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**ADP receptor P2Y12 is the capstone of the cross-talk between Ca<sup>2+</sup> mobilization pathways dependent on ATPases SERCA3 and SERCA2b in platelet.**

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**Running title**

P2Y12 & SERCA3/SERCA2b in platelet secretion

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## **Non-standard abbreviations and acronyms**

ARC: ARC69931MX

IP3: 1,4,5, inositol trisphosphate

MRS: MRS2179

NAADP: Nicotinic Acid Adenosine Dinucleotide Phosphate

P2Y1: P2-class purinergic receptor type Y subtype 1

P2Y12: P2-class purinergic receptor type Y subtype 12

PPACK: phenylalanyl-L-prolyl-L-arginine chloromethyl ketone

SERCA2b: Sarco-Endoplasmic Reticulum Calcium ATPase type 2b

SERCA3: Sarco-Endoplasmic Reticulum Calcium ATPase type 3

Tg: Thapsigargin

## Essentials

- NAADP mobilizes SERCA3-ATPase  $\text{Ca}^{2+}$  stores initiating early ADP secretion in activated platelets
- The role of P2Y1 and P2Y12 ADP receptors was studied
- SERCA3-dependent ADP early release activates P2Y12 (and not P2Y1) and SERCA2b-dependent release
- Full platelet secretion relies on coupling of SERCA3 and SERCA2b stores mobilization via P2Y12

## Abstract

Background: Blood platelet  $\text{Ca}^{2+}$  stores are regulated by two  $\text{Ca}^{2+}$ -ATPases (SERCA2b and SERCA3). Upon thrombin stimulation, NAADP mobilizes SERCA3-dependent stores, inducing early ADP secretion, potentiating later SERCA2b-dependent secretion.

Objectives: Our aim was to identify which ADP P2 purinergic receptor (P2Y1 and/or P2Y12) is(are) involved in the amplification of platelet secretion dependent on the SERCA3-dependent  $\text{Ca}^{2+}$  mobilization pathway (SERCA3 stores mobilization) as triggered by low concentration of thrombin.

Methods: The study used the pharmacological antagonists MRS2719 and AR-C69931MX, of respectively, the P2Y1 and the P2Y12, as well as *Serca3*<sup>-/-</sup> mice and mice exhibiting platelet lineage-specific inactivation of the P2Y1 or the P2Y12 genes.

Results: We found that in mouse platelets, pharmacological blockade or gene inactivation of P2Y12 but not of P2Y1 led to a marked inhibition of ADP secretion after platelet stimulation with low concentration of thrombin. Likewise, in human platelets, pharmacological inhibition of P2Y12 but not of P2Y1 alters amplification of thrombin-elicited secretion through SERCA2b stores mobilization. Finally, we show that early SERCA3 stores secretion of ADP is a dense granule secretion, based on parallel ATP and serotonin early secretion. Furthermore, early secretion involves a single granule, based on the amount of ATP released.

Conclusion: Altogether these results show that upon low concentration of thrombin SERCA3- and SERCA2b-dependent  $\text{Ca}^{2+}$  mobilization pathways cross-talk via ADP and activation of the P2Y12 and not the P2Y1 ADP receptor. The relevance in hemostasis of the coupling of the SERCA3 and the SERCA2b pathways is reviewed.

## Keywords

Blood platelets, P2 purinergic receptor (P2Y12, P2Y1), ADP, SERCA3, NAADP

## Introduction

Calcium ( $\text{Ca}^{2+}$ ) is central in cellular signaling, and requires fine-tuned regulation to control its cytosolic concentration and to shape signals.[1-3] Upon stimulation  $\text{Ca}^{2+}$  is imported from the extracellular medium (influx), or mobilized from internal stores (mobilization), leading to a cytosolic  $\text{Ca}^{2+}$  rise. The rise may be global or localized, and is counterbalanced by either export through the plasma membrane, or recapture into internal stores or mitochondria. The major actors in down-regulation of cytosolic  $\text{Ca}^{2+}$  are energy-dependent membrane ATPases which are able to drive  $\text{Ca}^{2+}$  export against a concentration gradient from 100 nmol/L in the cytosol to the mmol/L range in the extracellular milieu or in internal stores (mainly the endoplasmic reticulum, ER). PMCA (for Plasma Membrane Calcium ATPases) expel  $\text{Ca}^{2+}$  in the extracellular milieu, while SERCA (Sarco-Endoplasmic Reticulum Calcium ATPases) recapture cytosolic  $\text{Ca}^{2+}$  into internal stores. In platelets, mobilization from internal stores has been shown to regulate such primordial functions as adhesion and aggregation.[4, 5][6] The SERCA family is encoded by 3 genes (*ATP2A1*, *ATP2A2* and *ATP2A3*), which produce each 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.[7-10] SERCA maintain a  $\text{Ca}^{2+}$  concentration gradient between the cytosol (100 nmol/L) and the Endoplasmic Reticulum (ER) (1 mmol/L).[11] However, SERCA redundancy may not be explained only by  $\text{Ca}^{2+}$  gradient maintenance, but also by functional differences. While SERCA enzymes exhibit comparable structures, they possess also different activities including:  $\text{Ca}^{2+}$  affinity higher for SERCA2b ( $K_{1/2} \sim 0.27 \mu\text{mol/Lol/L}$ ) than for SERCA3 (1  $\mu\text{mol/Lol/L}$ ), but  $\text{Ca}^{2+}$  uptake lower for SERCA2b (7 nmol/min/mg) than SERCA3 (21 nmol/min/mg).[12, 13] Additionally, in platelets, subcellular localization studies by immuno-electron microscopy have suggested a differential topology for SERCA, central for SERCA2b and peripheral for SERCA3.[14, 15] Functional studies have pointed to involvement of SERCA3 in store operated  $\text{Ca}^{2+}$  entry (SOCE) via association with STIM1, a  $\text{Ca}^{2+}$  sensor participating in  $\text{Ca}^{2+}$  stores replenishing,[16] as well as with SERCA3 functional association with acidic  $\text{Ca}^{2+}$  stores.[17] Recently, we have provided evidence that the SERCA3-dependent  $\text{Ca}^{2+}$  mobilization pathway

