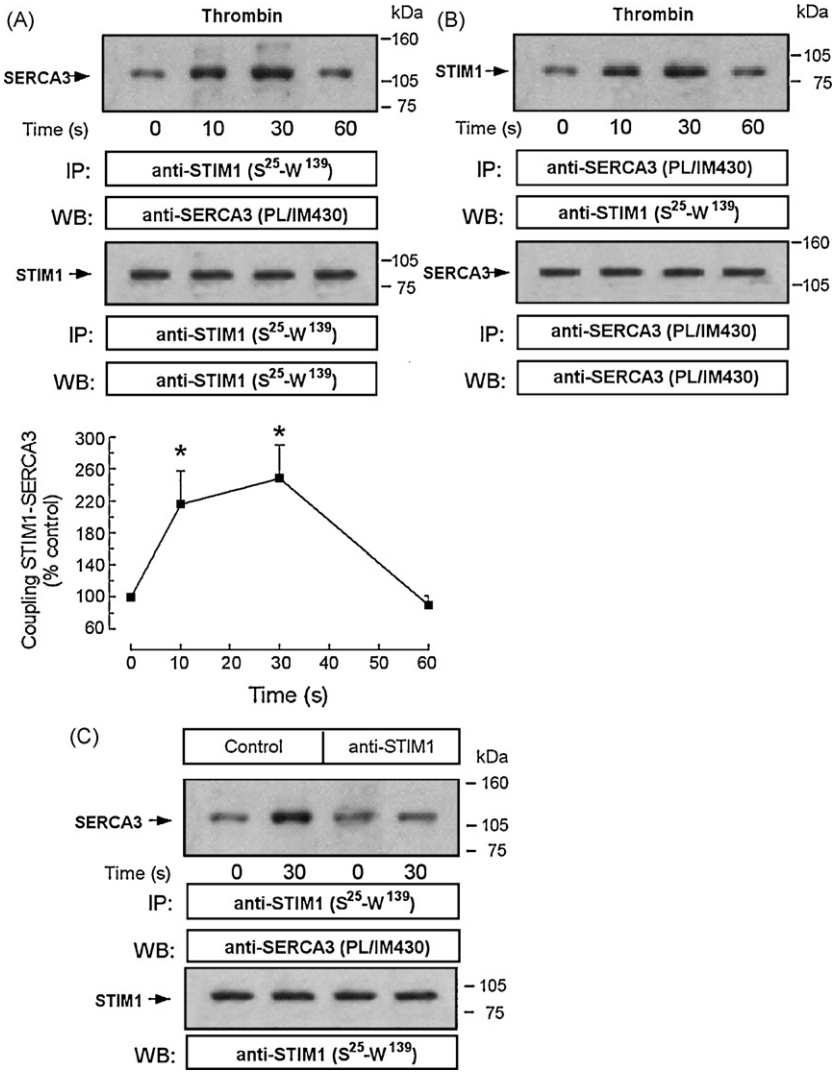


**Fig. 2 – Thrombin enhances the coupling between STIM1 and SERCA3 in human platelets. (A and B) Platelets were stimulated with 1 U/mL thrombin at 37 °C for various time periods (10–60 s) and lysed. Samples were immunoprecipitated with anti-STIM1 ($S^{25}$–$W^{139}$) (A) or anti-SERCA3 (B) antibody. Proteins were separated by SDS/PAGE followed by Western blotting with anti-SERCA3 (A, top panel) or anti-STIM1 (B, top panel) antibody and reprobed by Western blotting using anti-STIM1 (A, bottom panel) or anti-SERCA3 (B, bottom panel) antibody. Positions of molecular-mass markers are shown on the right. Graph represents the quantification of the amount of SERCA3 co-immunoprecipitated with STIM1. Values are mean ± S.E.M. of six separate experiments and expressed as the percentage of the amount of SERCA3 associated with STIM1 in resting cells. $^{*}p < 0.05$ compared to the resting level (ANOVA combined with Dunnet test). (C) Platelets were electropermeabilized and incubated in the absence or presence of 1 μg/mL anti-STIM1 antibody. Cells were then stimulated with 1 U/mL thrombin for 30 s and lysed. Western blotting was performed as described above.**

