Full activation of mouse platelets requires an ADP secretion pathway regulated by SERCA3 ATPase-dependent calcium stores

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Running title: Defective ADP secretion in SERCA3\(^{-/-}\) platelets.

Key Points: Defect in thrombus formation, platelet aggregation, and ADP secretion induced by ablation or inhibition of SERCA3\(^{-/-}\).
Abstract

The role of the sarco-endoplasmic reticulum calcium (Ca$^{2+}$) ATPase (SERCA)3 in platelet physiology remains poorly understood. Here, we show that SERCA3−/− mice exhibit prolonged tail bleeding time and re-bleeding. Thrombus formation was delayed both in arteries and venules in an in vivo ferric chloride-induced thrombosis model. Defective platelet adhesion and thrombus growth over collagen was confirmed in vitro. ADP removal by apyrase diminished adhesion and thrombus growth of control platelets to the level of SERCA3−/− platelets. Aggregation, dense granule secretion and Ca$^{2+}$ mobilization of SERCA3−/− platelets induced by low collagen or low thrombin concentration were weaker than controls. Accordingly, SERCA3−/− platelets exhibited a partial defect in total stored Ca$^{2+}$, and in Ca$^{2+}$ store re-uptake following thrombin stimulation. Importantly ADP but not serotonin, rescued aggregation, secretion and Ca$^{2+}$ mobilization in SERCA3−/− platelets, suggesting specificity. Dense granules appeared normal upon electron microscopy, mepacrine staining, and total serotonin content, ruling out a dense granule defect. ADP induced normal platelet aggregation, excluding a defect in ADP activation pathways. The SERCA3-specific inhibitor tBHQ diminished both Ca$^{2+}$ mobilization and secretion of control platelets, as opposed to the SERCA2b inhibitor thapsigargin. This confirmed the specific role of catalytically active SERCA3 in ADP secretion. Accordingly, SERCA3-dependent Ca$^{2+}$ stores appeared depleted in SERCA3−/− platelets. Finally $\alpha_{IIb}\beta_3$ integrin blockade did not affect SERCA3-dependent secretion, therefore proving independent of $\alpha_{IIb}\beta_3$ engagement. Altogether these results show that SERCA3-dependent Ca$^{2+}$ stores control a specific ADP secretion pathway required for full platelet secretion induced by agonists at low concentration, and independent of $\alpha_{IIb}\beta_3$. 
Introduction

Among regulatory mechanisms of Ca^{2+} intracellular signaling in platelets, the sarco-endoplasmic Ca^{2+} ATPases (SERCAs) which pump Ca^{2+} into intracellular stores are particularly relevant.\(^1\) SERCAs are encoded by 3 genes, \textit{ATP2A1}, \textit{ATP2A2} and \textit{ATP2A3}, which produce several alternate transcripts and protein isoforms: SERCA1a/b, SERCA2a-c, and SERCA3a-f. They are found in multiple tissues, but platelets exhibit only SERCA2b and SERCA3 isoforms.\(^2\,4\) SERCAs maintain a Ca^{2+} concentration gradient between the cytosol (100nM) and the ER (1mM), requiring degradation of ATP\(^5\) into ADP then released into the cytosol.\(^6\)

SERCA enzymes share similar structures with distinct intrinsic activities: higher Ca^{2+} affinity for SERCA2b than for SERCA3 (\(K_{1/2} \approx 0.27\) µM versus 1 µM) but a lower Ca^{2+} uptake (7 nmol/min/mg of protein versus 21 nmol/min/mg)\(^7\,8\), allowing cytosolic Ca^{2+} to be maintained at low levels in the resting cells.\(^9\)

Pathologies and mouse models provide insight into SERCA2b and SERCA3 functions. Mutations in the human \textit{ATP2A2} gene affecting SERCA2, lead to the Darier's syndrome in humans, a dermatological syndrome.\(^10,11\) SERCA mutations are associated with some cancers,\(^12\,-\,14\) suggesting involvement in cell differentiation.\(^15\) SERCA3 human mutations seem associated with type II diabetes.\(^16\) Mouse SERCA2 knockouts are not viable at the homozygous state, but heterozygotes exhibit SERCA2a- (defect in heart contractility and relaxation)\(^17\) and SERCA2b-type defects, evocative of the Darier's syndrome.\(^18\) Mouse SERCA3 knockouts exhibit no phenotypic alterations,\(^19\) except for an altered gustatory nerve response.\(^20\) Impaired relaxation of SERCA3\(^{-/-}\) aorta rings was reported, with defective relaxation of vascular smooth muscle cells, altered Ca^{2+} signaling and low NO production.\(^19\) \textit{In vitro}, low insulin secretion and altered Ca^{2+} oscillations were reported.\(^21\,-\,23\) Altogether these results point to a potential specific role for SERCA3 in Ca^{2+} signal modulation.

