

Mitochondrial Calcium and Reactive Oxygen Species Regulate Agonist-Initiated Platelet Phosphatidylserine Exposure

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Objective—To study the interactions of cytoplasmic calcium elevation, mitochondrial permeability transition pore (mPTP) formation, and reactive oxygen species formation in the regulation of phosphatidylserine (PS) exposure in platelets.

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

Conclusion—Sustained cytoplasmic calcium 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. (*Arterioscler Thromb Vasc Biol.* 2012;32:2946-2955.)

Key Words: calcium ■ mitochondria ■ mitochondrial permeability transition pore ■ phosphatidylserine ■ platelets

Platelet phosphatidylserine (PS) exposure amplifies thrombin generation by facilitating assembly of the tenase and prothrombinase complexes.¹ 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.² The importance of PS exposure in normal hemostasis is illustrated by the congenital disorder Scott syndrome, a bleeding diathesis characterized by delayed hemostasis and impaired wound healing.³ In Scott syndrome, absence of the transmembrane protein 16F (TMEM16F) results in a defect in agonist-initiated scramblase activity and platelet PS exposure.^{4,5}

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.⁶⁻⁸ 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.^{7,9} 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.¹⁰ The relative physiological 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 cytoplasmic calcium ($\text{Ca}^{2+}_{\text{cyt}}$) levels in the regulation of high-level PS exposure and scramblase activity is well appreciated,^{9,11} and both store-operated calcium-entry (SOCE) and noncapacitative calcium-entry mechanisms have been implicated in the regulation of agonist-initiated PS exposure.¹²⁻¹⁴ Potential determinants of prolonged $\text{Ca}^{2+}_{\text{cyt}}$ elevation have been identified, including tyrosine kinases, which potentiate,⁹ and isoforms of protein kinase C, which inhibit, calcium signal generation and procoagulant activity.¹⁵ Increased potassium efflux through Ca^{2+} -activated K⁺ channels may also facilitate PS exposure.¹⁶

Mitochondrial events have been implicated as important determinants of platelet PS exposure. The mitochondrial permeability transition pore (mPTP) is a nonselective multiprotein pore that spans the inner mitochondrial membrane, the formation of which causes a rapid loss of mitochondrial

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transmembrane potential ($\Delta\psi_m$).¹⁷ A key regulator of mPTP function is the peptidylprolylisomerase cyclophilin D (CypD), and in its absence mPTP formation is abrogated.^{18–20} In strongly stimulated platelets, mPTP formation is closely associated with high-level PS exposure,^{21,22} and in the absence of CypD 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.²¹ PS exposure can also be initiated by a BH3-protein mimetic. Although agonist-initiated PS exposure is unaffected in the absence of the mitochondrially localized BH3 proteins, Bax and Bax,²³ their absence ablates BH3-mimetic induced PS exposure. This BH3-protein regulated pathway is unaffected in the absence of CypD and has been implicated in platelet aging.^{23,24}

Important determinants of mPTP formation in other cell types include both calcium and reactive oxygen species (ROS).¹⁷ In ischemic myocytes subject to reperfusion, it is increased ROS production, not calcium elevation, that initiates mPTP formation.²⁵ Although ROS production occurs in platelets activated by a single agonist,²⁶ 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.

Materials and 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^{-/-} mice, which are homozygous for a targeted deletion of the *Ppif* gene, and control CypD^{+/+} mice were generated as described previously¹⁸ 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.²⁷ Isolation of human platelets is described in Methods in the online-only Data Supplement.

Flow Cytometry

Washed platelets in the Tyrode buffer with CaCl₂ were stimulated with agonist(s) and preincubated with the pharmacological agents as indicated. For experiments evaluating loss of $\Delta\psi_m$ and ROS generation, platelets were incubated with 500 nmol/L tetramethylrhodamine methyl ester²⁸ or 5 μ mol/L 5-(and 6-) chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate ethyl ester,²⁹ respectively, before stimulation. For experiments detecting mitochondrial superoxide generation, platelets were stimulated with the indicated agonist(s) and then incubated with 5 μ mol/L Mito-SOX. Labeled platelets were evaluated

by flow cytometry. See Methods in the online-only Data Supplement for details.