impacts the physiology of haemostasis as well as its pathophysiology: 1) SERCA3<sup>-/-</sup> mice exhibit prolonged tail-clip bleeding time, mainly associated with rebleeding, showing that the SERCA3-dependent Ca<sup>2+</sup> pathway is involved in haemostasis; 2) thrombosis of mesenteric vessel assay (FeCl<sub>3</sub>) induces unstable thrombi with emboli, indicating the SERCA3 pathway is involved in thrombus stabilization; [2] these effects have been correlated to the reduced activation of SERCA3<sup>-/-</sup> platelets stimulated with low concentration agonists such as thrombin, as shown by 3) diminished aggregation, consistent with a role for SERCA3-dependent Ca<sup>2+</sup> mobilization in  $\alpha$ IIb $\beta$ 3 integrin-mediated platelet-platelet interaction; and 4) lower secretion. [2, 18] We further established that an initial mobilization of Ca<sup>2+</sup> from internal stores specifically dependent on the Ca<sup>2+</sup> pump SERCA3, and not on Ca<sup>2+</sup> stores dependent on SERCA2b was necessary to obtain full platelet activation in these conditions. [2] Moreover we found that SERCA3 stores and SERCA2b stores were mobilized differentially since SERCA3 stores were specifically mobilized by the second messenger Nicotinic-Acid-Adenine-Dinucleotide-Phosphate (NAADP), as opposed to SERCA2b stores, mobilized only by IP<sub>3</sub>. [18] Additionally, SERCA3 store mobilization drove an initial ADP secretion responsible for co-stimulation with thrombin of SERCA2b store mobilization and secretion amplification, dependent on thromboxane A<sub>2</sub>. We also demonstrated a similar mechanism in human platelets [19]. Here, we keep characterizing the molecular mechanism underlying platelet Ca<sup>2+</sup> mobilization of SERCA2b and SERCA3 stores by providing evidence that in both mouse and human platelets, the P2Y<sub>12</sub>, but not the P2Y<sub>1</sub> ADP receptor, is engaged by ADP secreted via mobilization of SERCA3 stores, driving secretion via the mobilization of SERCA2b stores.

## Methods

### Materials

Adenosine 5'-diphosphate (ADP) was obtained from Chrono-log Corp (Havertown, PA, USA). ARC69931MX tetrasodium salt, Apyrase from potatoes (grade VII), bovine thrombin, MRS2179 ammonium salt hydrate, SERCA inhibitor; thapsigargin (Tg) are from Sigma (St Louis, MO, USA). The d-Phe-Pro-Arg chloromethylketone dihydrochloride (PPACK) and prostaglandin E<sub>1</sub> were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). BAPTA1-AM Oregon Green 488 was

purchased from Molecular Probes (Eugene, OR, USA). The NAADP antagonist, Ned-19 was from Tocris (Bristol, UK). The ATP determination kit was from Fisher Scientific (Illkirch, France), the ADP and the serotonin detection kits were from Abcam (Cambridge, UK). PAR1-AP (SFLLRN) and PAR4-AP (AYPGKF) were from BACHEM (Bubendorf, Switzerland).

## **Mice**

*Serca3*<sup>-/-</sup> mice were originally generated by Dr G.E. Shull (University of Cincinnati, OH),<sup>[20]</sup> and swiss black *Serca3*<sup>-/-</sup> mice were crossed with C57BL/6 mice and provided by Dr P. Gilon (University of Louvain, Belgium) with the authorization of Dr G.E. Shull. Transferred *Serca3*<sup>+/-</sup> heterozygous mice were obtained with the help of Charles River Laboratories (Saint Germain-Nuelles, France) and were intercrossed. Wild-type (WT) and *Serca3*<sup>-/-</sup> homozygote mice were detected by PCR, using published oligonucleotide primers.<sup>21</sup> Then, homozygote wild-type (WT) and *Serca3*<sup>-/-</sup> mice were kept apart and genotypes of breeding couples were verified every six months. C57BL/6-inbred *Platelet factor 4 (Pf4)-Cre*<sup>+</sup> mice [C57BL/6-Tg(*Pf4-icre*)Q3Rsko/J; The Jackson Laboratory] originally created by the Skoda's laboratory,<sup>[21]</sup> were a generous gift from Dr M-P Gratacap, U1048 Inserm, Toulouse, France. These mice are transgenics harboring a Cre recombinase gene driven by the platelet-lineage-specific promoter PF4, expressing the recombinase only in the platelet-megakaryocyte lineage. They were used in this study as controls for *PF4-P2Y1*<sup>-/-</sup> and *PF4-P2Y12*<sup>-/-</sup> knockout mice. *PF4-P2Y1*<sup>-/-</sup> and *PF4-P2Y12*<sup>-/-</sup> knockout mice exhibiting deletion of the P2Y1 or the P2Y12 receptor exclusively in the megakaryocytic lineage were generated by crossing of recombinant mice with floxed alleles (*P2Y1*<sup>flox/flox</sup> and *P2Y12*<sup>flox/flox</sup> mice) with C57BL/6-inbred *Platelet factor 4 (Pf4)-Cre*<sup>+</sup> mice [C57BL/6-Tg(*Pf4-icre*)Q3Rsko/J; The Jackson Laboratory]. Genotyping was performed on mouse tail DNA using a polymerase chain reaction (PCR) amplification method.(Hechler et al, in preparation).