Among other differences in platelets is a different topology, peripheral for SERCA3, more central for SERCA2b.\(^24\) SERCA3 is specifically associated with acidic Ca^{2+} stores,\(^25\) and with STIM1 (the Ca^{2+} sensor of the store operated Ca^{2+} entry (SOCE)).\(^26\) To assess the role of SERCA3 in platelets, we have
decided to assess the hemostasis status of SERCA3<sup>−/−</sup> mice. Here, we report the analysis of both in vivo and in vitro hemostasis features of SERCA3 knockout mice, and show that ablation of SERCA3 lowers in vivo hemostatic and thrombotic responses, as well as platelet adhesive and secretory functions in vitro. Moreover, we find that thrombin or collagen activation are affected, due to low dense granule secretion. Importantly, mobilization and secretion are rescued by ADP addition, consistent with a role for SERCA3 in ADP secretion. Confirming a specific role for SERCA3, pharmacological inhibition of SERCA3, but not of SERCA2b, in control platelets recapitulates the same defect in secretion in vitro, definitely pointing to SERCA3 as specifically involved in the regulation of such a secretion. Finally, SERCA3-dependent secretion appears independent of α<sub>IIb</sub>β<sub>3</sub> integrin engagement. These results thus point to an as yet unreported role of SERCA3 in hemostasis and in positive regulation of platelet dense granule and ADP secretion.
Material and Methods

Material

Fibrillar collagen (equine type I) and adenosine 5'-diphosphate (ADP) were obtained from Kordia (Leiden, The Netherlands). Apyrase (grade VII), rhodamine 6G, bovine thrombin, ferric chloride, indomethacin, the SERCA inhibitors thapsigargin and 2,5-di-( tert- butyl)-1,4-benzhydroquinone (tBHQ) were obtained from Sigma (St Louis, MO). We purchased d-Phe-Pro-Arg chloromethylketone dihydrochloride (PPACK) from Calbiochem-VWR (Fontenay-sous-Bois, France). The protease-activated receptor (PAR) agonist peptide (PAR4-AP; AYPGKF-NH₂) was purchased from Bachem (Weil am Rhein, Germany). Mepacrine (Quinacrine dihydrochloride) was from Sigma-Aldrich (Saint-Louis, MO). Alexa Fluor 488-labeled phalloidin were from Invitrogen (Cergy Pontoise, France). Phycoerythrin (PE)-labeled rat anti-mouse integrin α₁β₃ mAb (JON/A), fluorescein isothiocyanate (FITC)-labeled rat anti-mouse CD62P (P-selectin) mAb (Wug.E9) and purified rat anti-mouse integrin α₁β₃ mAb (Leo.H4) were from Emfret Analytics (Würzburg, Germany). Oregon Green 488 BAPTA1-AM was from Molecular Probes (Eugene, OR). Polyclonal antibodies specific for SERCA2b²⁷ and for SERCA3 (N89)²⁸ were generous gifts from Pr F. Wuytack (Katholieke Universiteit Leuven, Leuven, Belgium). The antibody directed against 14-3-3ζ, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-(Ser) PKC substrate antibody was from Cell Signaling Technology (Danvers, MA).

SERCA3 knockout mice

The swiss black SERCA3⁻/⁻ mice originally generated by Dr G.E. Shull (University of Cincinatti, OH)¹⁹ were crossed with C57BL/6 mice and kindly provided by Dr P. Gilon (University of Louvain, Belgium) with the authorization of Dr G.E. Shull. Wild-type litter mate mice were provided as well, and served as controls. Transferred wt/- heterozygous mice were intercrossed and homozygotes were detected by PCR, using published oligonucleotide primers.¹⁹ All experimental procedures were carried out in accordance with the European legislation concerning the use of laboratory animals and approved by the Animal Care and Ethical Committee of Université Paris-Sud (agreement # 00243-02).
**Hematologic analysis and bleeding time**

Blood counts were determined with an automatic cell counter (scil Vet abc Plus, Horiba Medical, France). Bleeding time assays were performed as previously described,\textsuperscript{29} on 8- to 12-week-old mice.

**Measurement of intracellular calcium**

Mouse platelets (3 x 10\textsuperscript{7} platelets/mL) were loaded with the Ca\textsuperscript{2+}-sensitive dye Oregon Green 488 BAPTA1-AM (1mM) for 45 minutes at 20°C. Ca\textsuperscript{2+} mobilization induced by thrombin was analyzed in Ca\textsuperscript{2+} free medium and in the presence of 0.5mM EGTA using an Accuri C6 flow cytometer. Changes in Ca\textsuperscript{2+} signal intensity were calculated as the ratios of fluorescence of activated over non-activated platelets and the area below the curve for 2 minutes after agonist addition was chosen as an indicator of the calcium response. Fluorescence calibration in pM Ca\textsuperscript{2+} was established as described in supplemental methods.

**In vitro thrombus formation under flow conditions**

Thrombus formation was evaluated in a whole blood perfusion assay on a fibrillar collagen matrix (50 μg/mL) at various shear rates (150s\textsuperscript{-1} and 1200s\textsuperscript{-1}) and recorded and analyzed as previously described.\textsuperscript{29} Thrombus formation was evaluated by assessment of platelet adhesion quantitated by measurement of the mean percentage of the total area covered by thrombi.

**Ferric chloride-induced thrombosis model**

Ferric chloride (FeCl\textsubscript{3}) injury was induced in 4- to 5-week-old mice, as previously described.\textsuperscript{30} Briefly, rhodamine 6G (3.3 mg/kg) was injected into the retro-orbital plexus of anesthetized mice (to label platelets). After topical deposition on the mesenteric vessels of FeCl\textsubscript{3} solution (10%), thrombus growth was monitored in real-time with an inverted epifluorescent microscope (×10) (Nikon Eclipse TE2000-U).
Serotonin assay

Platelet serotonin (5HT) was assessed using the Serotonin ELISA Kit assay from Abcam (Cambridge, UK). 5HT was assayed in platelets supernatants or in platelet lysate (after four freeze-thaw cycles) to determine platelet total 5HT content.

Statistical analysis

Statistical significance was evaluated with the Student t tests or 1-way ANOVA followed by Tukey pair wise test as indicated, using GraphPad Prism (San Diego, CA).

Results

Prolonged bleeding time and delayed in vivo thrombosis in SERCA3-/- mice.

