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## Mitochondrial calcium and reactive oxygen species regulate agonist-initiated platelet phosphatidylserine exposure

Hyo-Jung Choo<sup>1</sup>, Talib B. Saafir<sup>2</sup>, Laura Mkumba<sup>1</sup>, Mary B. Wagner<sup>2</sup>, and Shawn M. Jobe<sup>1</sup>

<sup>1</sup>Department of Pediatrics, Aflac Cancer Center and Blood Disorders Service, Emory University and Children's Healthcare of Atlanta

<sup>2</sup>Department of Pediatrics, Sibley Heart Center, Emory University and Children's Healthcare of Atlanta

### Abstract

**Objective**—To study the interactions of cytoplasmic calcium ( $\text{Ca}^{2+}_{\text{cyt}}$ ) elevation, mitochondrial permeability transition pore (mPTP) formation, and reactive oxygen species (ROS) formation in the regulation of phosphatidylserine (PS) exposure in platelets.

**Methods and results**—mPTP formation, but not the degree of  $\text{Ca}^{2+}_{\text{cyt}}$  elevation, was associated with PS exposure in wild-type, CypD null, ionomycin-treated and ROS-treated platelets. In the absence of the mPTP regulator cyclophilin D agonist-initiated mPTP formation and high-level PS exposure were markedly blunted, but  $\text{Ca}^{2+}_{\text{cyt}}$  transients were unchanged. Mitochondrial calcium ( $\text{Ca}^{2+}_{\text{mit}}$ ) transients and ROS, key regulators of mPTP formation, were examined in strongly-stimulated platelets. Increased ROS production occurred in strongly-stimulated platelets and was dependent on extracellular calcium entry, but not the presence of CypD.  $\text{Ca}^{2+}_{\text{mit}}$  increased significantly in strongly-stimulated platelets. Abrogation of  $\text{Ca}^{2+}_{\text{mit}}$  entry either by inhibition of the mitochondrial calcium uniporter or mitochondrial depolarization prevented mPTP formation and exposure, but not platelet aggregation or granule release.

**Conclusions**—Sustained  $\text{Ca}^{2+}_{\text{cyt}}$  levels are necessary, but not sufficient, for high-level PS exposure in response to agonists. Increased  $\text{Ca}^{2+}_{\text{mit}}$  levels are a key signal initiating mPTP formation and PS exposure. Blockade of  $\text{Ca}^{2+}_{\text{mit}}$  entry allows the specific inhibition of platelet procoagulant activity.

### Keywords

Mitochondria; calcium; phosphatidylserine; platelets; mPTP

### Introduction

Platelet phosphatidylserine (PS) exposure amplifies thrombin generation by facilitating assembly of the tenase and prothrombinase complexes.<sup>1</sup> In this process, PS, which had been limited to the platelet membrane inner leaflet, is rapidly equilibrated between the inner and outer leaflets of the platelet membrane. This results in the exposure of PS to the plasma milieu.<sup>2</sup> The importance of PS exposure in normal hemostasis is illustrated by the congenital disorder Scott syndrome, a bleeding diathesis characterized by delayed hemostasis and

Address correspondence to: Shawn M. Jobe, Emory Children's Center, 2015 Uppergate Drive, 4<sup>th</sup> floor, Atlanta, GA 30068, shawn.job@emory.edu, Phone: 404-727-2712, Fax: 404-727-4455.

#### **Conflict-of-interest disclosure**

The authors declare no competing financial interests.

impaired wound healing.<sup>3</sup> In Scott syndrome absence of the protein TMEM16F results in a defect in agonist-initiated scramblase activity and platelet PS exposure.<sup>4,5</sup>

Distinct mechanisms regulate agonist-initiated PS exposure in platelets relative to other platelet responses, such as platelet aggregation and granule release. Even when strongly-stimulated, only a subpopulation of platelets demonstrates high-level PS exposure.<sup>6-8</sup> And, relative to these other platelet responses, PS exposure is delayed. In fact, several minutes pass before high levels of PS can be detected on the activated platelet surface.<sup>7,9</sup> In addition to this high-level PS exposure, low level PS exposure is broadly present on stimulated platelets when PS exposure is analyzed using lactadherin.<sup>10</sup> The relative physiologic importance of low- and high-level PS exposure is uncertain.

Relatively little is known about the intracellular mechanisms that specifically regulate high-level agonist-initiated platelet PS exposure. The importance of extracellular calcium and elevated  $\text{Ca}^{2+}_{\text{cyt}}$  levels in the regulation of high-level PS exposure and scramblase activity is well appreciated,<sup>9,11</sup> and both store-operated (SOCE) and non-capacitative calcium-entry (NCCE) mechanisms have been implicated in the regulation of agonist-initiated PS exposure.<sup>12-14</sup> Potential determinants of prolonged  $\text{Ca}^{2+}_{\text{cyt}}$  elevation have been identified, including tyrosine kinases, which potentiate,<sup>9</sup> and isoforms of protein kinase C, which inhibit, calcium signal generation and procoagulant activity.<sup>15</sup> Increased potassium efflux through  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels may also facilitate PS exposure.<sup>16</sup>

Mitochondrial events have been implicated as important determinants of platelet PS exposure. The mitochondrial permeability transition pore (mPTP) is a non-selective multiprotein pore that spans the inner mitochondrial membrane, the formation of which causes a rapid loss of mitochondrial transmembrane potential ( $\Delta\psi_{\text{m}}$ ).<sup>17</sup> A key regulator of mPTP function is the peptidylprolyl isomerase cyclophilin D (CypD), and in its absence mPTP formation is abrogated.<sup>18-20</sup> In strongly-stimulated platelets, mPTP formation is closely associated with high-level PS exposure,<sup>21,22</sup> and in CypD's absence both mPTP formation and PS exposure are markedly abrogated indicating the critical role of mPTP formation in the regulation of agonist-initiated high-level PS exposure.<sup>21</sup> PS exposure can also be initiated by a BH3-protein mimetic. While agonist-initiated PS exposure is unaffected in the absence of the mitochondrially-localized BH3 proteins, Bax and Bcl-2,<sup>23</sup> their absence ablates BH3-mimetic induced PS exposure. This BH3 protein regulated pathway is unaffected in CypD's absence and has been implicated in platelet aging.<sup>23,24</sup>

Important determinants of mPTP formation in other cell types include both calcium and reactive oxygen species (ROS).<sup>17</sup> In ischemic myocytes subject to reperfusion, it is increased ROS production, not calcium elevation, that initiates mPTP formation.<sup>25</sup> Although ROS production occurs in platelets activated by a single agonist,<sup>26</sup> its role in the regulation of mPTP formation in platelets is not known. Here we closely examine the interactions of calcium, ROS, and mPTP formation in the regulation of high-level PS exposure. The outcomes of these studies provide novel insights into the roles of intracellular calcium and ROS in the regulation of platelet mPTP formation and PS exposure and identify mitochondrial calcium entry as a unique potential target for the specific inhibition of platelet procoagulant activity.

## Methods

### Mice

Animal protocols were approved by the Emory University and Veterans Affairs Animal Care and Use committees. C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). CypD<sup>-/-</sup> mice, which are homozygous for a targeted deletion of the *Ppif* gene,

and control CypD<sup>+/+</sup> mice were generated as described previously<sup>18</sup> and maintained on an inbred SV129 background.

### Human samples

Blood was obtained from healthy persons after informed consent was obtained in accordance with the Declaration of Helsinki and in compliance with the standards of the Emory Institutional Review Board.

### Preparation of washed platelets

Washed murine platelets were isolated as described previously.<sup>27</sup> Isolation of human platelets is described in supplemental methods.