Confocal Microscopy

Washed platelets in the mTyrode buffer were allowed to adhere to a fibrinogen-coated (0.1 mg/mL) coverglass for 30 minutes in the presence of 4 μ mol/L Fluo-4-AM and 5 μ mol/L Rhod-2-AM. Epifluorescence was continuously examined, and the buffer was sequentially changed to mTyrode buffer with calcium, and then thrombin convulxin (Thr/Cvx). Subsequently, FM1-43 was added to allow identification of PS-exposed platelets.³⁰ Data were normalized to the initial staining intensity before platelet stimulation. See Methods in the online-only Data Supplement for details.

Fluorometry

Washed platelets were stimulated with the indicated agonist(s) and evaluated by fluorometry for 5 minutes. For experiments evaluating calcium transients or ROS generation, platelets were preincubated with 1 μ mol/L Fluo-4-AM or 50 μ mol/L dichlorodihydrofluorescein diacetate for 30 minutes. Data were normalized to the initial staining intensity before platelet stimulation.

Statistical Analysis

Data are presented as mean \pm SD. Significant differences between means were determined by Student *t* test or ANOVA. Significance was set at *P* < 0.05.

Results

Calcium and Mitochondrial Events in Strongly Stimulated Platelets

Ca²⁺_{cyt} levels, mPTP formation, and PS exposure were examined in suspended and adherent platelets. Stimulation with thrombin caused a sudden increase in Ca²⁺_{cyt}; the glycoprotein VI (GPVI) agonist convulxin caused a gradual, but more sustained, increase in Ca²⁺_{cyt} levels (Figure 1A); and simultaneous stimulation with Thr/Cvx caused a rapid and sustained increase in Ca²⁺_{cyt} in both suspended and adherent platelets (Figure 1A and 1B). By using adherent platelets, we were able to continuously analyze Ca²⁺_{cyt} transients in individual platelets. This allowed a retrospective comparison of Ca²⁺_{cyt} transients in platelets that became PS⁺ (\approx 50%) versus those that remained PS⁻. Initial Ca²⁺_{cyt} peak levels differed minimally in PS⁺ and PS⁻ subpopulations. Over time a gradual decrease in Ca²⁺_{cyt} was observed in PS⁻ platelets, whereas in PS⁺ platelets the elevation in Ca²⁺_{cyt} was more sustained.

To simultaneously evaluate Ca²⁺_{cyt}, $\Delta\psi_m$, and PS exposure, multicolor flow cytometry was used (Figure 1C and 1D). Thr/Cvx stimulation caused a rapid increase in Ca²⁺_{cyt} levels that was maximal within 20 seconds (unpublished results), a result consistent with that observed using fluorometry and confocal microscopy (Figure 1A and 1B). Examination of $\Delta\psi_m$ in strongly stimulated platelets revealed a rapid increase in tetramethylrhodamine methyl ester fluorescence consistent with mitochondrial hyperpolarization (increased $\Delta\psi_m$). This was followed 300 to 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 and 1D). These experiments demonstrate that sustained elevations in Ca²⁺_{cyt} 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.