We first confirmed in our mouse colony the deletion of Atp2a3, the mouse SERCA3 gene, by PCR (data not shown) as well as the absence of the SERCA3 protein in platelets by Western blotting (figure 1A). Note that platelet SERCA2b is expressed at the same level than controls. The tail clipping bleeding time assay was found significantly increased in SERCA3-/- versus wild type mice (142 ± 12 seconds for SERCA3-/- mice versus 67 ± 5 seconds for controls, P<0.001 figure 1B). Moreover, a marked rebleeding tendency was noted with 75% rebleeding within 1 minute of bleeding arrest, versus 10% for controls (figure 1C). Platelets were normal morphologically (figure 1D), slightly larger in size (5.82 ± 0.08 compared to 4.99 ± 0.04fL, Table 1) and in slightly lower numbers than controls (average 778.2 ± 0.31 x 10^9/L versus 914.5 ± 0.33 x 10^9/L, n = 21 and 18, respectively, Table 1), not low enough to explain the prolonged bleeding time. Other blood cell counts were normal.

Ferric chloride-induction of thrombosis in mesenteric vessels, showed delayed thrombus formation (often no occlusion at 60 minutes) in both venules and arterioles of SERCA3-/- mice compared to controls (30 minutes occlusion time for controls, figure 1E and 1F). Thrombus instability in SERCA3-/-
mice was frequent with 3 times more venule or arteriole emboli than in controls (figure 1G). These results indicate that SERCA3 ablation affects hemostasis, thrombus formation and stability in vivo.

**Evidence for an ADP secretion defect of SERCA3−/− platelets in in vitro flow adhesion and aggregation.**

To confirm a platelet defect, we next assessed adhesion and thrombus formation of SERCA3−/− platelets on a collagen matrix in both low (150s−1, figure 2A, 2B) and high (1200s−1, figure 2C, 2D) shear conditions, which were both significantly lower in SERCA3−/− platelets compared to controls. Adhesion and thrombus size (in both low and high shear conditions) of control platelets was diminished to the level of SERCA3−/− platelets after secretion inhibition by apyrase and indomethacin. Altogether these results indicate that SERCA3 ablation affected platelets, most likely through alteration of ADP secretion. Confirming platelet involvement, aggregation of SERCA3−/− platelets was impaired, when induced by low levels of collagen (0.8 µg/mL) (figure 3A), thrombin (40 mU/mL) (figure 3B), or PAR4-AP peptide (activator peptide of the PAR-4 thrombin receptor, a G-protein coupled receptor, GPCR) (figure S2A). Higher doses of agonist essentially normalized aggregation levels. ADP scavenging by apyrase abolished aggregation responses of both control and SERCA3−/− platelets (figures 3C and S2B). This strengthened the hypothesis of an ADP secretion defect in SERCA3−/− platelets. Moreover aggregation of SERCA3−/− platelets was rescued by 10 µM ADP (not promoting aggregation alone in absence of added fibrinogen, not shown) addition to either collagen or thrombin (Fig 3D).

Secretion of the ADP storage organelles dense granules, as monitored by ATP release during aggregation appeared strongly diminished in SERCA3−/− compared to control platelets (figures 4A, 4B and S2C). Confirming a dense granule secretion defect, serotonin (5HT) release was also markedly altered in SERCA3−/− platelets (figure S3C). Conversely, dense granule secretion was almost completely rescued by addition of 10µM ADP, as assessed by ATP or 5HT secretion (figures 4A and S3C, respectively). ADP alone (Fig 4A,B), did not elicit dense granule secretion in control or SERCA3−/− platelets. Thus SERCA3−/− platelets exhibit a defect due to alteration of dense granule secretion.
P-selectin exposure of stimulated SERCA3+/− platelets (figures 4C or S2E) was significantly diminished compared to control platelets. Not shown, a defect in P-selectin exposure was also observed in convulxin stimulated SERCA3+/− unstirred platelets. Apyrase treatment nearly suppressed P-selectin exposure of control and SERCA3+/− platelets, stimulated with low thrombin concentration while ADP addition to thrombin restored normal P-selectin exposure on SERCA3+/− platelets (figure 4C). This indicated that α-granule exocytosis is likely to be secondary to ADP secretion and thus only indirectly dependent on SERCA3.

Finally, aggregation to ADP of SERCA3+/− platelets (in PRP, to provide fibrinogen), was normal compared to control (figure 5C). Thus, SERCA3+/− ablation does not affect platelet activation by ADP, but specifically acts on ADP secretion.

**Dense granule content is not affected in SERCA3+/− platelets**

To check that defective secretion was not due to a dense granule defect, platelets were labeled with the fluorescent reporter mepacrine which accumulates specifically in dense granules. Flow cytometry showed that mepacrine was stored to the same extent in control and SERCA3+/− platelets (figure S3A), strongly suggesting a normal content in dense granules. In addition ATP release after maximal platelet stimulation (thrombin 2U/mL) of SERCA3+/− platelets reached approximately 70% of controls (figure S3B), but reached 100% ATP secretion compared to control platelets upon ADP addition to thrombin. Most significantly, total 5HT content was identical between control and SERCA3+/− platelets (figure S3C).

Thus the dense granule secretion defect observed in SERCA3+/− platelets is not due to a defect in number, or content.

**5HT does not restore the functional defect of SERCA3+/− platelets.**

To test whether rescue is specific for ADP, and may not be induced just by any weak agonist, we analyzed aggregation and secretion rescue induced by 5HT (another weak agonist and dense granule
cargo). At concentrations within the same range than ADP, 5HT did not induce aggregation (figure S3D), nor did it rescue aggregation (figure S3E), ATP secretion (figure S3F), αIIbβ3 activation or P-selectin exposure (data not shown) elicited by 40 mU/mL thrombin. These data, together with the defects induced by apyrase (flow adhesion, platelet aggregation and secretion) in control platelets, confirm that ADP is most likely the only agonist involved in SERCA3−/− platelet defect.