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-Saclay (APAFIS#14745-20180418165532119 v3).

## **Blood collection and mouse platelet preparation**

Blood was collected by cardiac puncture of anesthetized (intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg)) mice and was added 160  $\mu\text{mol/L}$  D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) and 10% (vol/vol) ACD-C buffer (124 mmol/L sodium citrate, 130 mmol/L citric acid, 110 mmol/L dextrose, pH6.5). Washed platelets were isolated by centrifugation as previously described.[2]

### ***Blood collection and human platelet preparation***

In accordance with the declaration of Helsinki, all healthy voluntary donors (men and women), gave written informed consent before blood collection. Human blood was collected in 10% (vol/vol) acid citric-citrate-dextrose A (ACD-A) buffer. Platelet-rich plasma (PRP) was obtained by centrifugation (120g for 15 minutes at 20°C). Washed platelets were prepared as previously described.[2] Briefly, platelets were isolated from PRP (10% (vol/vol) ACD-A, 100 mU/mL apyrase and 1  $\mu\text{mol/Lol/L}$  prostaglandin E1 by centrifugation (1200g for 12 minutes at 20°C) and washed 2 times with wash buffer in presence of apyrase (100 mU/mL) and prostaglandin E1 (1  $\mu\text{mol/Lol/L}$ ) to minimize platelet activation. Platelets were adjusted to  $3 \times 10^8$  platelets/mL in calcium free Tyrode's buffer.

### ***Platelet aggregation***

Platelet aggregation was carried out as previously described,[2] with washed platelets stimulated with thrombin. Light transmission was measured through the stirred suspension of platelets ( $3 \times 10^8$  platelets/mL) during 3 minutes using a Chronolog aggregometer (Chrono-Log Corporation, USA).

### ***Platelet dense granule secretion***

Dense granule secretion was quantified by assessment of ATP release using an ATP determination kit (Molecular Probes, Saint-Aubin, France), as previously described.[2] All experiments were run with triplicate samples. Serotonin secretion was assessed in platelet supernatants with the serotonin ELISA kit (Abcam, Cambridge, UK).

### ***Assay of cytosolic calcium by flow cytometry***

Mouse or human platelets ( $3 \times 10^8$  platelets/mL) were washed as indicated, resuspended in  $\text{Ca}^{2+}$  free (EGTA 0.1 mmol/L) Tyrode's buffer and incubated at room temperature for 30 to 45 minutes with 1  $\mu\text{mol/L}$  BAPTA-Oregon green 488, platelets were then diluted to  $6 \times 10^5$  platelets/mL in  $\text{Ca}^{2+}$  free Tyrode's buffer and  $\text{Ca}^{2+}$  mobilization was analyzed using an Accuri C6 flow cytometer (BD Biosciences; Le Pont de Claix, France). Flow cytometer was setup to low speed (10 $\mu\text{L/min}$ ) in order to analyze approximately 100 platelets/sec. Platelets were activated with 5  $\mu\text{L}$  agonists by gentle stirring with the pipet tip. Basal fluorescence before activation was arbitrarily set as 1 and variation of fluorescence intensity ratio over basal fluorescence represented evolution of  $\text{Ca}^{2+}$  concentration.  $\text{Ca}^{2+}$  mobilization was quantitated by area under the curve for 1 min after stimulation and presented as arbitrary units (A.U.).

### ***Statistical analysis and data presentation***

Analyses of statistical significance were performed with GraphPad Prism software (version 7.04; GraphPad, Inc, San Diego, CA). Normal data distribution for platelet secretions and  $\text{Ca}^{2+}$  mobilization in response to thrombin was observed in previous studies[2, 16, 22] and in the present study, and was assumed so all along the study. Depending on the conditions, values were analyzed with 1-way, 2-way or 3-way ANOVA or mixed-effects analysis (for groups with different sizes) followed by Tukey's pair wise test or Bonferroni's multiple comparisons test, as indicated in figure legends, and presented *P* values have been adjusted for multiple tests correction within experiments, and have not been adjusted across tests. Results are presented either as graphs (mainly dot plots with means and S.E.M), or as representative images or tracings that were selected to illustrate data presented in the corresponding graph.