incubated with mouse IgG or anti-STIM1, respectively; $p < 0.05$ Student's *t*-test; $n = 7$). Electrotransjection with anti-STIM1 antibody significantly reduced subsequent $Ca^{2+}$ release induced by TBHQ (the integral of the rise in $[Ca^{2+}]_c$ above basal for 2 min after the addition of TBHQ was 996 ± 328 nM s; Fig. 1D; $p < 0.05$; $n = 7$). In cells electrotransjected with a mouse IgG the integral of the rise in $[Ca^{2+}]_c$ above basal for 2 min after the addition of TBHQ was 3281 ± 587 nM s; Fig. 1B; $n = 7$; thus suggesting that this effect was not mediated by electrotransjection of a mouse IgG in platelets. These findings suggest that the amino acid sequence recognized by the anti-STIM1 antibody might be essential for acidic store refilling.

### 3.2. Association between STIM1 and SERCA3

Two mechanisms have been proposed to maintain a $Ca^{2+}$ gradient across the acidic stores membrane, the proton gradient generated by the vacuolar $H^+$-ATPase and $Ca^{2+}$ reuptake by SERCA3; however, only SERCA3 is involved in store refilling after agonist stimulation [12]. Since STIM1 is required for acidic store refilling we have tested for the interaction between STIM1 and SERCA3 by looking for co-immunoprecipitation from platelet lysates. Immunoprecipitation and subsequent SDS-PAGE and Western blotting were conducted using control platelets and platelets stimulated with 1 U/mL thrombin for several times (10–60 s). After immunoprecipitation with anti-STIM1 ($S^{25}$–$W^{139}$) antibody, Western blotting revealed the presence of SERCA3 in samples from resting platelets (Fig. 2A). Similar results were observed when platelet lysates were immunoprecipitated with anti-SERCA3 antibody followed by Western blotting with anti-STIM1 antibody (Fig. 2B). Thrombin increased the association between STIM1 and SERCA3 in a time-dependent manner, reaching a maximum after 30 s with a 248 ± 41% of control (resting cells), and returning to basal levels after 60 s of stimulation (Fig. 2A, $p < 0.05$ ANOVA; $n = 6$). Electrotransjection of the anti-STIM1 antibody, which impairs refilling of the acidic store as described above, reduces the interaction between STIM1 and SERCA3 stimulated by thrombin (Fig. 2B). The interaction between these two proteins in resting cells was not affected by electrotransjection of the anti-STIM1 antibody. These might be attributed to a lack of interaction of the anti-STIM1 antibody with SERCA-linked STIM1 in resting cells, while the antibody prevents further association between SERCA3 and STIM1 induced by thrombin. Altogether, these findings suggest that STIM1 is required for acidic store refilling, which is likely mediated by interaction with SERCA3.

We have also tested for the association between STIM1 and SERCA3 after extensive store depletion of the intracellular stores by treatment with TG (1 μM) and Iono (50 nM) in the absence of extracellular $Ca^{2+}$ (100 μM EGTA; [17,16]. As demonstrated by co-immunoprecipitation treatment of platelets with TG + Iono enhanced the coupling between SERCA3 and STIM1 in a time-dependent manner, reaching a maximum after 30 s of treatment with a 209 ± 25% of control (Fig. 3; $p < 0.05$ ANOVA; $n = 6$). Western blotting of the same membranes with anti-STIM1 antibody confirmed a similar content of this protein in all lanes (Figs. 2 and 3, lower panel).