**SERCA3 ablation alters ADP dependent αIIbβ3 activation**

Platelet secretion is elicited by agonist-induced receptor activation, and αIIbβ3 integrin engagement (outside-in signaling). To examine a potential link between SERCA3-dependent ADP secretion and αIIbβ3, SERCA3−/− platelets were activated by thrombin in the presence of the monoclonal antibody JON/A, specific for the active form of mouse αIIbβ3,32 and analyzed by flow cytometry. SERCA3−/− platelets exhibited lower αIIbβ3 activation [but normal total level (figure S1)] than controls (figure 5A). ADP scavenging by apyrase almost completely abrogated αIIbβ3 activation in both control and SERCA3−/− platelets (figure 5A). Conversely addition of ADP rescued αIIbβ3 activation in both cases, confirming that the lower activation level of αIIbβ3 in SERCA3−/− platelets is due to reduced ADP secretion. Blockade of αIIbβ3 by the antibody Leo.H433 which prevents αIIbβ3 engagement, reduced secretion equally in both control and SERCA3−/− platelets, corresponding to αIIbβ3-dependent secretion, but the difference in secretion remained unchanged (figure 5B). Importantly, ADP addition rescued secretion in SERCA3−/− platelets (unchanged in control platelets) despite αIIbβ3 blockade. Thus the altered secretion in SERCA3−/− platelets is αIIbβ3-independent.

**SERCA3 ablation or inhibition alters Ca2+ signaling and dense granule secretion.**

SERCA3 and SERCA2b,34 regulate Ca2+ mobilization from intracellular stores, essential to platelet activation and secretion.35 Flow cytometry of unstirred Oregon-Green BAPTA1-AM loaded platelets pre-incubated with EGTA (no extracellular Ca2+ to avoid Ca2+ influx) and stimulated with thrombin showed a lower Ca2+ mobilization (50%, as measured by the area below the curve for 2 min) in
SERCA3−/− platelets compared to controls, at 40 mU/mL (figure 6A, left panel), but not at 100mU/mL (figure S4A). Ca2+ influx induced by extracellular Ca2+ (1 mM) was stronger in SERCA3−/− (30 to 40% increase as assessed by areas under curves) than control platelets (figures 6A,B and S4A). Interestingly, stimulation of SERCA3−/− platelets with thrombin in the presence of extra-cellular Ca2+ showed a similar fluorescence increase compared to control platelets, suggesting compensation of low Ca2+ mobilization by high Ca2+ influx (Fig 6A, right panel). Importantly, in the presence of apyrase, Ca2+ mobilization of control platelets at 40 mU/mL of thrombin was lowered to the level of SERCA3−/− platelets, unaffected by ADP scavenging (figure 6B). Conversely, addition of ADP to thrombin raised Ca2+ mobilization in SERCA3−/− platelets to the level of control platelets (figure 6B). In contrast, Ca2+ influx remained unaffected by apyrase pretreatment or after addition of ADP, for both control and SERCA3−/− platelets suggesting that, contrary to mobilization, influx is independent of ADP. These results are thus consistent with SERCA3-dependent Ca2+ mobilization in low agonist conditions being secondary to secreted ADP.

To confirm the role of SERCA3 and its catalytic activity, platelets were challenged with 2,5-di-(tert-butyl)-1,4-benzohydroquinone (tBHQ) specific for SERCA3.3 In control platelets, 10µM tBHQ (specific for SERCA3, since inducing Ca2+ mobilization in control but not in SERCA3−/− platelets, figure S4C) lowered thrombin-induced Ca2+ mobilization (figure 6C) comparatively to control (figure 6A). Ratios of areas under curves of wild type over SERCA3−/− Ca2+ mobilization dropped from 2 in absence of tBHQ to 1 in the presence of the antagonist. tBHQ elicited partial inhibition of thrombin-induced aggregation (figure 6E) and secretion (figure 6F) of SERCA3−/− platelets, that were both rescued by ADP. In contrast specific inhibition of SERCA2b with 200nM thapsigargin (figure S5A), which partially inhibited thrombin-induced Ca2+ mobilization in control platelets (presumably leaving SERCA3-dependent Ca2+ stores unaffected) but completely inhibited mobilization in SERCA3−/− platelets, indicating complete inhibition of SERCA2b (figure 6D). Importantly, specific inhibition of SERCA2b did not affect ATP release, contrary to tBHQ-mediated SERCA3 inhibition (figure 6F). Altogether these results thus demonstrate that catalytically active SERCA3 and not SERCA2b, is involved in dense granule secretion, through its Ca2+ pump activity and SERCA3-dependent Ca2+ stores.
**Ca\textsuperscript{2+} stores are defective in SERCA3\textsuperscript{-/-} platelets.**