### Flow cytometry

Washed platelets in Tyrode's buffer with CaCl<sub>2</sub> were stimulated with agonist(s) and preincubated with the pharmacologic agents as indicated. For experiments evaluating loss of  $\Delta\psi_m$  and ROS generation, platelets were incubated with 500 nM tetramethylrhodamine methyl ester (TMRM)<sup>28</sup> or 5  $\mu$ M 5-(and 6-) chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate ethyl ester (CM-DCFDA),<sup>29</sup> respectively, prior to stimulation. For experiments detecting mitochondrial superoxide generation, platelets were stimulated with the indicated agonist(s) and then incubated with 5  $\mu$ M MitoSOX<sup>TM</sup>. Labeled platelets were evaluated by flow cytometry. See supplemental methods for details.

### Confocal Microscopy

Washed platelets in mTyrode's buffer were allowed to adhere to a fibrinogen (0.1 mg/ml) coated coverglass for 30 minutes in the presence of 4  $\mu$ M Fluo-4-AM and 5  $\mu$ M Rhod-2-AM. Epifluorescence was continually examined, and the buffer was sequentially to mTyrode's buffer with calcium, then Thr/Cvx. Subsequently, FM1-43 was added to allow identification of PS-exposed platelets.<sup>30</sup> Data were normalized to the initial staining intensity prior to platelet stimulation. See supplemental methods for details.

### Fluorometry

Washed platelets were stimulated with the indicated agonist(s) and evaluated by fluorometry for five minutes. For experiments evaluating calcium transients or ROS generation, platelets were pre-incubated with 1  $\mu$ M Fluo-4-AM or 50  $\mu$ M DCFDA for thirty minutes. Data were normalized to the initial staining intensity prior to platelet stimulation.

### Statistical analysis

Data are presented as means  $\pm$  SD. Significant differences between means were determined by Student's t- test or ANOVA. Significance was set at  $P < .05$ .

## Results

### Calcium and mitochondrial events in strongly-stimulated platelets

Ca<sup>2+</sup><sub>cyt</sub> levels, mPTP formation, and PS exposure were examined in suspended and adherent platelets. Stimulation with thrombin caused a sudden increase in Ca<sup>2+</sup><sub>cyt</sub>; the GPVI agonist convulxin caused a gradual, but more sustained, increase in Ca<sup>2+</sup><sub>cyt</sub> levels (Figure 1A); and simultaneous stimulation with thrombin and convulxin caused a rapid and sustained increase in Ca<sup>2+</sup><sub>cyt</sub> in both suspended and adherent platelets (Figure 1A and 1B). By using adherent platelets, we were able to continuously analyze Ca<sup>2+</sup><sub>cyt</sub> transients in individual platelets. This allowed a retrospective comparison of Ca<sup>2+</sup><sub>cyt</sub> transients in platelets that became PS<sup>+</sup>

(approximately 50%) versus those that remained PS<sup>-</sup>. Initial Ca<sup>2+</sup><sub>cyt</sub> peak levels differed minimally in PS<sup>+</sup> and PS<sup>-</sup> subpopulations. Over time a gradual decrease in Ca<sup>2+</sup><sub>cyt</sub> was observed in PS<sup>-</sup> platelets, while in PS<sup>+</sup> platelets the elevation in Ca<sup>2+</sup><sub>cyt</sub> was more sustained.

To simultaneously evaluate Ca<sup>2+</sup><sub>cyt</sub>,  $\Delta\psi_m$ , and PS exposure, multi-color flow cytometry was utilized (Figure 1 C, D). Thrombin plus convulxin stimulation caused a rapid increase in Ca<sup>2+</sup><sub>cyt</sub> levels that was maximal within twenty seconds (unpublished results), a result consistent with that observed using fluorometry and confocal microscopy (Figure 1A, B). Examination of  $\Delta\psi_m$  in strongly-stimulated platelets revealed a rapid increase in TMRM fluorescence consistent with mitochondrial hyperpolarization (increased  $\Delta\psi_m$ ). This was followed 300–400 seconds later by  $\Delta\psi_m$  loss (Figure 1C).  $\Delta\psi_m$  loss and PS exposure were closely correlated at all-time points examined (Figure 1C, D). These experiments demonstrate that sustained elevations in Ca<sup>2+</sup><sub>cyt</sub> along with a transient increase in  $\Delta\psi_m$  precede  $\Delta\psi_m$  loss, and that this loss of  $\Delta\psi_m$  in individual platelets is temporally associated with PS exposure.

### Agonist-initiated loss of $\Delta\psi_m$ , but not Ca<sup>2+</sup><sub>cyt</sub> elevation, is abrogated in CypD<sup>-/-</sup> platelets

We next sought to determine the relative roles of mPTP formation and Ca<sup>2+</sup><sub>cyt</sub> transients in the regulation of PS exposure. The observation that higher Ca<sup>2+</sup><sub>cyt</sub> transients are observed in PS<sup>+</sup> platelets (Figure 1B) suggests two alternative hypotheses. By eliminating the mitochondrion's Ca<sup>2+</sup><sub>cyt</sub> buffering capacity,<sup>31</sup> mPTP formation might mediate Ca<sup>2+</sup><sub>cyt</sub> elevation, and thus facilitate PS exposure. Alternatively, Ca<sup>2+</sup><sub>cyt</sub> elevation might act through the mPTP to regulate PS exposure, and the presence of higher Ca<sup>2+</sup><sub>cyt</sub> levels in PS-expressing cells might merely reflect the greater likelihood of these high Ca<sup>2+</sup><sub>cyt</sub> containing cells to undergo mPTP formation.<sup>32</sup> These alternative hypotheses were tested using CypD<sup>-/-</sup> platelets, in which mPTP formation and PS exposure are markedly abrogated.<sup>21</sup>

In stark contrast to the pronounced effect of CypD's absence on mPTP formation and PS exposure, no significant difference in Ca<sup>2+</sup><sub>cyt</sub> transients was observed between agonist-stimulated CypD<sup>+/+</sup> and CypD<sup>-/-</sup> platelets, either suspended (Figure 2A) or fibrinogen-adherent (Figure 2B). This CypD independence of Ca<sup>2+</sup><sub>cyt</sub> elevation effectively negates the hypothesis that the role of the mPTP in regulating PS exposure is mediated through its effects on Ca<sup>2+</sup><sub>cyt</sub>. Instead, these results indicate that a threshold Ca<sup>2+</sup><sub>cyt</sub> elevation is essential in initiating mPTP formation and PS exposure in platelets. However, sustained Ca<sup>2+</sup><sub>cyt</sub> transients are neither sufficient for, nor are they the sole determinant of PS exposure, a conclusion starkly demonstrated by the similar Ca<sup>2+</sup><sub>cyt</sub> transients in CypD<sup>+/+</sup> and CypD<sup>-/-</sup> platelets.

We also tested the relationship of mPTP formation and low-level PS exposure using the non-Ca<sup>2+</sup> dependent PS binding protein C2-lactadherin (Supplemental Figure 1).<sup>10</sup> Unlike high-level PS exposure low-level PS exposure was relatively unaffected by CypD's absence indicating the primary role of mPTP formation in high-, but not low-level PS exposure in the activated platelet.