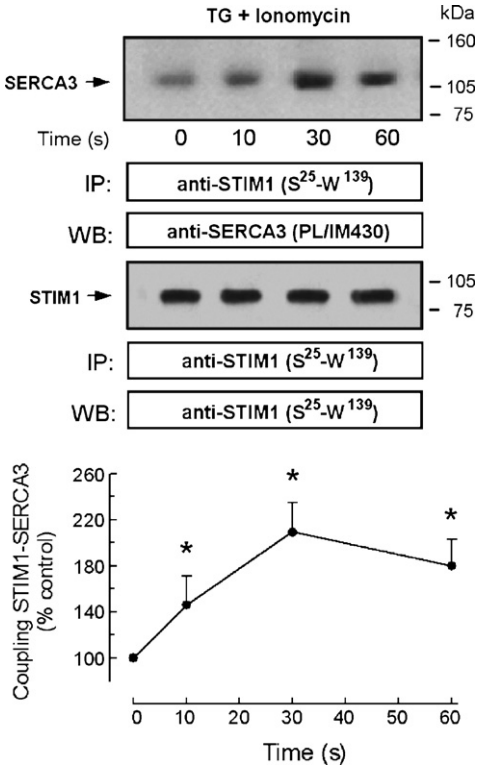


**Fig. 3 – Extensive store depletion increases the coupling between STIM1 and SERCA3. Platelets were stimulated with 1 μM TG + 50 nM ionomycin for various time periods (10–60 s) and lysed. Samples were immunoprecipitated with anti-STIM1 ($S^{25}$–$W^{139}$) antibody. Proteins were separated by SDS/PAGE followed by Western blotting with anti-SERCA3 antibody (top panel) and reprobed by Western blotting using anti-STIM1 ($S^{25}$–$W^{139}$) antibody (bottom panel). Positions of molecular-mass markers are shown on the right. Graph represents the quantification of the amount of SERCA3 co-immunoprecipitated with STIM1. Values are mean ± S.E.M. of six separate experiments and expressed as the percentage of the coupling in resting cells. *$p < 0.05$ compared to the resting level (ANOVA combined with Dunnet test).**

### 3.3. Association between STIM1 and SERCA3 and acidic store refilling is reduced in platelets from type 2 diabetic donors

$Ca^{2+}$ homeostasis has been shown to be altered in platelets from diabetic patients [5,26,27]. Higher resting $[Ca^{2+}]_c$ [28] and reduced $Ca^{2+}$ extrusion has been reported in platelets from diabetic donors [29,30]. Now we have investigated acidic store refilling in these patients. First of all, we have explored whether the interaction between STIM1 and SERCA3 is altered. As described above for control cells, immunoprecipitation and Western blotting were conducted in control platelets and cells stimulated with thrombin or TG + Iono for several times (10–60 s). Our results indicate that in platelets from diabetic donors the ability of thrombin to induce association between STIM1 and SERCA3 was abolished, while that of TG + Iono was

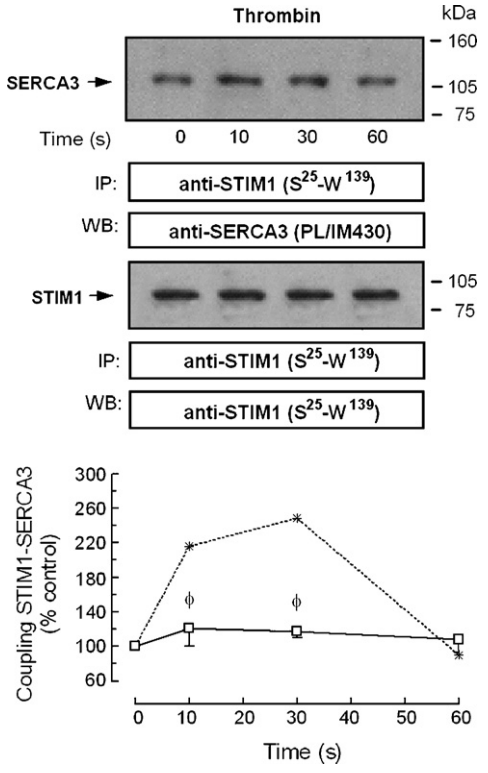


**Fig. 4 – The ability of thrombin to enhance the coupling between STIM1 and SERCA3 is abolished in platelets from type 2 diabetics. Platelets from type 2 diabetics were stimulated with 1 U/mL thrombin for various time periods (10–60 s) and lysed. Samples were immunoprecipitated with anti-STIM1 ($S^{25}$–$W^{139}$) antibody. Proteins were separated by SDS/PAGE followed by Western blotting with anti-SERCA3 antibody (top panel) and reprobed by Western blotting using anti-STIM1 ($S^{25}$–$W^{139}$) antibody (bottom panel). Positions of molecular-mass markers are shown on the right. Graph represents the quantification of the amount of SERCA3 co-immunoprecipitated with STIM1. Values are mean ± S.E.M. of separate experiments and expressed as the percentage of the coupling in resting cells. Dashed line represents the effect of thrombin in platelets from healthy donors as shown in Fig. 2. $^{\Phi}p < 0.05$ compared to healthy donors.**