SERCA3 depletion is expected to lead to depletion of SERCA3-dependent Ca\textsuperscript{2+} stores, and hence to altered Ca\textsuperscript{2+} mobilization. Platelets were subjected to ionomycin which permeabilizes inner membranes and empties Ca\textsuperscript{2+} stores, and to thapsigargin at high concentration (1µM) to prevent Ca\textsuperscript{2+} store re-uptake by both SERCA3 and SERCA2b (figure S4B). SERCA3\textsuperscript{-/-} platelets exhibited a weaker cytosolic Ca\textsuperscript{2+} release signal than control platelets (0.65 relative ratio assessed by areas under the curves) consistent with partially depleted Ca\textsuperscript{2+} stores in SERCA3\textsuperscript{-/-} platelets. Conversely, Ca\textsuperscript{2+} re-uptake was assessed in control and SERCA3\textsuperscript{-/-} platelets by Ca\textsuperscript{2+} mobilization triggered by thrombin, followed after 3 minutes of stimulation (allowing Ca\textsuperscript{2+} store refilling) by thapsigargin (1µM, inhibiting both SERCAs) to let stored Ca\textsuperscript{2+} "leak" into the cytosol (figure S5C). Thapsigargin triggered significantly less Ca\textsuperscript{2+} release in SERCA3\textsuperscript{-/-} platelets than in control platelets, consistent with less efficient Ca\textsuperscript{2+} re-uptake in SERCA3\textsuperscript{-/-} platelets than in controls, underlining the functional relevance of SERCA3. Both the lower levels of Ca\textsuperscript{2+} store release and the low Ca\textsuperscript{2+} store re-uptake in SERCA3\textsuperscript{-/-} platelets strongly suggest that SERCA3-dependent Ca\textsuperscript{2+} stores are involved, at least in part, in dense granule secretion.

**Discussion**

We have analyzed SERCA3\textsuperscript{-/-} mice and found a significant prolonged bleeding time as well as defective thrombosis *in vivo*. Functional assessment of SERCA3\textsuperscript{-/-} platelets *in vitro* has confirmed a defect in adhesion, thrombus formation over collagen, as well as aggregation elicited by either collagen or thrombin. This indicated that platelets were directly affected by SERCA3 ablation, and that the defect was independent of the stimulus pathway. Most importantly we found that the defect could be tracked down to a markedly reduced secretion as assessed by ATP measurement, and thus presumably reduced dense granule exocytosis. Importantly all affected functions in platelet SERCA3\textsuperscript{-/-}, including adhesion under flow, aggregation and secretion were reversed by exogenous ADP, adding more support to the idea that ablation of SERCA3 leads to an ADP secretion defect. Experiments conducted with 5HT
showed 1) that this weak agonist also stored in dense granules is defective to the same extent than ATP, confirming that the defect lies in the release mechanism of dense granules, 2) like ATP, it reaches normal secretion when ADP is added to thrombin but 3) unlike ADP it is unable to restore normal aggregation or secretion of SERCA3<sup>−/−</sup> platelets upon thrombin stimulation. This strengthens the hypothesis that SERCA3 depletion does affect release of a fraction of dense granules, and that this release is specifically dependent upon ADP co-stimulation, and not co-stimulation by just any weak agonist.

In addition this SERCA3-dependent secretion is independent of α<sub>IIbβ3</sub> engagement and only dependent on primary platelet activation. Importantly, aggregation to ADP of SERCA3<sup>−/−</sup> platelets was normal indicating that ADP-dependent activation pathways of platelets were not altered by SERCA3 ablation. Our results indicate also that SERCA3 ablation does not affect dense granules, as evidenced by normal mepacrine content, normal serotonin content or electron microscopy imaging. It follows that SERCA3 ablation is responsible for alteration of dense granule secretory rather than storage pathways.