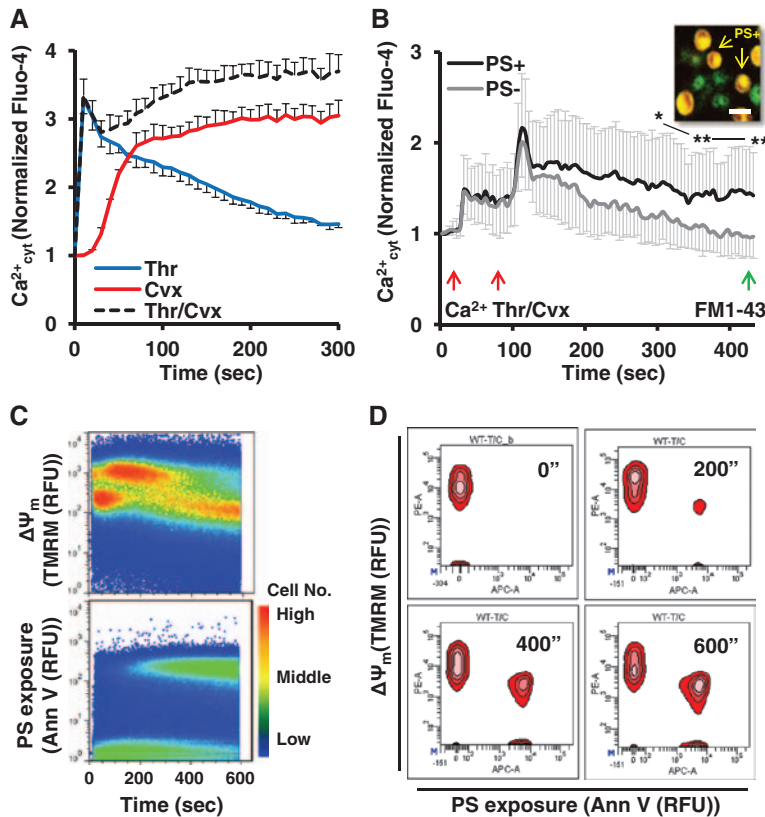


Figure 1. Calcium and mitochondrial events in strongly stimulated platelets. **A**, Cytoplasmic calcium ($\text{Ca}^{2+}_{\text{cyt}}$) transients in stimulated platelets. Murine platelets in suspension were loaded with the calcium indicator Fluo-4-AM, stimulated with the indicated agonist(s), and analyzed by fluorimetry. $n=3$. **B**, $\text{Ca}^{2+}_{\text{cyt}}$ 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 thrombin (Thr; 0.5 U/mL) and convulxin (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 phosphatidylserine (PS) exposure (enclosed image). FM1-43⁺ and FM1-43⁻ platelets are identified as PS⁺ and PS⁻, respectively. **C** and **D**, Mitochondrial transmembrane potential ($\Delta\psi_m$) and PS exposure in strongly stimulated platelets. **C**, Murine platelets were labeled with the $\Delta\psi_m$ indicator tetramethylrhodamine methyl ester (TMRM) and Annexin V (Ann V) to detect PS exposure. Platelets were stimulated with thrombin and convulxin (Thr/Cvx) and then analyzed by flow cytometry for 600 seconds. Pseudocolors are used to indicate population density. Representative of 4 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 used to indicate population density. * $P<0.05$, ** $P<0.01$. RFU indicates relative fluorescent units.

Agonist-Initiated Loss of $\Delta\psi_m$, But Not $\text{Ca}^{2+}_{\text{cyt}}$ Elevation, Is Abrogated in $\text{CypD}^{-/-}$ Platelets

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

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

We also tested the relationship of mPTP formation and low-level PS exposure using the non- Ca^{2+} -dependent PS binding protein C2-lactadherin (Figure I in the online-only Data Supplement).¹⁰ Unlike high-level PS exposure, low-level PS exposure was relatively unaffected by the absence of CypD, 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,¹⁹ the effects of calcium-ionophore stimulation on $\text{Ca}^{2+}_{\text{cyt}}$, mPTP formation and PS exposure were examined in $\text{CypD}^{+/+}$ and $\text{CypD}^{-/-}$ platelets. Experiments were performed in which either ionomycin (Figure 2C) or extracellular calcium concentrations (Figure 2D) were varied, and the effects of the absence of CypD 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 physiological calcium concentration, maximal PS exposure occurred with the addition of 0.6 $\mu\text{mol/L}$ and 1.0 $\mu\text{mol/L}$ ionomycin for $\text{CypD}^{+/+}$ and $\text{CypD}^{-/-}$ platelets, respectively (Figure 2C). When extracellular calcium concentration was varied and fixed amount of ionomycin was used, maximal PS exposure occurred in the presence of 1 mmol/L and 2 mmol/L extracellular calcium for $\text{CypD}^{+/+}$ and $\text{CypD}^{-/-}$ platelets, respectively (Figure 2D). No significant differences in $\text{Ca}^{2+}_{\text{cyt}}$ were observed between $\text{CypD}^{+/+}$ and $\text{CypD}^{-/-}$ platelets using either maneuver, and regardless of genotype or maneuver, mPTP formation was closely associated with PS exposure (Figure 2E and unpublished observations). $\text{Ca}^{2+}_{\text{cyt}}$ elevation caused by Thr/Cvx