To examine whether the effects of CypD deletion on platelet PS exposure were caused by altered calcium sensitivity of the mPTP,<sup>19</sup> the effects of calcium-ionophore stimulation on Ca<sup>2+</sup><sub>cyt</sub>, mPTP formation, and PS exposure were examined in CypD<sup>+/+</sup> and CypD<sup>-/-</sup> platelets. Experiments were performed in which either ionomycin (Figure 2C) or extracellular calcium concentrations (Figure 2D) were varied, and the effects of CypD's absence on mPTP formation and PS exposure were examined. In both conditions, the platelet PS response to ionomycin was significantly decreased in the absence of CypD. In the presence of a physiologic calcium concentration, maximal PS exposure occurred with the addition of 0.6  $\mu$ M and 1.0  $\mu$ M ionomycin for CypD<sup>+/+</sup> and CypD<sup>-/-</sup> platelets,

respectively (Figure 2 C). When extracellular calcium concentration was varied and fixed amount of ionomycin was utilized, maximal PS exposure occurred in the presence of 1 mM and 2 mM extracellular calcium for CypD<sup>+/+</sup> and CypD<sup>-/-</sup> platelets, respectively (Figure 2D). No significant differences in Ca<sup>2+</sup><sub>cyt</sub> were observed between CypD<sup>+/+</sup> and CypD<sup>-/-</sup> platelets using either maneuver, and regardless of genotype or maneuver, mPTP formation was closely associated with PS exposure (Figure 2E and unpublished observations). Ca<sup>2+</sup><sub>cyt</sub> elevation caused by thrombin/convulxin stimulation was plotted versus PS exposure using the ionomycin response curves (triangles in Figure 2C). Interestingly, Ca<sup>2+</sup><sub>cyt</sub> elevation and PS exposure generated by these physiologic agonists corresponded with the PS response observed when a similar Ca<sup>2+</sup><sub>cyt</sub> elevation was generated using ionomycin. These results indicate that CypD's absence affects platelet PS exposure by altering the calcium sensitivity of the mPTP, and suggest the mPTP's calcium-sensitivity as an important determinant of Thr/Cvx-initiated PS exposure.

### Reactive oxygen species positively modulate PS exposure independent of the magnitude of Ca<sup>2+</sup><sub>cyt</sub> elevation

In addition to Ca<sup>2+</sup><sub>cyt</sub>, another key determinant of mPTP formation is ROS.<sup>17</sup> The effects of ROS on mPTP formation are calcium-independent and are mediated by thiol oxidation of mPTP regulatory components.<sup>33</sup> Given this independent and important role in the regulation of mPTP formation, the effects of ROS on ionomycin- and thrombin-initiated PS exposure in platelets and their relationship to mPTP formation and Ca<sup>2+</sup><sub>cyt</sub> were examined.

In platelets treated with phenylarsine oxide (PAO), an oxidant with potent effects on mPTP formation,<sup>34</sup> platelet sensitivity to ionomycin stimulation was markedly increased (Figure 3A). Similarly, increasing amounts of the physiologic oxidant H<sub>2</sub>O<sub>2</sub> potentiated thrombin-initiated PS exposure (<sup>21</sup> and Figure 3B). Whether the effects of these oxidants are mediated through Ca<sup>2+</sup><sub>cyt</sub> elevation or mPTP formation has not previously been examined. mPTP formation was potentiated by PAO (Figure 3C) or H<sub>2</sub>O<sub>2</sub> (Figure 3D) treatment, and loss of Δψ<sub>m</sub> and PS exposure were closely associated (compare Figures 3A, B with Figures 3C, D). In contrast, when Ca<sup>2+</sup><sub>cyt</sub> levels were examined, no significant difference was noted between PAO-treated and untreated platelets (Figure 3E). Similarly, in Thr/H<sub>2</sub>O<sub>2</sub>-stimulated platelets (compare Figure 3B and Figure 3F), Ca<sup>2+</sup><sub>cyt</sub> elevation and PS exposure were poorly correlated although here the presence of H<sub>2</sub>O<sub>2</sub> positively impacted thrombin-initiated Ca<sup>2+</sup><sub>cyt</sub> at a concentration as low as 50 μM. These results demonstrate that ROS can increase the platelet's potential for PS exposure independent of its effects on Ca<sup>2+</sup><sub>cyt</sub>. The close association of mPTP formation, but not Ca<sup>2+</sup><sub>cyt</sub> elevation, with PS exposure induced in the presence of ROS provides further evidence that Ca<sup>2+</sup><sub>cyt</sub> primarily affects PS exposure indirectly through its effects on mPTP formation, and not by acting directly on scramblase to initiate platelet PS exposure.

### Increased ROS production in strongly-stimulated platelets is dependent on extracellular calcium, but not mPTP formation

Since ROS profoundly impacted platelet mPTP formation and PS exposure (Figure 3), their production in strongly-stimulated platelets was examined. Although many studies have examined ROS production in platelets stimulated with a single agonist, ROS production in strongly-stimulated platelets has received little attention.<sup>26</sup> ROS formation was investigated in platelets activated with either single or dual agonists using the ROS-sensitive dye DCFDA. Five minutes after stimulation a pronounced increase in ROS production was noted in platelets stimulated with thrombin and convulxin together relative to platelets stimulated with either agonist alone (Figure 4A).

Key sources of ROS production within the platelet include NADPH oxidases<sup>26</sup> and mPTP formation.<sup>35</sup> Addition of the NADPH oxidase inhibitor DPI blunted Thr/Cvx initiated-ROS production, while the absence of CypD, did not significantly affect ROS production (Figure 4B). To further investigate a role for mitochondria as the potential ROS source apart from mPTP formation, mitochondrial superoxide production was investigated using the mitochondrially-localized ROS sensitive dye Mito-SOX. Increased generation of mitochondrial superoxide was noted following stimulation with either convulxin, Thr/Cvx, or Thr/H<sub>2</sub>O<sub>2</sub> (Figure 4B). However, this increase in mitochondrial superoxide production, unlike the generalized ROS production measured by DCFDA, was CypD-dependent. This result indicates that platelet mPTP formation initiates mitochondrial superoxide production in strongly-stimulated platelets, but this mitochondrial ROS production contributes only minimally to the ROS burst as detected by DCFDA.

Since sustained elevation of Ca<sup>2+</sup><sub>cyt</sub> is prominent in strongly-stimulated platelets (Figure 1), the importance of extracellular Ca<sup>2+</sup> in the regulation of ROS production in strongly-stimulated platelets was investigated. ROS production required the presence of extracellular Ca<sup>2+</sup>, and addition of the calcium chelators EGTA or BAPTA prevented ROS elevation (Figure 4C). The importance of store-operated (SOCE) and non-capacitative calcium entry (NCCE) in the ROS burst was examined. Addition of either SKF96365, an inhibitor of both SOCE and NCCE,<sup>12</sup> or 2-ABP, an inhibitor of SOCE,<sup>36</sup> partially blocked ROS production in strongly-stimulated platelets. These results indicate the importance of extracellular calcium entry in the regulation of ROS production in strongly-stimulated platelets.

### Mitochondrial calcium (Ca<sup>2+</sup><sub>mit</sub>) regulates agonist-initiated PS exposure

The close communication between Ca<sup>2+</sup><sub>cyt</sub> and Ca<sup>2+</sup><sub>mit</sub> led us to examine the possibility that sustained Ca<sup>2+</sup><sub>cyt</sub> elevations may affect mPTP formation and PS exposure by increasing Ca<sup>2+</sup><sub>mit</sub>.<sup>32</sup> Ca<sup>2+</sup><sub>mit</sub> levels were examined in adherent strongly-stimulated platelets using the mitochondrial-specific calcium indicator Rhod-2 (Supplemental Figure 2). Thr/Cvx stimulation caused a rapid increase in Ca<sup>2+</sup><sub>mit</sub> (Figure 5A, B) with higher Ca<sup>2+</sup><sub>mit</sub> levels observed in PS<sup>+</sup> platelets. Interestingly, when Ca<sup>2+</sup><sub>mit</sub> was examined in Thr/H<sub>2</sub>O<sub>2</sub>-stimulated platelets, Ca<sup>2+</sup><sub>mit</sub>, unlike Ca<sup>2+</sup><sub>cyt</sub>, was closely correlated with PS exposure (Figure 5B). The relationship of Ca<sup>2+</sup><sub>mit</sub> elevation to mPTP formation and PS exposure was studied in strongly-stimulated CypD<sup>+/+</sup> and CypD<sup>-/-</sup> platelets. As with Ca<sup>2+</sup><sub>cyt</sub>,<sup>21</sup> Ca<sup>2+</sup><sub>mit</sub> levels were similar between CypD<sup>+/+</sup> and CypD<sup>-/-</sup> platelets consistent with mPTP formation acting downstream of Ca<sup>2+</sup><sub>mit</sub> elevation to regulate agonist-initiated PS exposure (Figure 5C). Together, these results suggest that increased Ca<sup>2+</sup><sub>mit</sub> levels, influenced either by Ca<sup>2+</sup><sub>cyt</sub> elevation or an ROS-mediated signal, might act as a primary driver of agonist-initiated PS exposure.