Importantly pharmacological inhibition of SERCA3 but not of SERCA2b recapitulated the ADP secretory defect of SERCA3 genetic ablation, clearly showing a specific role for SERCA3 in secretion. Moreover, this demonstrates that SERCA3 catalytic activity is required for secretion, pointing to SERCA3-dependent Ca<sup>2+</sup> storage and/or signaling as involved in secretion regulation. Of note, when platelets were maximally stimulated with 2U/mL thrombin, SERCA3<sup>−/−</sup> platelets still exhibited a significant differential secretion compared with controls, whether assessed by ATP or 5HT release (figures S3B and S3C, respectively), and only compensated for by added ADP. This secretion resistance to strong stimulation argues in favor of a pool of dense granules present but not releasable in SERCA3-deleted platelets. This would thus be consistent with two physically and/or functionally separate ADP secretory pathways, possibly corresponding to distinct populations of dense granules, and/or to different exocytosis pathways, one involving SERCA3 (and its Ca<sup>2+</sup> stores) and not the other. An attractive hypothesis could be that this subpopulation of dense granules correspond to SERCA3-dependent Ca<sup>2+</sup>-stores, explaining the link between ADP release and SERCA3. However we found no co-localization
between SERCA3 and dense granules by confocal microscopy (data not shown): we conclude that
SERCA3-dependent Ca\(^{2+}\) stores are distinct from dense granules, but regulate exocytosis of a
subpopulation of dense granules, for example proximal to the plasma membrane, allowing early release
of ADP.
We noted that α-granule secretion, in absence of α\(_{IIb}\)β\(_3\) engagement, is almost completely dependent on
ADP release. This is consistent with an earlier report showing that dense granules are mobilized earlier
than α-granules,\(^{36}\) and with two recent reports showing that mouse models of Hermansky-Pudlak
syndrome, which are defective in dense granules exhibit, upon low agonist stimulation or laser-induced
injury in vivo, a defect in α-granule (and lysosome) secretion secondary to the lack of ADP
secretion.\(^{37,38}\) Moreover their observations of an autocrine ADP secretion,\(^{39}\) are consistent with our
contention that ADP secretion (here SERCA3-dependent) reinforces platelet activation by other agonists.
Interestingly there was an increased level of SOCE in SERCA3\(^{-/-}\) platelets, apparently compensating for
the low level of Ca\(^{2+}\) mobilization (such that the overall Ca\(^{2+}\) cytosolic rise upon agonist stimulation in
the presence of external Ca\(^{2+}\) was normal). This compensating SOCE is consistent with the low levels of
Ca\(^{2+}\) stores in SERCA3\(^{-/-}\) platelets, known to induce recruitment of STIM1 and STIM2 by Orai-1.\(^{40}\) Of
note, this sustained SOCE is not modulated by ADP scavenging or addition, strengthening the idea that
it is only driven by Ca\(^{2+}\) store depletion, and not via an ADP-dependent pathway. Thus SOCE appears
as a compensatory mechanism in the context of SERCA3 ablation (or inhibition), possibly explaining
the limited hemostasis impact on SERCA3\(^{-/-}\) mice. Interestingly Harper et al have shown that NC(K)X
Ca\(^{2+}\) exchangers drive initial SOCE upon Tg-induced Ca\(^{2+}\) stores depletion, triggering dense granule
secretion, which potentiated activation through ADP, ATP and 5HT pathways.\(^{41}\) While this observation
points to a link between Ca\(^{2+}\) regulation and an autocrine platelet activation amplification, it clearly acts
in a different manner, since involving NC(K)X and SOCE, and not being specific for ADP.
Importantly, tBHQ-mediated SERCA3 inhibition in human platelets leads to defective aggregation, ATP
secretion and α\(_{IIb}\)β\(_3\) activation (unpublished). Thus SERCA3 plays the same role in human and mouse
platelets.
Finally, SERCA3 depletion or inhibition appears to lead to defective SERCA3-dependent Ca\(^{2+}\) storing. This conclusion stems from several convergent observations: first, mobilization of Ca\(^{2+}\) from intracellular compartments was clearly affected in SERCA3\(^{−/−}\) platelets, independent of the agonist used. Second, SERCA3\(^{−/−}\) platelets exhibit stored Ca\(^{2+}\) levels lower than control platelets, as evidenced by experiments assessing release of total Ca\(^{2+}\) stores by ionomycin and Tg (figure S4B). Third, blockade by the SERCA3-specific pharmacological inhibitor tBHQ (leading to Ca\(^{2+}\) "leakage" from SERCA3-dependent Ca\(^{2+}\) stores), in conditions (10µM) where it did elicit Ca\(^{2+}\) release in the cytosol of control but not of SERCA3\(^{−/−}\) platelets, reproduced the defect in Ca\(^{2+}\) mobilization, as well as in secretion. This effect was SERCA3-specific since not observed with 200 nM Tg, specific for SERCA2b. One can thus conclude that a Ca\(^{2+}\) storage pool dependent on SERCA3 and not on SERCA2b seems to be required for a release of ADP, itself seemingly important to full platelet activation in conditions of low agonist concentration.

In conclusion, our data provide evidence that platelet activation seems to involve a release of ADP through a secretory pathway under the control of SERCA3-dependent Ca\(^{2+}\) stores, independently from \(α_{IIbβ3}\) integrin engagement. The link between SERCA3-dependent Ca\(^{2+}\) stores and SERCA3-dependent ADP stores remains to be established.

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Disclosure of conflicts-of-interest

The authors declare no conflict-of-interest.

References


Figure legends

Figure 1

Characterization of hemostasis and in vivo thrombosis in SERCA3+/− mice

(A) Western blot of SERCAs in mouse platelets. Control (WT) and SERCA3+/− mouse platelets were collected and solubilized and subjected to SDS-PAGE, prior to transfer to nitrocellulose and detection by antibodies specific for SERCA3 or SERCA2b. After addition of a secondary antibody coupled to horse radish peroxidase, bands were revealed by chemiluminescence. The 14-3-3ζ adaptor was used as an internal standard for normalization. Note the absence of SERCA3 in SERCA3+/− platelets, and the same levels of SERCA2b in both control and SERCA3+/− platelets. (B) Tail bleeding time. Tail bleeding was performed as indicated in Methods and bleeding time assessed both on control (WT) and SERCA3+/− mice. Results are presented as mean ± SEM, using the Student’s t test; *** P< 0.001. (C) Rebleeding was assessed for one minute following initial bleeding arrest. A total of 18 control and 21 SERCA3+/− mice were used. (D) Transmission electron microscopy of resting control and SERCA3+/− platelets. Platelets were subjected to standard transmission electron microscopy. Upper panel, control (WT), lower panel, SERCA3+/− platelets. The scale bar (0.5µm) is shown in the lower left corner of the WT panel. (E) Kinetics of in vivo ferric chloride-induced thrombosis of mesenteric vessels. Venules (v) or arterioles (a) are shown by fluorescence microscopy (limits outlined with white dashed lines), thrombi being visualized by rhodamine 6G-labeled platelets. Images at 0, 30, and 60 minutes are shown. (F) Quantification of thrombus formation. Time to occlusion was noted for 18 control (WT, closed circles) and 21 SERCA3+/− (open circles) mice up to 60 minutes, the maximal time assessed. Results were analyzed using the 1-way ANOVA followed by Tukey's multiple comparison test; *** P<0.001. (G) Quantification of emboli. The number of emboli shedding from thrombi was assessed for 60 minutes, both in venules and arterioles of control and SERCA3+/− mice.

Figure 2

In vitro thrombus formation of SERCA3+/− platelets compared to controls
Rhodamine 6G-labeled platelets were injected into capillaries precoated with collagen (50 µg/mL) at low (150 s\(^{-1}\); A) or at high (1200 s\(^{-1}\); C) shear rates in absence or in presence of both apyrase (5U/mL) and indomethacin (5µM), noted "Apy-Indo". Images show platelet adhesion and thrombus formation after 3 minutes of perfusion, and plots (B, D) represent the quantification of platelet adhesion, expressed as covered surface area relative to controls given as 100%. Absence or presence of Apy-Indo is noted "-" or "+" below plots. Data were calculated using the 1-way ANOVA followed by Tukey's multiple comparison test; ns, not significant; *** P<0.001.