## **Results**

### **Pharmacological inhibition of the ADP receptor P2Y12, but not P2Y1, alters thrombin-stimulated SERCA2b-dependent $\text{Ca}^{2+}$ mobilization and secretion**

To better understand how ADP released by mobilization of SERCA3-dependent  $\text{Ca}^{2+}$  stores ("SERCA3 stores") stimulates secretion driven by mobilization of SERCA2b stores, we asked which of the P2Y1 and/or the P2Y12 ADP receptor is(are) activated. The effect of pharmacological inhibition of the ADP receptors on the

SERCA3-dependent activation of platelets was assessed using the P2Y1 and P2Y12 antagonists, MRS2179 (MRS) and ARC69931MX (ARC), respectively, the efficacy of which was checked by  $\text{Ca}^{2+}$  mobilization by ADP (Supplemental Figure 1). First, thrombin-elicited  $\text{Ca}^{2+}$  mobilization in WT platelets as assessed by flow cytometry was not significantly diminished in the presence of the P2Y1 antagonist MRS. In contrast, the P2Y12 antagonist ARC induced a marked and significant reduction in  $\text{Ca}^{2+}$  mobilization, almost paralleling  $\text{Ca}^{2+}$  mobilization in *Serca3*<sup>-/-</sup> platelets or in WT platelets treated with the ADP scavenger apyrase (Figure 1). Addition of MRS to ARC did not reinforce the antagonistic effect of ARC.

Most importantly, the secretion kinetics of WT platelets stimulated with thrombin was not affected by either ADP receptor antagonist at 5 s, confirming no role for ADP or its receptors in initial secretion driven by SERCA3 stores mobilization. Accordingly, *Serca3*<sup>-/-</sup> platelets did not secrete within this time frame (Figure 2A). At 10 s and thereafter, the P2Y12 antagonist ARC did strongly inhibit secretion, while MRS, the P2Y1 antagonist did show very little inhibition if any (Figure 2B–D). The inhibitory effect of ARC was not increased by addition of MRS. These results suggest that P2Y12, but not P2Y1, is involved in delayed but not in early secretion induced by the thrombin-stimulated SERCA3 store mobilization.

We have previously shown that exogenous ADP added simultaneously to other agonists, restored defective  $\text{Ca}^{2+}$  mobilization and secretion in *Serca3*<sup>-/-</sup> platelets, via an IP3-SERCA2b pathway.[2, 18] To verify that P2Y12 was the actual ADP receptor involved, ADP was added to thrombin, and secretion was assessed in the presence of the ADP receptor antagonists. In the presence of the P2Y1 antagonist MRS, addition of ADP (10  $\mu\text{mol/L}$ ) to thrombin did restore secretion in *Serca3*<sup>-/-</sup> platelets almost to the same level than in WT platelets (Figure 2E & 2F), consistent with P2Y1 not being the principal ADP receptor involved. In contrast, no restoration of secretion by addition of ADP to thrombin was observed in the presence of the P2Y12 antagonist ARC, either in WT or *Serca3*<sup>-/-</sup> platelets (Figure 2E & 2F). This result strongly argues in favor of P2Y12 being the main ADP receptor triggered by early ADP released by the SERCA3 stores mobilization.

**Genetic inactivation of the ADP receptor P2Y12 alters thrombin-stimulated cross-talk between SERCA3 and SERCA2b-dependent  $\text{Ca}^{2+}$  mobilization pathway.**

To confirm the role of P2Y12 we examined thrombin or PAR4-AP stimulation of platelets from *P2Y1<sup>-/-</sup>*;PF4Cre (heretofore noted *P2Y1<sup>-/-</sup>*) and *P2Y12<sup>-/-</sup>*;PF4Cre mice (*P2Y12<sup>-/-</sup>*) in which the gene of the corresponding ADP receptor was inactivated in the platelet-megakaryocyte lineage (see Methods).  $\text{Ca}^{2+}$  mobilization in thrombin-stimulated *P2Y1<sup>-/-</sup>* platelets was only minimally altered, contrary to *P2Y12<sup>-/-</sup>* platelets where  $\text{Ca}^{2+}$  mobilization was severely diminished (Figure 3A & 3B). Moreover, pretreatment with the NAADP antagonist Ned19 inhibited thrombin-induced  $\text{Ca}^{2+}$  mobilization in *P2Y1<sup>-/-</sup>* platelets to the same extent than WT platelets (approximately 40%), and exhibited also a strong inhibition on *P2Y12<sup>-/-</sup>* platelets (Figure 3C). This latter result suggests that diminished mobilization in *P2Y12<sup>-/-</sup>* platelets in response to thrombin is not due to alteration of SERCA3 store mobilization, and is thus likely due to inhibition of the SERCA2b pathway. Secretion kinetics confirmed that while early secretion at 5s, only dependent on the SERCA3 store mobilization, was comparable to WT, both in *P2Y1<sup>-/-</sup>* as well as in *P2Y12<sup>-/-</sup>* platelets, no secretion inhibition was observed at later time points with *P2Y1<sup>-/-</sup>* (Figure 3D). In contrast, beyond 10 s, *P2Y12<sup>-/-</sup>* platelets exhibited very low secretion. Similarly, early serotonin secretion at 5 s was not impaired in *P2Y12<sup>-/-</sup>* platelets, but delayed serotonin secretion was completely blocked (Figure 3E). These results suggest that while P2Y1 is not involved in either SERCA3- or SERCA2b-dependent phases of thrombin-stimulated secretion, P2Y12, though not involved in the initial SERCA3-dependent phase of secretion, is essential to the SERCA2b-dependent phase of secretion.