Calcium influx through the ion-impermeable inner mitochondrial membrane (IMM) occurs as the result of passive flow of Ca<sup>2+</sup><sub>cyt</sub> down its electrical and chemical gradient through the mitochondrial calcium uniporter (MCU).<sup>37-39</sup> The mitochondrial electrical gradient ( $\Delta\psi_m$ ) is generated by the proton-motive force of the mitochondrial respiratory complexes. The importance of Ca<sup>2+</sup><sub>mit</sub> levels in PS exposure was tested in two ways, by using Ru360, a specific MCU inhibitor, and by disruption of the proton-motive force using mitochondrial depolarizing agents.<sup>38</sup> Due to its relative membrane impermeability, the effects of Ru360 have typically been ascertained following incubation with the agent for 6–12 hours, however this incubation period was not compatible with platelets. To overcome this limitation relatively high concentrations of Ru360 were required to adequately block Ca<sup>2+</sup><sub>mit</sub> entry. To rule out potential off-target effects of Ru360 on other calcium entry pathways, Ca<sup>2+</sup><sub>cyt</sub> and Ca<sup>2+</sup><sub>mit</sub> were both assessed. Treatment of platelets with Ru360 effectively blocked the agonist-initiated increase in Ca<sup>2+</sup><sub>mit</sub>, and this decreased Ca<sup>2+</sup><sub>mit</sub> elevation was associated with a significant decrease in platelet PS exposure (Figure 5D). Consistent with a specific

effect of Ru360 on  $\text{Ca}^{2+}_{\text{mit}}$ ,  $\text{Ca}^{2+}_{\text{cyt}}$  levels were not substantially affected in Ru360-treated platelets. To further assess the importance of  $\text{Ca}^{2+}_{\text{mit}}$ , the effects of mitochondrial depolarizing agents were examined. FCCP is a protonophore, while metformin blocks complex I of the mitochondrial respiratory chain.<sup>40</sup> Mitochondrial depolarization effectively blocked uptake of calcium into the mitochondria in Thr/Cvx stimulated platelets, and PS exposure was markedly abrogated consistent with the observed decrease in  $\text{Ca}^{2+}_{\text{mit}}$  (Figure 5E).  $\text{Ca}^{2+}_{\text{cyt}}$  transients were unaffected by either depolarizing agent, and it was confirmed that pre-treatment of platelets with either of these agents caused mitochondrial depolarization (Figure 5F). Together, these results indicate the importance of  $\text{Ca}^{2+}_{\text{mit}}$  elevation in regulating platelet PS exposure, and identify the mitochondrial calcium uniporter and the mitochondrial electrical gradient as key mediators regulating mitochondrial calcium entry in the activated platelet.

### **Inhibition of $\text{Ca}^{2+}_{\text{mit}}$ uptake blocks PS exposure, but not integrin $\alpha_{\text{IIb}}\beta_3$ activation or granule release**

$\text{Ca}^{2+}_{\text{cyt}}$  transients are central to the regulation of platelet function. Consequently inhibition of SOCE or other mediators of  $\text{Ca}^{2+}_{\text{cyt}}$  elevations block multiple platelet functions.<sup>12–14</sup> We hypothesized that, unlike inhibition of  $\text{Ca}^{2+}_{\text{cyt}}$  increase, blockade of  $\text{Ca}^{2+}_{\text{mit}}$  elevation would affect platelet PS exposure specifically. To test the utility of inhibitors of  $\text{Ca}^{2+}_{\text{mit}}$  entry, including the clinically utilized agent metformin, to act as specific inhibitors of PS exposure, their effects on human platelets were assessed. PS exposure, granule release and integrin  $\alpha_{\text{IIb}}\beta_3$  activation were assessed in Thr/Cvx-stimulated platelets treated with various inhibitors of  $\text{Ca}^{2+}_{\text{mit}}$  elevation. While each of the agents effectively inhibited PS exposure (Figure 6A), none affected either granule release, as measured by P-selection exposure (Figure 6B), or the initial activation of integrin  $\alpha_{\text{IIb}}\beta_3$  (Figure 6C), as measured by PAC-1. A similar effect of these pharmacologic agents on platelet activation was observed using murine platelets (Supplemental Figure 3).

## **Discussion**

In strongly-stimulating environments, platelet activation evokes a procoagulant response in a subpopulation of platelets.<sup>6–8</sup> Here we investigated how calcium, ROS, and mitochondrial events contribute temporally and substantially to initiate platelet procoagulant activity in strongly-stimulated platelets. To parse these events, we visualized calcium and ROS fluxes and investigated their relationship with each other and with  $\Delta\psi_{\text{m}}$  in several experimental conditions and in *CypD*<sup>-/-</sup> platelets.

Whether mPTP formation impacts agonist-initiated PS exposure through effects on  $\text{Ca}^{2+}_{\text{cyt}}$  transients has been uncertain. Elevated  $\text{Ca}^{2+}_{\text{cyt}}$  transients were found to precede mPTP formation and PS exposure by as much as 3–5 minutes, and definitive evidence that mPTP formation does not mediate PS exposure through changes in  $\text{Ca}^{2+}_{\text{cyt}}$  was provided by the almost identical  $\text{Ca}^{2+}_{\text{cyt}}$  transients observed in *CypD*<sup>+/+</sup> and *CypD*<sup>-/-</sup> platelets. Furthermore, in response to diverse stimuli mPTP formation, not  $\text{Ca}^{2+}_{\text{cyt}}$  elevation, was found to be most closely associated with PS exposure. Conditions that inhibited mPTP formation, namely *CypD*'s absence or inhibition of mitochondrial calcium uptake, inhibited PS exposure; while agents that accentuated mPTP formation, namely PAO, accentuated PS exposure. None of these agents affected  $\text{Ca}^{2+}_{\text{cyt}}$  elevation providing further evidence of the role of mPTP formation as an essential down-stream mediator of high-level PS exposure.

Although elevated  $\text{Ca}^{2+}_{\text{cyt}}$  levels have been hypothesized to act directly on scramblase to initiate PS exposure,<sup>1</sup> other studies have demonstrated an inconsistent association between PS exposure and the height of  $\text{Ca}^{2+}_{\text{cyt}}$  elevation, a finding that led these investigators to propose a role for intermediate mediators in mediating the PS response.<sup>9, 11</sup> The results

presented here indicate that though sustained elevations in  $\text{Ca}^{2+}_{\text{cyt}}$  are an essential initial signal for scramblase activation and platelet PS exposure,<sup>12, 14</sup> they are not sufficient. In agonist-stimulated platelets, there is a threshold  $\text{Ca}^{2+}_{\text{cyt}}$  elevation that must be met for initiation of mPTP formation and subsequent PS exposure, as indicated by the different  $\text{Ca}^{2+}_{\text{cyt}}$  elevations in PS+ and PS- platelets. By altering the sensitivity of the mPTP, by CypD deletion or oxidant exposure, this threshold can be changed. Whether the role of  $\text{Ca}^{2+}_{\text{cyt}}$  elevation in PS exposure is solely to initiate  $\text{Ca}^{2+}_{\text{mit}}$  elevation and mPTP formation, or whether it acts together with a mPTP-initiated signal to regulate PS exposure remains to be determined.

Our results contradict a previous study that concluded that mitochondrial events have only a minimal role in agonist-initiated PS exposure.<sup>41</sup> However, this study was limited by the investigators use of cyclosporine to investigate the mPTP's role and their use of Jurkat cells to investigate a platelet process. Cyclosporine, in addition to inhibiting the mitochondrial cyclophilin CypD, inhibits multiple other cytoplasmic cyclophilins. Among these is Cyclophilin A, which interacts with several calcium-regulatory proteins in platelets, including sarcoendoplasmic calcium adenosine triphosphatase 2b (SERCA2b) and STIM1.<sup>42, 43</sup> Here, by using CypD null platelets, these key limitations are overcome.