**Figure 3**

**Aggregation of washed platelets from control or SERCA3\(^{-/-}\) mice**

(A) Washed platelets from controls (WT, black line) or SERCA3\(^{-/-}\) (gray line) mice were stimulated with collagen (0.8 or 1.2 µg/mL) or (B), with thrombin (40 and 100 mU/mL) and recorded for aggregation for 3 minutes. Aggregation intensities are expressed as percent of light transmitted, 100% corresponding to buffer alone. Note the low aggregation rate of SERCA3\(^{-/-}\) platelets at 0.8 µg/mL of collagen and 40mU/mL thrombin. These tracings are representative of 5 experiments. (C) Aggregation induced by collagen (0.8 µg/mL) or thrombin (40mU/mL) of control (WT) and SERCA3\(^{-/-}\) platelets was carried out in the presence of apyrase (5 U/mL, noted "Apy"). (D) Aggregation rescue was conducted on control and SERCA3\(^{-/-}\) washed platelets by addition of 10µM ADP following stimulation by either collagen (0.8µg/mL) or thrombin (40mU/mL). These tracings are representative of 3 experiments.

**Figure 4**

**Platelet secretion of washed platelets from control or SERCA3\(^{-/-}\) mice**

(A) Dense granule secretion from platelets aggregated in the presence of collagen (0.8 or 1.2 µg/mL) or (B), thrombin (40 or 100 mU/mL), with the addition (+) or not (-) of 10µM ADP, was assessed by measuring ATP release in pmoles (calculated for 10\(^{7}\) platelets) in control (WT, black bars) and SERCA3\(^{-/-}\) (white bars) platelets. A total of 3 experiments was conducted and presented as mean ± SEM,
using the 1-way ANOVA followed by Tukey's multiple comparison test; ns, not significant; *** $p < 0.001$. (C) The expression of the $\alpha$-granule membrane marker P-selectin following thrombin platelet stimulation was assessed by flow cytometry on control (black bars) or SERCA3$^{-/-}$ (white bars) platelets. The same experiment was conducted in the presence of apyrase (5U/mL, noted "+Apy") or added ADP (10 $\mu$M, noted "+ADP") on control (WT, closed squares, dashed line) or SERCA3$^{-/-}$ (open squares, dashed line) platelets. Data presented are the means of 3 separate experiments in duplicates, as means ± SEM, as assessed by the 1-way ANOVA followed by Tukey's multiple comparison test; ns, not significant; ** $P<0.01$.

**Figure 5**

Assessment of $\alpha_{IIb}\beta_3$ activation and engagement in washed platelets from control or SERCA3$^{-/-}$ mice and aggregation to ADP.

(A) Quantitation of activated $\alpha_{IIb}\beta_3$ integrin at the surface of washed platelets was assessed by flow cytometry by binding of the specific monoclonal antibody JON/A to control (black bars) or SERCA3$^{-/-}$ (white bars) platelets upon activation with thrombin at 40 or 100 mU/mL. The same experiments were conducted in the presence of 5U/mL apyrase (noted "+ Apy"), or after addition of 10$\mu$M ADP ("+ADP"). Statistical significance was established with the 1-way ANOVA followed by Tukey's multiple comparison test; ns, not significant; **$P<0.01$. (B) Assessment of the role of $\alpha_{IIb}\beta_3$ engagement in dense granule secretion in SERCA3$^{-/-}$ platelets. Platelets stimulated with either 40 or 100 mU/mL of thrombin were subjected to aggregation, in the absence (-) or the presence (+) of the blocking monoclonal antibody Leo.H4 (20 $\mu$g/mL) specific for mouse $\alpha_{IIb}\beta_3$, as well as in the absence (-) or the presence (+) of 10 $\mu$M ADP. Secretion was assessed by ATP measurement in the supernatant. Using WT as control, statistical significance was established with the 1-way ANOVA followed by Tukey's multiple comparison test; ns, not significant; ** $P<0.01$; *** $P<0.001$. (C) Aggregation to ADP was assessed at 0.25, 0.5, 1, 2.5, 5, 10 $\mu$M in control (WT) or SERCA3$^{-/-}$ platelet rich plasma.
Figure 6

Effect of SERCA3 deletion or pharmacological inhibition on Ca\(^{2+}\) mobilization, Ca\(^{2+}\) influx, aggregation and secretion in washed platelets.