Examination of the respective effects of Ned-19 which blocks SERCA3 stores mobilization and thapsigargin (Tg), an inhibitor of SERCA2b which leads to the emptying of SERCA2b-dependent  $\text{Ca}^{2+}$  stores, showed that upon 3 minutes of thrombin stimulation, Ned-19-pretreated *P2Y12<sup>-/-</sup>* platelets exhibited virtually no secretion compared to Ned-19-pretreated WT platelets (Figure 3F). In contrast, Tg inhibited about 50% of ATP secretion in WT platelets, but did not further affect *P2Y12<sup>-/-</sup>* platelet secretion, reaching the same level than Tg-inhibited WT platelets. Our results thus confirm that upon low thrombin stimulation, P2Y12 is not involved in the initial SERCA3-dependent secretion, but drives the secondary SERCA2b-dependent secretion. P2Y1 does not exhibit any role in the SERCA3/SERCA2b secretory mechanism stimulated by low concentration of thrombin.

## **P2Y12 also controls the SERCA2b-dependent secretion amplification step in human platelets**

We next examined the role of the ADP receptor P2Y12 in  $\text{Ca}^{2+}$  mobilization induced by thrombin in human platelets. In Figure 4, upon thrombin stimulation of platelets, inhibition of P2Y12 by ARC, scavenging ADP with apyrase, or both, led to a marked diminution of  $\text{Ca}^{2+}$  mobilization. Ned-19 and the blocking of SERCA3 store mobilization lowered  $\text{Ca}^{2+}$  mobilization within the same range (60% inhibition) of ARC or apyrase, and addition of either agent to Ned-19 did not show any additive effect (Figure 4H). Thus, global  $\text{Ca}^{2+}$  mobilization by thrombin involves SERCA3 stores mobilization, secreted ADP and P2Y12 but not P2Y1. Similar results were obtained for serotonin (Supplemental Figure 2A-C)

Next, we examined the role of P2Y12 in secretion elicited by thrombin. Secretion assessment upon thrombin stimulation (40mU/mL) of human platelets showed that inhibition of P2Y12 by ARC did not affect secretion till 3 s, after which, it almost completely blunted secretion till 3 minutes (Figure 4I). This suggests that, like in mouse platelets, human platelet P2Y12 is not involved in the first wave of secretion elicited by thrombin, and is consistent with the initial secretion driven by the SERCA3 being independent of P2Y12. A drop in secreted ATP level between 3 and 4 seconds when P2Y12 is blocked by ARC, suggests that beyond this time point, in absence of active P2Y12, ATP (and probably ADP) undergoes either degradation or uptake by platelets. Altogether these results confirm that thrombin-induced early secretion corresponding to the SERCA3-dependent secretion is independent of P2Y12, while delayed secretion is dependent on P2Y12, and is likely to correspond to the SERCA2-dependent secretion.

## **Discussion**

We have previously observed that the absence of SERCA3 in mice or the reduced expression of SERCA3 protein in human platelets is associated with decreased platelet activation in response to low concentrations of agonists, whereas platelets normally respond to higher doses. [2, 19]

Considering the interest for a way to reduce unwanted activation of platelets without completely inhibiting their function, we studied the signaling pathways involved.

We have then shown that the two distinct platelet  $\text{Ca}^{2+}$  mobilization pathways involving stores controlled by the  $\text{Ca}^{2+}$ -ATPases SERCA2b and SERCA3,

respectively, are regulated differently and most importantly control two distinct phases of ADP secretion. Of note, we found, both in mouse and human platelets, that the early phase of ADP secretion dependent on SERCA3 stores potentiated the delayed phase of ADP secretion controlled by SERCA2b stores. Thus, the SERCA3 and SERCA2b store mobilization pathways cross-talk via ADP. Here, we have addressed the next question: which of the P2Y1 and/or P2Y12 ADP receptors is(are) involved in this cross-talk? We show that ADP released by SERCA3 store mobilization potentiates thrombin-stimulated secretion dependent on the SERCA2b store mobilization via the P2Y12 and not the P2Y1 ADP receptor.

These results raise a number of questions, one of which is why does P2Y12 appear as the main ADP receptor activating the SERCA2b pathway, P2Y1 playing a very minor or no role? In fact, of the two platelet ADP receptors, P2Y12 is considered as playing a more relevant role than P2Y1 in platelet physiology,[23] and this is due to the much lower number of P2Y1 compared to P2Y12 receptors,[24] and the ensuing weaker signaling, probably moreover "swamped" by P2Y12 as well as thrombin signaling. Alternatively, since P2Y1 (but not P2Y12) desensitization is PKC-dependent,[25] it may be desensitized by thrombin activation. At any rate, our finding that P2Y12 is the predominant ADP receptor acting in the potentiation step following SERCA3 store  $Ca^{2+}$  mobilization and early secretion is consistent with this view.

The involvement of P2Y12 in the potentiation of SERCA2b store mobilization may appear counter-intuitive since P2Y12 does not activate phospholipase C[26] and thus cannot directly mobilize SERCA2b stores via IP3 release. However, we have previously established that SERCA3 store mobilization potentiated SERCA2b store-dependent secretion via the thromboxane A2 pathway, at least in part (about 30%).[18] Since the Gq-dependent platelet thromboxane A2 receptor  $TP\alpha$  activates phospholipase C and releases IP3,[27] we conclude that the thromboxane A2 pathway is one of the pathways triggered by P2Y12 leading to further SERCA2b store mobilization (see scheme Fig 5), although another IP3-producing pathway yet unidentified, is likely to be involved.