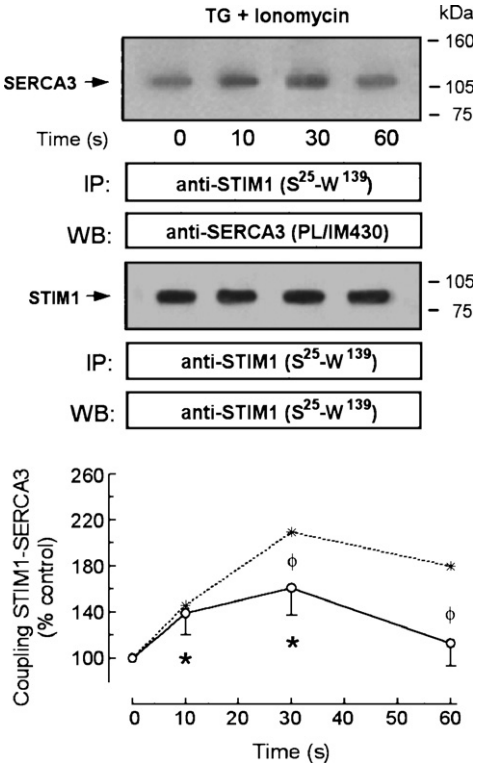


**Fig. 5 – Store depletion-induced coupling between STIM1 and SERCA3 is reduced in platelets from type 2 diabetics. Platelets from type 2 diabetics were stimulated with 1 μM TG + 50 nM ionomycin for various time periods (10–60 s) and lysed. Samples were immunoprecipitated with anti-STIM1 ($S^{25}$–$W^{139}$) antibody. Proteins were separated by SDS/PAGE followed by Western blotting with anti-SERCA3 antibody (top panel) and reprobed by Western blotting using anti-STIM1 ($S^{25}$–$W^{139}$) antibody (bottom panel). Positions of molecular-mass markers are shown on the right. Graph represents the quantification of the amount of SERCA3 co-immunoprecipitated with STIM1. Values are mean ± S.E.M. of separate experiments and expressed as the percentage of the coupling in resting cells. Dashed line represents the effect of TG + Iono in platelets from healthy donors as shown in Fig. 3. $^{\Phi}p < 0.05$ compared to healthy donors. $^{*}p < 0.05$ compared to the resting level.**

significantly reduced (Figs. 4 and 5, upper panel; $p < 0.05$ ANOVA; $n = 6$). Hence, we have investigated acidic store refilling by SERCA3 in platelets from type 2 diabetic donors after store depletion using the agonist thrombin. Platelet stimulation with 1 U/mL thrombin induced a transient rise in $[Ca^{2+}]_c$ due to depletion of the intracellular stores in platelets, subsequent refilling of the acidic stores was estimated by treatment with 20 μM TBHQ. TBHQ-induced elevation in $[Ca^{2+}]_c$ was found to be significantly smaller in platelets from diabetic donors (the integral of the rise in $[Ca^{2+}]_c$ above basal for 2 min after the addition of TBHQ was 4261 ± 408 nM s in healthy donors and 2295 ± 352 nM s in diabetics; Fig. 6; $p < 0.05$ Student's *t*-test; $n = 10$), despite the fact that the size of the acidic stores is similar in diabetics and healthy donors [31].

## 4. Discussion

Platelets, anucleated cells that circulate in the blood checking the integrity of the vascular wall, activate in response to a number of agonists that mobilizes intracellular $Ca^{2+}$. STIM1 has been presented as a central regulator of platelet function [16,32]. Here we show that functional knockdown of STIM1 by electrotransjection of cells with anti-STIM1 antibody impairs acidic store refilling, which indicates that STIM1 is involved in the regulation of $Ca^{2+}$ reuptake into the acidic stores. This conclusion is further supported by the association of STIM1 with SERCA3, the $Ca^{2+}$-ATPase involved in $Ca^{2+}$ reuptake into the acidic stores [11–13]. The association between STIM1 and SERCA3 is enhanced after stimulation with thrombin or by extensive $Ca^{2+}$ store depletion in a time-dependent manner,