Since the events that occur upstream and downstream of mPTP formation in the initiation of platelet PS exposure remain largely undefined, the potential role of ROS in these processes was examined in strongly-stimulated platelets. ROS mediate mPTP formation in reperfused, ischemic tissues,<sup>25, 44</sup> and we demonstrate here that exogenous addition of ROS can similarly impact mPTP formation, and subsequently PS exposure in platelets. Previous studies have primarily focused on ROS production in platelets stimulated with single agonists. Here we demonstrate a substantial increase in ROS production in platelets stimulated simultaneously with thrombin and convulxin, consistent with the recent findings of Arthur et al.<sup>45</sup> The ROS burst was independent of mPTP formation and required extracellular calcium entry. This suggests the possibility that ROS production, induced by heightened extracellular calcium levels, might potentiate mPTP formation and PS exposure in strongly-stimulated platelets. Future investigation into the source and role of this ROS burst in strongly-stimulated platelets may provide additional insights into how mPTP formation and PS exposure are initiated.

Elevated calcium levels within the mitochondrial matrix are a key trigger of mPTP formation,<sup>32</sup> and there is close communication of the mitochondria with the calcium-rich endoplasmic reticulum.<sup>46</sup> In strongly-stimulated platelets  $\text{Ca}^{2+}_{\text{mit}}$  was increased. To demonstrate the importance of  $\text{Ca}^{2+}_{\text{mit}}$  elevation in PS exposure, two complementary approaches were utilized, one focused on blockade of the mitochondrial calcium uniporter (MCU),<sup>38, 39</sup> the second focused on disruption of the electrical gradient driving calcium entry through the MCU. Inhibition of  $\text{Ca}^{2+}_{\text{mit}}$  entry using either of these approaches effectively abrogated  $\text{Ca}^{2+}_{\text{mit}}$  elevation, mPTP formation, and PS exposure, but did not affect  $\text{Ca}^{2+}_{\text{cyt}}$  transients, initial integrin  $\alpha_{\text{IIb}}\beta_3$  activation, or granule release.

Our findings suggest the potential utility of MCU inhibitors as specific antagonists of platelet procoagulant activity. Intriguingly, a recent study reported that inhibitors of mitochondrial respiration inhibited platelet-activated blood coagulation.<sup>47</sup> These respiratory inhibitors, due to their effects on mitochondrial respiration, would also be expected to inhibit  $\text{Ca}^{2+}_{\text{mit}}$  entry and PS exposure, as a result of their depolarizing effects. Metformin, a first line anti-diabetic drug, acts in a similar fashion to inhibit mitochondrial respiration and calcium uptake, a finding specifically demonstrated here for platelets. Previous studies of platelet function in metformin-treated patients have only examined its effects on the aggregatory response, and have not examined PS exposure.<sup>48</sup> It is tempting to postulate that

metformin's effectiveness in the prevention of cardiovascular disease in diabetes, above and beyond its antihyperglycemic actions,<sup>49</sup> can be attributed in part to its anti-procoagulant effect in platelets. Inhibition of mitochondrial calcium entry, either through inhibition of the MCU or through the use of reversible mitochondrial depolarizing agents, such as metformin,<sup>47</sup> may provide an attractive therapeutic target in the treatment and prevention of thrombosis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

H.C. designed and performed the experiments, analyzed the data and wrote the manuscript; T.B.S. performed and assisted in the design of confocal microscopy experiments; L.M. performed experiments; M.B.W. assisted in the design and analysis of confocal microscopy experiments; S.M.J. participated in experimental design and data analysis and wrote the manuscript.