Interestingly, the SERCA3-NAADP signaling pathway appears to be relevant only for full platelet activation under conditions of low stimulation. In the presence of a high dose of thrombin, full platelet secretion or platelet aggregation is still reached even in conditions of absence or inhibition of this SERCA3-NAADP pathway. This may thus be interpreted as the SERCA3-NAADP pathway being an accessory, dispensable

pathway. However, the physiological and pathological impact of its absence or inhibition strongly suggest the opposite, not to mention the evolutionary conservation of the pathway from mice to humans. We thus infer from these considerations that the SERCA3-NAADP pathway provides fine tuning for platelet activation, allowing may be a more gradual response. In the scheme proposed in Fig. 5, we suggest that a high level of stimulation could produce enough IP3 to completely mobilize  $\text{Ca}^{2+}$  from SERCA2b-dependent stores, whereas ADP-dependent IP3 production would only be required in the presence of a low level of agonist. This would imply an autocrine role for ADP secretion. It is also possible that the early secreted ADP has a paracrine action contributing to the activation of platelets that are not at the site of activation, and thus helping to build a stronger thrombus. This may be in agreement with our initial in vivo observations in SERCA3-deficient animals, where we observed significantly more rebleeding or embolism. [2] At this point we cannot tell which mechanism is really at play.

One of the questions relating to early secretion driven by the SERCA3 store mobilization is whether it corresponds to actual dense granule secretion or to another non-vesicular secretory mechanism, such as export through the ATP transporter pannexin1.[28] We tested this question by the assessment of serotonin secretion, not exported by pannexin1, but co-secreted with ATP or ADP from dense granules. We found parallel secretion kinetics between ATP and serotonin in mouse platelets (Figure 3E) and in human platelets (Supplemental Figure 2A). Moreover, serotonin secretion within the first 5 seconds of secretion behaved like ATP and was inhibited by Ned-19, and not by the SERCA2b inhibitor Tg (Supplemental Figure 2B). These results show that serotonin and ATP are co-secreted via SERCA3 store mobilization, and are thus consistent with dense granule secretion. Interestingly SERCA3-dependent secretion represents about 5 pmoles of ATP (for  $3 \times 10^6$  platelets in our assay) within the first seconds of activation (for example see Figure 4). 5 pmoles ATP represent approximately 10% of the total of releasable ATP (50 pmoles) assessed with thrombin at high concentration (2 U/mL) after 3 minutes.[2, 29] Since mouse platelets exhibit 8-10 granules,[30] 10% might correspond to a single granule. This is close to 2 granules released by 0.1 U/mL thrombin in 10 seconds observed by Eckly et al,[30] the difference being probably due to our milder conditions (0.04 U/mL in 5 seconds). Whether this granule is specifically associated (functionally or

physically) with SERCA3 store mobilization, or is randomly recruited by proximity with SERCA3-dependent stores and the plasma membrane, remains to be established. Finally, the pathway of SERCA3 store mobilization being a platelet activation booster, it may be a pertinent anti-thrombotic target with limited impact on normal hemostasis, deserving close experimental assessment.

### **Authorship**

M.F. participated in research design, performed research, participated in data analysis and in manuscript writing and editing. F.A. participated in flow cytometry and aggregation experiments as well as in editing of the manuscript. B.H., A.E. and C.G. generated the P2Y platelet-specific knockout mice and participated in manuscript editing. C.V.D. participated in manuscript editing. A.K. and M.B. supervised secretion, flow cytometry and aggregation experiments, and helped with manuscript editing. R.B. and J.-P.R. designed experiments, analyzed data, wrote and edited the manuscript.

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## Figure Legends

### Figure 1. Effect of P2Y1 and P2Y12 antagonists on Ca<sup>2+</sup> mobilization in WT and *Serca3*<sup>-/-</sup> mouse platelets.

A) Washed WT (blue tracings) or *Serca3*<sup>-/-</sup> (orange tracings) platelets resuspended in the presence of EGTA (100 μmol/L) to prevent Ca<sup>2+</sup> influx, were preincubated with the Ca<sup>2+</sup> fluorophore BAPTA for 30 minutes, then stimulated, in absence of external Ca<sup>2+</sup> (100 μmol/L EGTA), with Thr (40 mU/mL) for 5 minutes, and fluorescence induced by Ca<sup>2+</sup> mobilization assessed by cell sorting. The ratio of fluorescence of activated versus resting platelets (set as 1) is expressed as a function of time in seconds. Then, platelets were preincubated for 10 minutes with B) the inhibitor of P2Y1 (MRS, 100 μmol/L) or C) the inhibitor of P2Y12 (ARC, 20 μmol/L) or D) both, or E) the ADP scavenger apyrase (5 U/mL) before stimulation with Thr (40 mU/mL). F) Area under curves for 1 minute was assessed in arbitrary units for WT (blue) and *Serca3*<sup>-/-</sup> (orange) platelets (n=3). Data are expressed as mean ± SEM, and probability *P* calculated by the 2 way ANOVA using the Tukey's multiple comparison test between all means; *P* ≤ 0.05, \*; ≤ 0.01, \*\*.

### Figure 2. Inhibition of the P2Y1 and P2Y12 ADP receptors and effect on secretion of WT and *Serca3*<sup>-/-</sup> platelets.