(A) Ca\(^{2+}\) mobilization was assessed in unstimulated control (WT, black tracings) and SERCA3\(^{-/-}\) (gray tracings) platelets pre-incubated with the cytosolic Ca\(^{2+}\) fluorescent probe Oregon-Green-BAPTA-AM after stimulation with 40 mU/mL thrombin ("Thr") by flow cytometry in conditions of no external Ca\(^{2+}\) (1mM EGTA). Ca\(^{2+}\) influx was assessed after 4 minutes by addition of 1 mM CaCl\(_2\) ("Ca\(^{2+}\)“). Global Ca\(^{2+}\) signaling was also assessed by addition of 1 mM Ca\(^{2+}\) together with thrombin ("Thr + Ca\(^{2+}\)“) (right tracing). Data are expressed as nM Ca\(^{2+}\), as calculated from calibration experiments (see Supplemental Data). (B) Ca\(^{2+}\) mobilization was assessed in the same conditions than in (A), but in the presence of apyrase (5U/mL) ("+ Apy") or ADP (1µM) ("+ ADP"). (C) Ca\(^{2+}\) mobilization by 40 mU/mL thrombin was assessed after pre-incubation with the SERCA3-specific inhibitor tBHQ (10µM), or (D) with thapsigargin (Tg) at a concentration affecting only SERCA2b (200nM, see figure S5A). (E) Maximal Ca\(^{2+}\) mobilization and Ca\(^{2+}\) influx from experiments in A (stimulation with 40 mU/mL thrombin in the presence of 100 µM EGTA, and Ca\(^{2+}\) influx after CaCl\(_2\) (300µM) addition), and in B (same as in A, but in the presence of 5U/mL apyrase (Thr + Apy), or of 10µM ADP (Thr + ADP)). Values were calculated after subtraction of unstimulated Ca\(^{2+}\) level (ΔCa\(^{2+}\) nM). Data presented are means ± SEM, n = 3, using the 1-way ANOVA followed by Tukey's multiple comparison test: ns, not significant; ** P<0.01; *** P<0.001.

(F) Washed control platelets were pre-incubated with DMSO (control), or tBHQ 10µM (tBHQ), or tBHQ and ADP (10µM each) for 4 minutes prior to addition of 40 mU/mL thrombin (Thr). (G) Washed control (WT, black bars) or SERCA3\(^{-/-}\) (white bars) platelets were pre-incubated with either buffer alone "-", tBHQ (10µM) or Tg (200nM), then added either buffer ("0") , thrombin 40mU/mL, or thrombin and 10µM ADP ("+ADP") and incubated further for 3 minutes. ATP secretion was then measured in supernatants. Data presented are means ± SEM, n = 3, using the 1-way ANOVA followed by Tukey's multiple comparison test: ns, not significant; *** P<0.001.
Table 1. Blood cell analysis of SERCA3⁻/⁻ mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>SERCA3⁻/⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes (10⁹/L)</td>
<td>5.63 ± 0.30</td>
<td>6.35 ± 0.33</td>
</tr>
<tr>
<td>Red blood cells (10¹²/L)</td>
<td>10.84 ± 0.16</td>
<td>10.33 ± 0.25</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>48.24 ± 0.7</td>
<td>44.57 ± 1.15</td>
</tr>
<tr>
<td>Platelets (10⁹/L)</td>
<td>914.5 ± 0.33</td>
<td>778.2 ± 0.31</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>4.99 ± 0.04</td>
<td>5.82 ± 0.08</td>
</tr>
</tbody>
</table>

Cell counts in whole blood were determined by an automated cell counter (see Methods), and values are expressed as means ± SEM. WT: wild type, SERCA3⁻/⁻, knock out mice. Units are shown in the right column in parentheses. MPV: mean platelet volume.
Figure 1

A

B

C

D

E

F

G

SERCA3

SERCA2b

14-3-3 ζ

WT SERCA3−/−

WT

WT

Occluded

0 min

30 min

0 min

30 min

60 min

Occluded

0 min

30 min

60 min

WT

SERCA3−/−

WT

SERCA3−/−

WT

SERCA3−/−

Venule Arteriole

Venule Arteriole

0

10

20

30

40

50

60

> 60

Occlusion time (min)

number of emboli per mouse

Venule Arteriole

0 1 2 3 4 5

WT SERCA3−/−

WT SERCA3−/−

WT SERCA3−/−

WT SERCA3−/−

WT SERCA3−/−
Figure 2

A

150 s⁻¹

WT

SERCA3⁻/⁻

Apyn-Indo +

B

**WT**  
**SERCA3⁻/⁻**

Relative adhesion (%)  
0 20 60 100 80 40 120

Apyn-Indo - + ***** ns

C

1200 s⁻¹

WT

SERCA3⁻/⁻

Apyn-Indo +

D

**WT**  
**SERCA3⁻/⁻**

Relative adhesion (%)  
0 20 60 100 80 40 120

Apyn-Indo - + ***** ns

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Figure 4

A

WT ATP release (pmole)

0 10 20 30 40 50

+ADP

Collagen (µg/mL)

B

WT ATP release (pmole)

0 10 20 30 40

+ADP

Thrombin (mU/mL)

C

WT Relative P-sel expression

0 40 100

+ Apy + Apy + ADP + ADP
Figure 5

A

![Graph A]

Relative αIIbβ3 activation

WT
SERCA3−/−

Thrombin (mU/mL)

B

![Graph B]

ATP release (pmole)

WT
SERCA3−/−

Leo-H4
ADP

Thrombin (mU/mL)

C

![Graph C]

ADP [µM]

WT
SERCA3−/−

Aggregation (%)
**Figure 6**

A

- **WT**
- **SERCA3**

Ca\(^{2+}\) concentration (nM) over time (1 min)

B

+ **Apy**
+ **ADP**

Ca\(^{2+}\) concentration (nM) over time (1 min)

C

- **WT**
- **SERCA3**

TBHQ effect on Ca\(^{2+}\) concentration (nM) over time (1 min)

D

- **WT**
- **SERCA3**

TBHQ effect on Ca\(^{2+}\) concentration (nM) over time (1 min)

E

- **WT**
- **SERCA3**

Ca\(^{2+}\) mobilization and influx

F

- **Aggregation (%)**
- **Thr**
- **TBHQ**
- **tBHQ + ADP**
- **Control**

G

- **WT**
- **SERCA3**

ATP release (pmole) over thrombin concentration (mU/mL)

Legend:

- ***
- **
- ns

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Full activation of mouse platelets requires an ADP secretion pathway regulated by SERCA3 ATPase-dependent calcium stores

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