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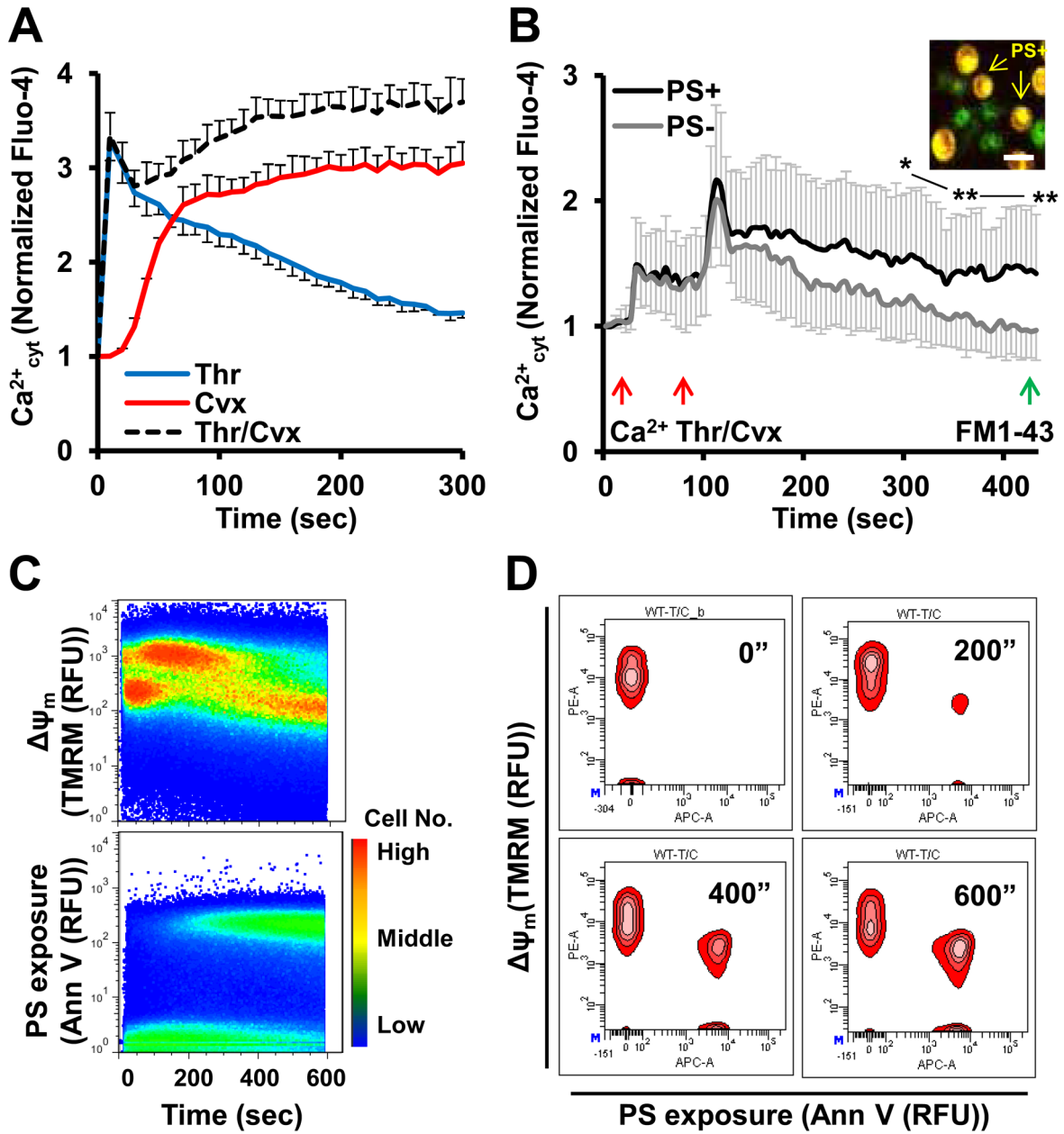
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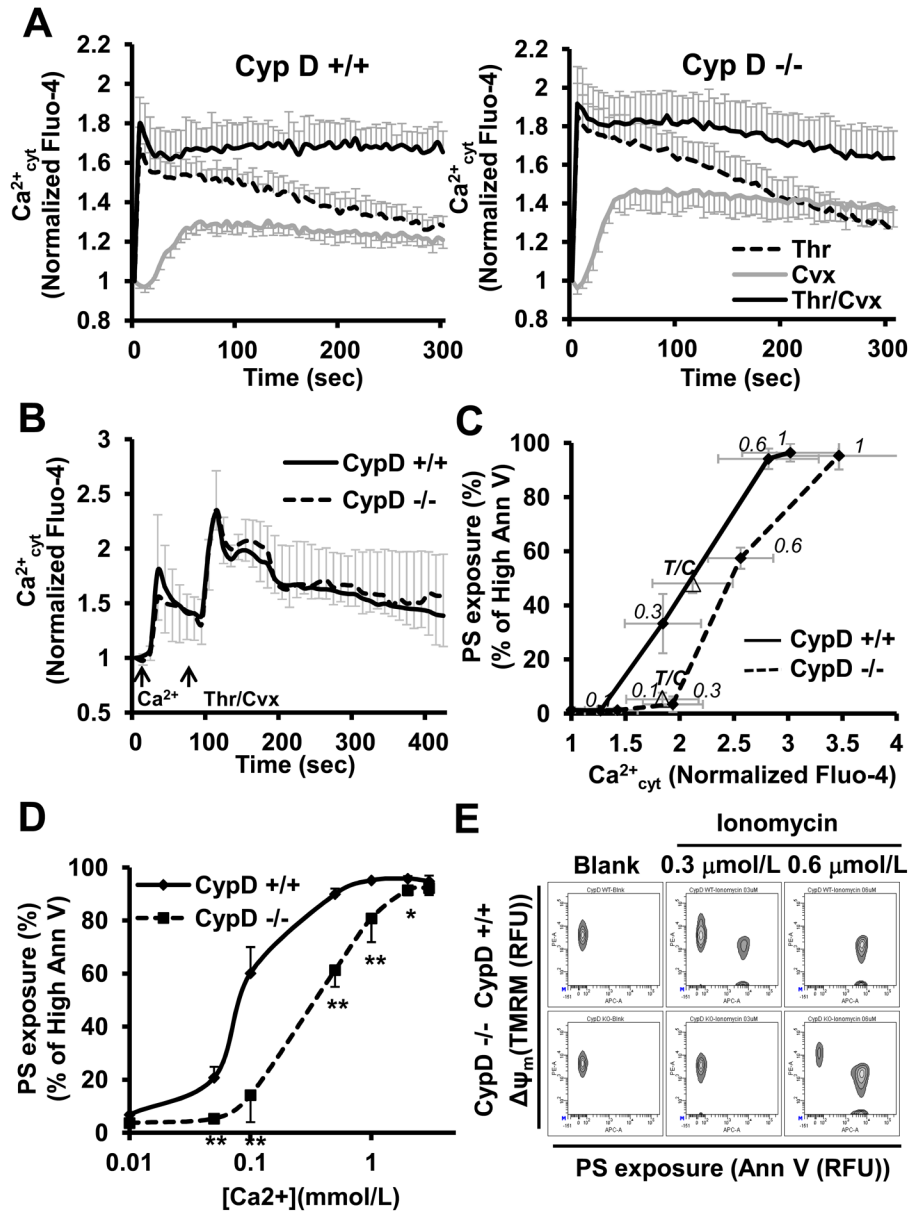
**Figure 1. Calcium and mitochondrial events in strongly-stimulated platelets**  
 (A)  $Ca^{2+}_{cyt}$  transients in stimulated platelets. Murine platelets in suspension were loaded with the calcium indicator Fluo-4-AM, stimulated with the indicated agonist(s), and analyzed by fluorometry.  $n=3$ . (B)  $Ca^{2+}_{cyt}$  transients in strongly-stimulated adherent platelets. Murine platelets were allowed to adhere on a fibrinogen-coated coverglass and loaded with Fluo-4-AM. Calcium then Thr (0.5 U/ml) and Cvx (100 ng/ml) were flowed over the adherent platelets at the indicated time points. FM1-43 was added at the indicated time point to detect PS exposure (enclosed image). FM1-43<sup>+</sup> and FM1-43<sup>-</sup> platelets are identified as PS<sup>+</sup> and PS<sup>-</sup>, respectively. (C, D)  $\Delta\psi_m$  and PS exposure in strongly-stimulated platelets. (C) Murine platelets were labeled with the  $\Delta\psi_m$  indicator TMRM and Ann V to detect PS exposure. Platelets were stimulated with thrombin and convulxin and then analyzed by flow cytometry for 600 seconds. Pseudo-colors are used to indicate population

density. Representative of four separate experiments. (D) Murine platelets were labeled with TMRM and Ann V, stimulated with Thr and Cvx, and analyzed by flow cytometry at 200, 400 and 600 seconds. Contour plots are utilized to indicate population density. \* $p < 0.05$ , \*\* $p < 0.01$

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**Figure 2. Agonist-initiated loss of  $\Delta\psi_m$ , but not  $Ca^{2+}_{cyt}$  elevation, is abrogated in  $CypD^{-/-}$  platelets**

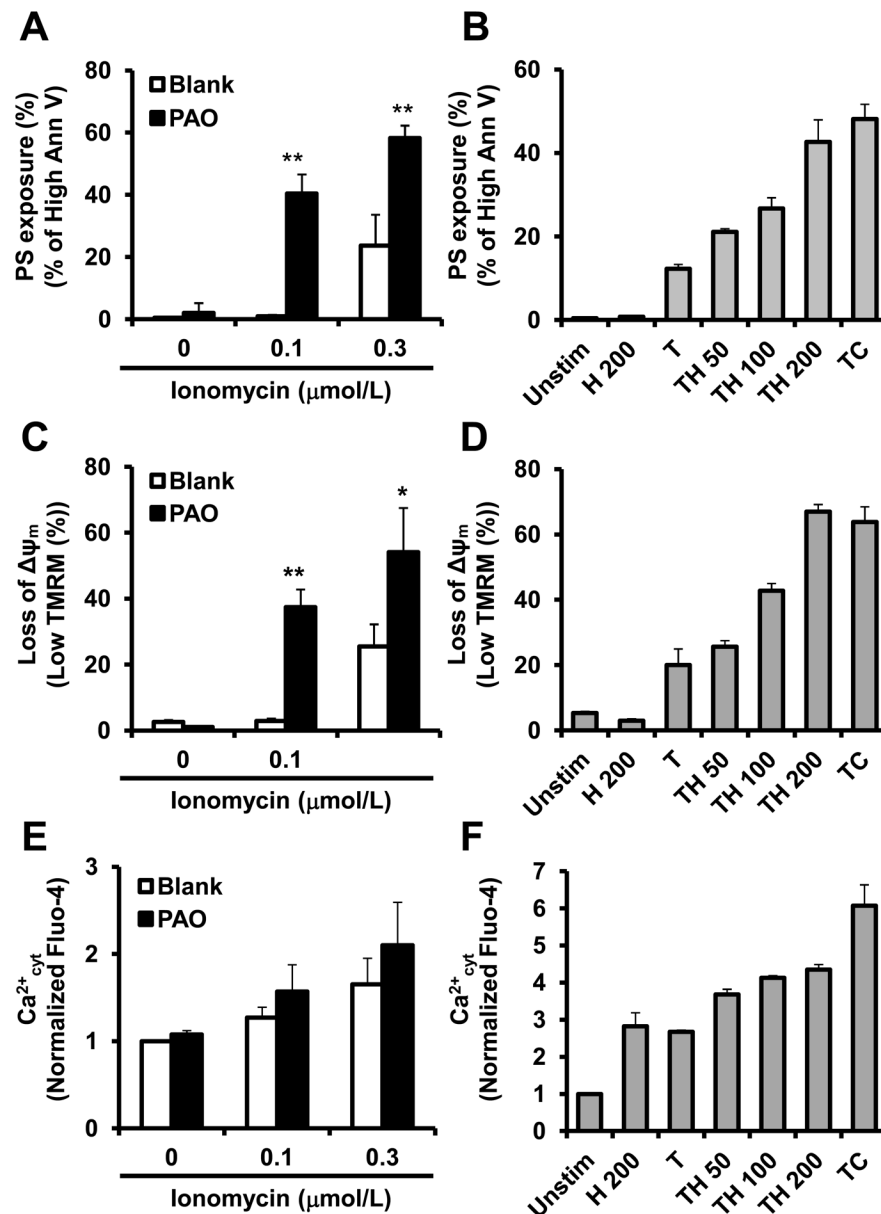
(A, B)  $Ca^{2+}_{cyt}$  transients in agonist-stimulated  $CypD^{+/+}$  and  $CypD^{-/-}$  platelets (A)  $CypD^{+/+}$  and  $CypD^{-/-}$  platelets were stained with TMRM and Ann V, stimulated with thrombin and convulxin (Thr/Cvx), and analyzed by flow cytometry for five minutes.  $n = 5$ . (B) Fibrinogen-adherent Fluo-4-AM stained  $CypD^{+/+}$  and  $CypD^{-/-}$  platelets were analyzed by confocal microscopy following stimulation with calcium, then Thr/Cvx. (C–E) Calcium sensitivity of  $CypD^{+/+}$  and  $CypD^{-/-}$  platelets. (C)  $CypD^{+/+}$  and  $CypD^{-/-}$  platelets were stained with Fluo-4-AM and Ann V, stimulated with the indicated concentration of ionomycin (circles with ionomycin concentration indicated in italics) or Thr/Cvx (triangles) and analyzed by flow cytometry.  $n=4$ . (D)  $CypD^{+/+}$  and  $CypD^{-/-}$  platelets were stained with AnnV and stimulated with 1  $\mu M$  ionomycin in the presence of the indicated extracellular calcium concentration. (E)  $CypD^{+/+}$  and  $CypD^{-/-}$  platelets were stained with TMRM and