Mouse WT (blue) or *Serca3*<sup>-/-</sup> (orange) platelets were washed, resuspended at 3 x 10<sup>8</sup> platelets/mL in Tyrode's buffer without Ca<sup>2+</sup>, and preincubated with buffer (-) or with the antagonists (+) MRS (P2Y1 specific, 100 μmol/L), ARC (P2Y12 specific, 20 μmol/L) or both for 10 minutes. Platelets were then stimulated with Thr (40 mU/mL) in absence of stirring, for 5 s (A), 10 s (B), 1 min (C) or 3 min (D), prior to stopping secretion by addition of PPACK (100 μmol/L) and immediately chilling at 0°C for 5 min, recovering supernatants by centrifugation and assessing ATP concentrations (see Methods). Data are expressed after subtraction of ATP values obtained in absence of thrombin stimulation. In other experiments, Thr (40 mU/mL) (E), Thr (40 mU/mL) and ADP (10 μmol/L) (F), were added (+) or not (-), and incubated for 3 minutes before stopping activation. Data are means ± SEM, and probability *P* calculated with 2 way ANOVA Bonferroni multiple comparison test (A-D, n=6) or the Tukey's multiple comparison test between all means (E, F, n=3); not significant, ns; *P* ≤ 0.05, \*; ≤ 0.01, \*\*; ≤ 0.001, \*\*\*; ≤ 0.0001, \*\*\*\*.

**Figure 3. Potentiation of SERCA2b-dependent secretion by SERCA3-dependent ADP is altered in P2Y12- but not in P2Y1-defective mouse platelets.**

A) Washed WT (blue tracings), *P2Y1*<sup>-/-</sup> (grey tracings), or *P2Y12*<sup>-/-</sup> (green tracings) platelets resuspended in the presence of EGTA (100 μmol/L) to prevent Ca<sup>2+</sup> influx, were preincubated with the Ca<sup>2+</sup> fluorophore Oregon-green 488 BAPTA for 30 minutes, then stimulated with Thr (40 mU/mL) for 5 minutes, and fluorescence induced by Ca<sup>2+</sup> mobilization assessed by flow cytometry. The ratio of fluorescence of activated versus resting platelets (set as 1) is expressed as a function of time in seconds. B) Area under curves for 1 minute was assessed in arbitrary units (A.U.) for WT (blue), *P2Y1*<sup>-/-</sup> (grey), or *P2Y12*<sup>-/-</sup> (green) platelets (n=7). C) Quantification of Ca<sup>2+</sup> mobilization in the absence (-) or the presence of the NAADP antagonist Ned-19 (10 μmol/L) is shown as area under curves for 2 minutes, assessed in arbitrary units (A.U.) for WT (blue), *P2Y1*<sup>-/-</sup> (grey), or *P2Y12*<sup>-/-</sup> (green) platelets after stimulation with Thr (40 mU/mL) (n=3). D) Kinetics of secretion of WT (blue dots), *P2Y1*<sup>-/-</sup> (grey), or *P2Y12*<sup>-/-</sup> (green) platelets were assessed after stimulation with thrombin (40 mU/mL) in absence of stirring, for 5, 10, 30, 60 or 180 seconds, prior to stopping secretion by addition of PPACK (100 μmol/L) and immediately chilling at 0°C for 5 min, recovering supernatants by centrifugation and assessing ATP concentrations (see Methods) (n=4). E) WT (blue), *Serca3*<sup>-/-</sup> (orange) or *P2Y12*<sup>-/-</sup> (green) mouse platelets were washed, resuspended at 3 x 10<sup>8</sup> platelets/mL in Tyrode's buffer without Ca<sup>2+</sup>, and stimulated with thrombin (40 mU/mL) in absence of stirring, for indicated times (5, 10 and 180 sec), prior to stopping secretion by addition of PPACK (100 μmol/L) and immediately chilling at 0°C for 5 min, recovering supernatants by centrifugation and assessing serotonin concentration (see Methods)(n=3). F) WT (blue) or *P2Y12*<sup>-/-</sup> (green) platelets were preincubated with diluent (-) or (+) either Ned-19 (10 μmol/L) for 10 minutes, Tg (200 nmol/L) for 15 minutes or both, and stimulated (+) or not (-) with Thr (40 mU/mL) for 3 minutes. ATP secretion was measured as described (Methods) (n=5). Data are expressed as mean ± SEM, and probability *P* calculated by the 2 way ANOVA using the Tukey's multiple comparison test between all means except for F), where was used the Bonferroni multiple comparison test between WT & *P2Y12*<sup>-/-</sup>; not significant, ns; *P* ≤ 0.05, \*; ≤ 0.01, \*\*; ≤ 0.001, \*\*\*; ≤ 0.0001, \*\*\*\*.