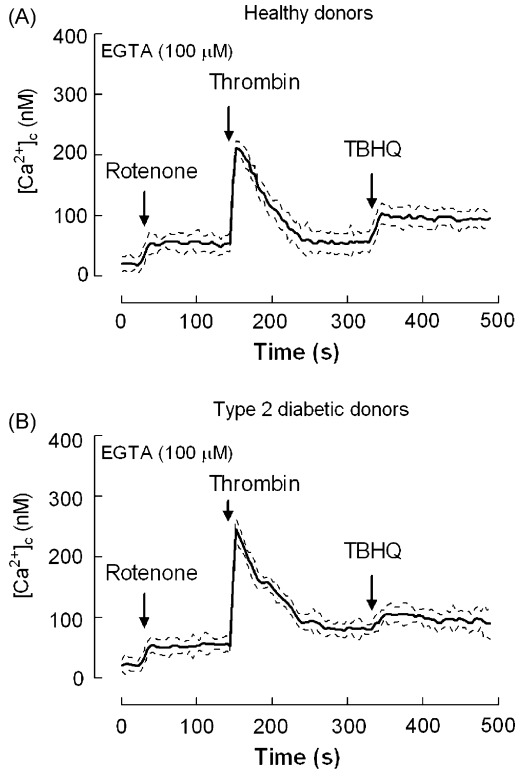


**Fig. 6 – Acidic store refilling is reduced in type 2 diabetic platelets. Platelets from healthy (A) and type 2 diabetics (B) were treated with 10 μM rotenone and 2 min latter stimulated with 1 U/mL thrombin followed by addition of 20 μM TBHQ 3.5 min later. Changes in fura-2 fluorescence were monitored using the 340/380-nm ratio and calibrated in terms of $[Ca^{2+}]_c$. Data are presented as mean ± S.E.M. of ten experiments performed in different donors.**

an effect that was detectable after 10 s of treatment, maximal after 30 s and then returns to basal levels, following the pattern of thrombin- or TG + Iono-evoked $Ca^{2+}$ mobilization. Electrotransjection of anti-STIM1 ($Y^{231}$–$K^{243}$) antibody prevented thrombin-evoked interaction between STIM1 and SERCA3, which suggests that the sequence $Y^{231}$–$K^{243}$ might be required for this interaction.

A recent study has reported the formation of microdomains containing store-operated $Ca^{2+}$ (SOC) channels in the plasma membrane, STIM proteins and SERCA pumps in the endoplasmic reticulum and mitochondria involved in the refilling of the intracellular $Ca^{2+}$ stores. The role of STIM1 in these microdomains consists of the regulation of the activity of SOC channels without modifying the activity of $Ca^{2+}$ transporters, which are able to refill the endoplasmic reticulum in STIM1 knockdown cells [33]. SOCE is a process where STIM1 plays a key role in platelets and other cells [12,14–16]; however, in our study, we have described for the first time the role of STIM1 in acidic store refilling independently of SOCE, which was prevented by performing the experiments in a $Ca^{2+}$-free medium. STIM1 has been proposed to communicate the intracellular stores with SOC channels in the plasma membrane [12,14–16]. We have recently demonstrated the formation of microdomains containing hTRPC1, hTRPC6, Orai1, the type II $IP_3$ receptor, SERCA3 and STIM1 in human platelets after depletion of the intracellular $Ca^{2+}$ stores [16,22]. We propose that STIM1 might be a point of convergence that communicates the filling state of the acidic $Ca^{2+}$ store to SERCA3 to regulate store refilling, as it does with SOC channels to modulate SOCE.

In platelets from patients with type 2 diabetes mellitus $Ca^{2+}$ homeostasis is altered, resulting in an elevated resting $[Ca^{2+}]_c$ and $Ca^{2+}$ mobilization, which leads to hyperactivity and hyperaggregability [26,28–31]. We have found that acidic store refilling is clearly reduced in platelets from type 2 diabetics, an effect that might be mediated by the impairment of the interaction between SERCA3 and STIM1 stimulated by thrombin or extensive store depletion in these cells. The reduced $Ca^{2+}$ store refilling, together with the limited $Ca^{2+}$ extrusion in platelets from diabetics [29,30], might be responsible for the elevated resting $[Ca^{2+}]_c$ observed in diabetic platelets.

In summary, we have shown for the first time that STIM1 is required for acidic store refilling through its interaction with SERCA3. This interaction might regulate the activity of SERCA3, which has been shown to depend on the rate of $Ca^{2+}$ release [34]. The interaction between STIM1 and SERCA3 is impaired in platelets from type 2 diabetic patients, which, in turn, might be the base for platelet hyperactivity and the cardiovascular complications associated to type 2 diabetes mellitus.

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