Ann V, stimulated with the indicated concentration of ionomycin, and analyzed by flow cytometry. Contour plots are utilized to indicate population density. \* $p < 0.05$ , \*\* $p < 0.01$

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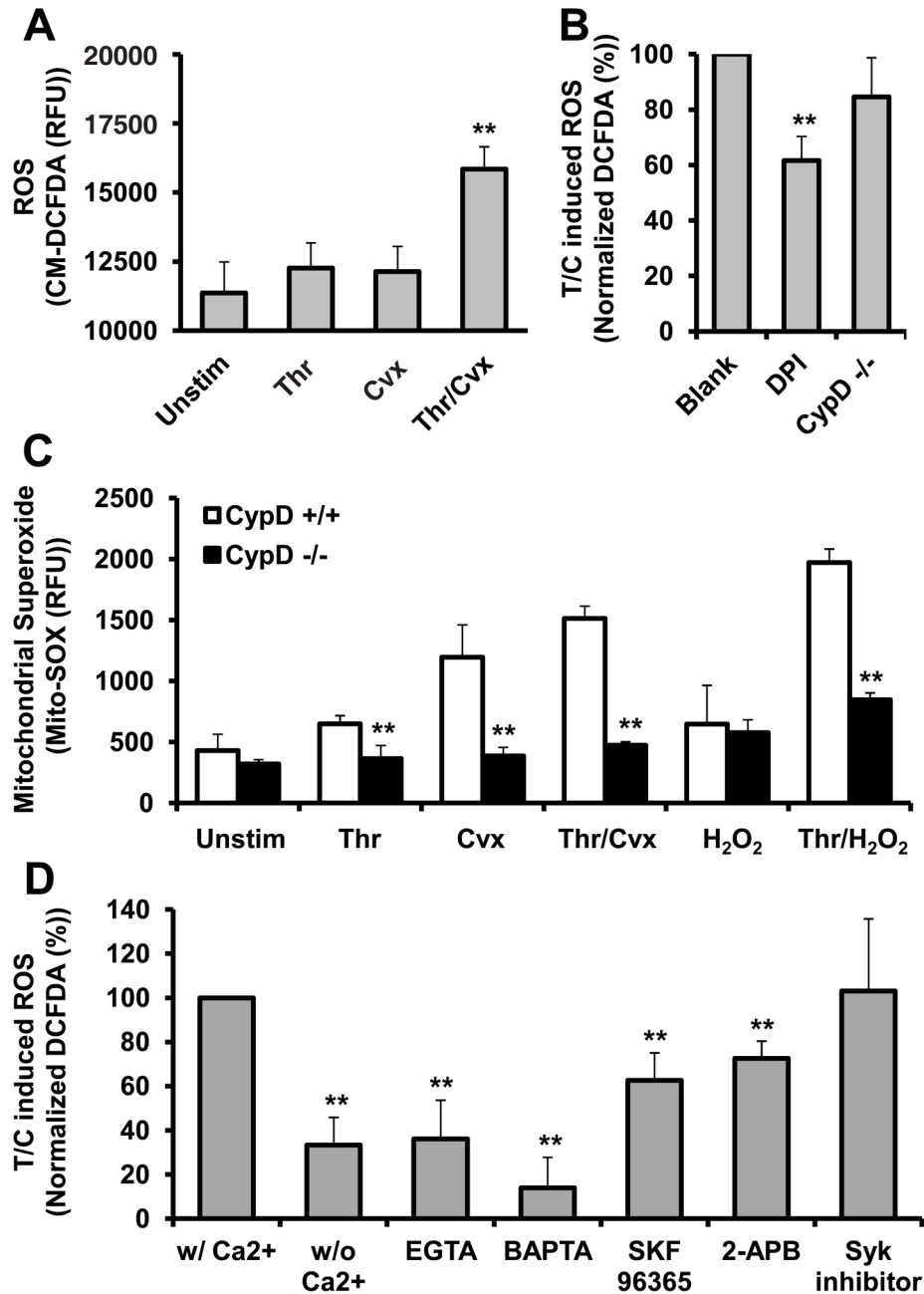
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**Figure 3. Reactive oxygen species modulate agonist-initiated PS exposure independent of the magnitude of  $Ca^{2+}_{cyt}$  elevation**

PS exposure,  $\Delta\psi_m$ , or  $Ca^{2+}_{cyt}$  were analyzed by flow cytometry. (A, B) Murine platelets were stimulated with the indicated doses of (A) ionomycin in the presence or absence of 25  $\mu$ M phenylarsine oxide (PAO) or with (B) thrombin (T)  $\pm$  indicated dose of hydrogen peroxide ( $\mu$ M)(H) or convulxin (100 ng/mL)(C) and labeled with APC-Ann V. (C, D) Using agonists as indicated,  $\Delta\psi_m$  was analyzed using TMRM. (E, F) Using agonists as indicated,  $Ca^{2+}_{cyt}$  was analyzed using Fluo-4-AM. n = 4-5. \*p<0.05, \*\*p<0.01



**Figure 4. Increased ROS production in strongly-stimulated platelets is dependent on extracellular calcium, but not mPTP formation**