**Figure 4. P2Y12, not P2Y1, is involved in Ca<sup>2+</sup> mobilization elicited by Thrombin and in late secretion.**

Washed control human platelets resuspended in the presence of EGTA (100 µmol/L) to prevent Ca<sup>2+</sup> influx, were preincubated with the Ca<sup>2+</sup> fluorophore Oregon-green 488 BAPTA for 30 minutes, then stimulated with thrombin (40mU/mL) for 5 minutes, and fluorescence induced by Ca<sup>2+</sup> mobilization assessed by flow cytometry. The ratio of fluorescence of activated versus resting platelets (set as 1) is expressed as a function of time in seconds. Prior activation, platelets were preincubated for 10 minutes with buffer (A), the P2Y12 antagonist ARC (20 µmol/L, B), the nucleoside phosphate scavenger apyrase (5 U/mL, C), apyrase and ARC (D), the NAADP antagonist Ned-19 (10 µmol/L) (E), Ned-19 and ARC (F), apyrase and Ned19 (G), Area under curves for 1 minute was assessed in arbitrary units (A.U.) after stimulation with Thrombin, in the absence (-) or the presence (+) of ARC, apyrase or Ned-19 (n=3). H), Tracings illustrated in the figure are representative of three independent experiments. I) Kinetics of secretion of washed human platelets was assessed after pre-incubation for 10 minutes with Tyrode's buffer (blue) or the P2Y12 antagonist (ARC) (orange) and stimulation with Thr (40 mU/mL) in absence of stirring or of added Ca<sup>2+</sup>, for 2, 3, 4, 5, 10, 30, 60 seconds or 3 minutes, prior to stopping secretion by addition of PPACK (100 µmol/L) and immediate chilling at 0°C for 5 min, recovering supernatants by centrifugation and assessing ATP concentrations (see Methods) (n=3). Data are expressed as mean ± SEM, and probability *P* calculated by ANOVA; *P* ≥ 0.05, ns; ≤ 0.05, \*; ≤ 0.01, \*\*; ≤ 0.001, \*\*\*; ≤ 0.0001, \*\*\*\*.

**Figure 5. Illustration of the links between SERCA3- and SERCA2b-dependent pathway in platelet activation**

Thrombin stimulation at low concentration activates two signaling pathways, the first one leading to the production of NAADP promoting SERCA3 stores mobilization and the second one activating phospholipase C and production of IP<sub>3</sub>, releasing Ca<sup>2+</sup> from SERCA2b stores. The first pathway results in a rapid secretion of ADP (red arrow) that potentiates the second SERCA2b-dependent pathway via the ADP receptor P2Y<sub>12</sub> (not P2Y<sub>1</sub>). [18] Note that P2Y<sub>12</sub> cannot activate Phospholipase C directly, but probably indirectly via TxA<sub>2</sub> and its receptor TP<sub>α</sub>, which activate phospholipase C and the release of IP<sub>3</sub>, as suggested by the fact that the

potentiation step is indomethacin-sensitive.[18] Note that ADP released via this pathway (blue arrow) may also activate P2Y12.

## Legends to supplemental figures

### **Supplemental Figure 1. Platelet Ca<sup>2+</sup> mobilization by ADP at 5μM (A-D) or 10μM (E-H) in the presence of the P2Y1 and P2Y12 pharmacological antagonists.**

The efficacy of MRS2179 (MRS), the P2Y1 antagonist, and of ARC69931MX (ARC), the P2Y12 antagonist was checked by assessment of Ca<sup>2+</sup> mobilization inhibition in platelets activated by ADP. Washed mouse platelets were loaded with the fluorescent free Ca<sup>2+</sup> probe BAPTA-Oregon Green 488 and preincubated for 10 minutes with buffer, MRS (100μM)(B,F), ARC (20μM)(C, G) or both antagonists (D, H) before stimulation with ADP 5μM (A-D) or 10 μM (E-H). Resultant free cytosolic Ca<sup>2+</sup> mobilization was detected by flow cytometry at 488nm, plotting fluorescence as a ratio over background level of fluorescence in absence of activation, set to 1. Quantitation was assessed by area under curves after 1 minute of activation with ADP 5μM (I) or 10μM (J) and plotted as bars. Data are expressed as mean ± SEM, n=3 and probability *P* calculated by ANOVA: *P* ≤ 0.05, \*; ≤ 0.01, \*\*; ≤ 0.001, \*\*\*; ≤ 0.0001, \*\*\*\*.

### **Supplemental Figure 2. Serotonin follows the same secretory pathways than ATP in human platelets**

A) Washed human platelets were stimulated with thrombin (Thr, 40mU/mL), for 5, 10 or 180 seconds. Activation was stopped by addition of PPACK (100μM) and immediate chilling at 0°C for 5 min, recovering supernatants by centrifugation, before serotonin (blue) or ATP (grey) concentrations were assessed (see Methods). B) Washed human platelets pre-incubated with (+) or without (-) Ned-19 (10μM), apyrase (5U/mL) or ARC (20μM) for 10 minutes, Tg (200nM) for 15 minutes were stimulated by thrombin (40mU/mL) for 0 or 5s and serotonin concentration was assessed in supernatants. C) Washed human platelets pre-incubated with Tg (200nM, orange) for 15 minutes and either Ned-19 (10μM, grey), ARC (20μM, yellow), apyrase (5U/mL, black) or ARC + apyrase (white) for 10 minutes and stimulated by thrombin (40mU/mL). Activation was stopped at 0, 5, 10 or 180 seconds by PPACK and chilling at 0°C, and serotonin concentration assessed in

supernatants. Data are expressed as mean  $\pm$  SEM, and probability  $P$  calculated by the 2 way ANOVA using the Tukey multiple comparison test between all means; not significant, ns;  $P \leq 0.01$ , \*\*;  $\leq 0.001$ , \*\*\*;  $\leq 0.0001$ , \*\*\*\*.