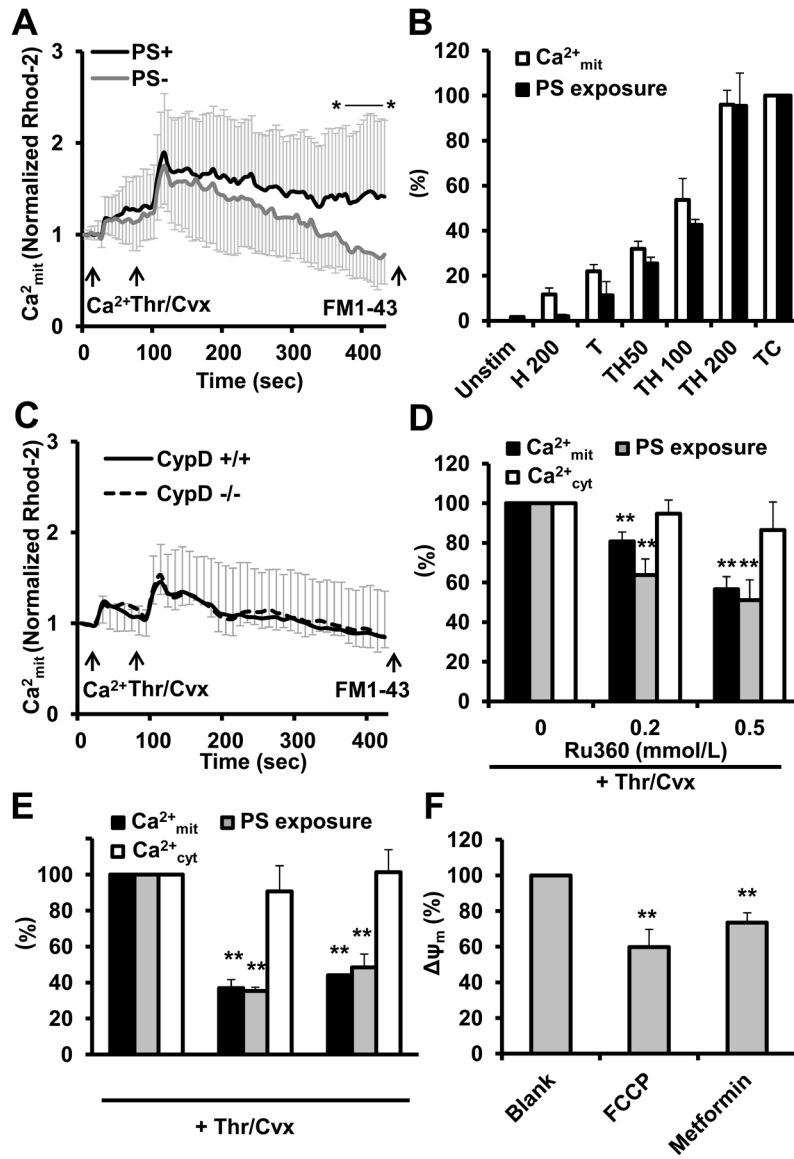
(A) Murine platelets were stained with CM-DCFDA (5  $\mu$ M) for thirty minutes and stimulated with the indicated agonists for seven minutes and analyzed by flow cytometry. n=4. (B) Untreated or DPI (10  $\mu$ M) treated platelets or CypD<sup>-/-</sup> platelets were stained with H<sub>2</sub>-DCFDA (50  $\mu$ M) for thirty minutes, stimulated with the Thr/Cvx and analyzed by fluorometry at five minutes. n=4. (C) CypD<sup>+/+</sup> and CypD<sup>-/-</sup> platelets were stimulated with the indicated agonists for five minutes, stained with Mito-SOX (5  $\mu$ M) for five minutes, and analyzed by flow cytometry. n=4. (D) Murine platelets were stained with H<sub>2</sub>-DCFDA (50  $\mu$ M) for thirty minutes in the presence of EGTA (5mM), BAPTA-AM (10  $\mu$ M), SKF96365

(25  $\mu\text{M}$ ), 2-APB (10  $\mu\text{M}$ ), Syk inhibitor (2  $\mu\text{M}$ ). Platelets were then stimulated with Thr/Cvx and analyzed by fluorometry after five minutes. n = 4. \*\*p<0.01

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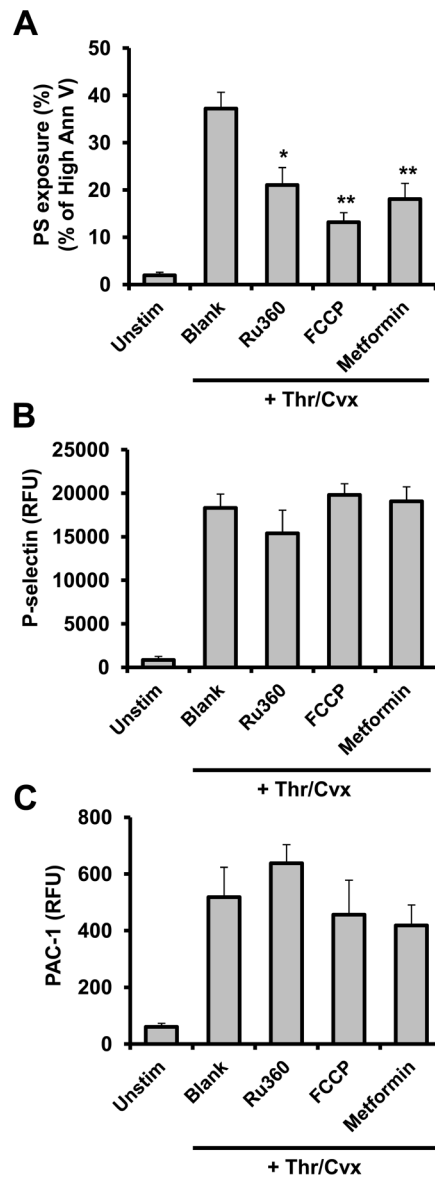
**Figure 5. Mitochondrial calcium regulates agonist-initiated PS exposure**  
 (A–C)  $Ca^{2+}_{mit}$  in stimulated platelets. (A) Fibrinogen-adherent, Rhod-2-AM stained, murine platelets were analyzed by confocal microscopy. Calcium, Thr/Cvx, and FM1-43 were added at the indicated time points. (B)  $Ca^{2+}_{mit}$  elevations and PS exposure in stimulated platelets. Murine platelets were stimulated with the indicated agonists (0.5 U/ml Thr  $\pm$  indicated dose of hydrogen peroxide ( $\mu$ M) or 100 ng/mL convulxin), labeled with APC-Ann V and Rhod-2-AM and analyzed by flow cytometry. Data were normalized to the intensity of Thr/Cvx-stimulated platelets. n=4. (C) Fibrinogen-adherent and Rhod-2-AM stained CypD<sup>+/+</sup> and CypD<sup>-/-</sup> platelets were analyzed by confocal microscopy. Calcium and Thr/Cvx were added at the indicated time points. (D–E) Human platelets were preincubated with Ru360 (30 minutes (D)), FCCCP (2  $\mu$ M, 5 minutes (E)) or metformin (10 mM, 30 minutes (E)), and stained with Rhod-2-AM and APC-Ann V or Fluo-4-AM. Labeled platelets were then stimulated with Thr/Cvx and analyzed by flow cytometry after seven minutes. n=3. Fluo-4-AM labeled platelets were stimulated with Thr/Cvx and analyzed by fluorometry after five minutes. Data were normalized to the intensity of Thr/Cvx-stimulated platelets.

n=3. (F) Human platelets were pretreated with FCCP (2  $\mu$ M) or metformin (10 mM) for thirty minutes and stained with TMRM to determine platelet  $\Delta\psi_m$  prior to stimulation. Data were normalized to the TMRM intensity of unstimulated platelets. n=3. \*p<0.05, \*\*p<0.01.

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**Figure 6. Inhibition of  $\text{Ca}^{2+}_{\text{mit}}$  uptake blocks PS exposure, but not integrin  $\alpha_{\text{IIb}}\beta_3$  activation or granule release**

(A) Human platelets were pre-treated with Ru360 (0.5 mM, 30 minutes), FCCP (2  $\mu\text{M}$ , 5 minutes) or metformin (10 mM, 30 minutes), labeled with APC-Annexin V, stimulated with Thr/Cvx, and analyzed by flow cytometry after seven minutes.  $n=3$ . (B, C) Human platelets were pre-treated with Ru360 (0.5 mM, 30 minutes), FCCP (2  $\mu\text{M}$ , 5 minutes) or metformin (10 mM, 30 minutes), labeled with PE-P-selectin and FITC-PAC-1, stimulated with Thr and Cvx, and analyzed by flow cytometry after one minute. Unstim means unstimulated platelets.  $n=3$ . \* $p<0.05$ , \*\* $p<0.01$