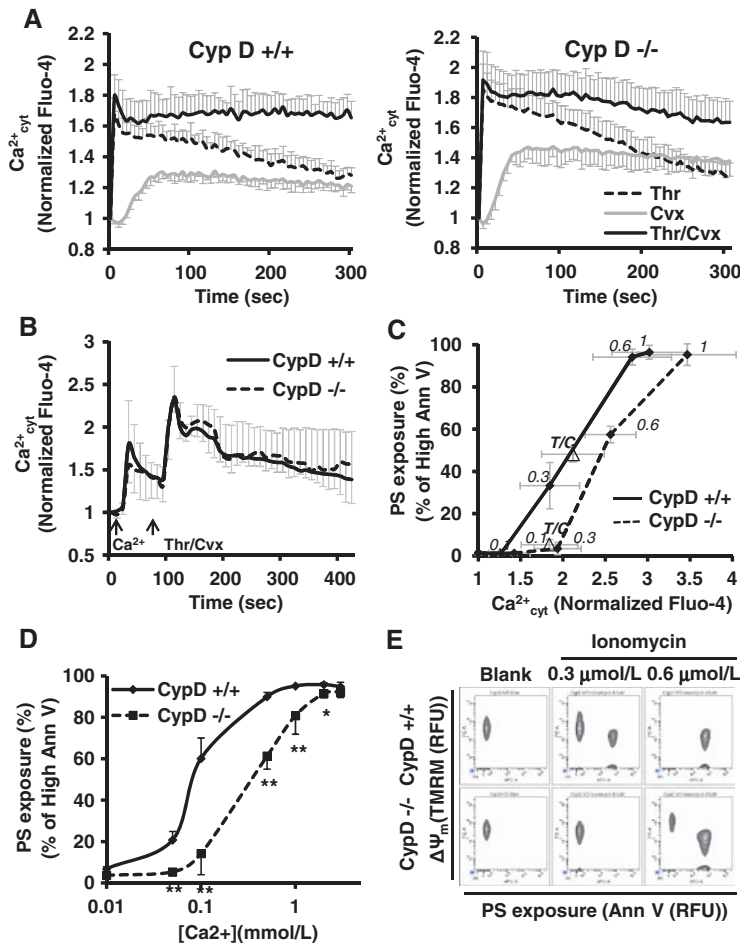


Figure 2. Agonist-initiated loss of mitochondrial transmembrane potential ($\Delta\psi_m$), but not cytoplasmic calcium ($\text{Ca}^{2+}_{\text{cyt}}$) elevation, is abrogated in cyclophilin D (CypD) $^{-/-}$ platelets. **A** and **B**, $\text{Ca}^{2+}_{\text{cyt}}$ transients in agonist-stimulated CypD $^{+/+}$ and CypD $^{-/-}$ platelets (**A**) CypD $^{+/+}$ and CypD $^{-/-}$ platelets were stained with tetramethylrhodamine methyl ester (TMRM) and Annexin V (Ann V), stimulated with thrombin and convulxin (Thr/Cvx), and analyzed by flow cytometry for 5 minutes. **B**, Fibrinogen-adherent Fluo-4-AM-stained CypD $^{+/+}$ and CypD $^{-/-}$ platelets were analyzed by confocal microscopy after stimulation with calcium, then Thr/Cvx. **C–E**, Calcium sensitivity of cyclophilin D (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. **D**, CypD $^{+/+}$ and CypD $^{-/-}$ platelets were stained with AnnV and stimulated with 1 $\mu\text{mol/L}$ 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 used to indicate population density. * $P < 0.05$, ** $P < 0.01$. RFU indicates relative fluorescence units.

stimulation was plotted versus PS exposure using the ionomycin response curves (triangles in Figure 2C). Interestingly, $\text{Ca}^{2+}_{\text{cyt}}$ elevation and PS exposure generated by these physiological agonists corresponded with the PS response observed when a similar $\text{Ca}^{2+}_{\text{cyt}}$ elevation was generated using ionomycin. These results indicate that the absence of CypD affects platelet PS exposure by altering the calcium sensitivity of the mPTP, and suggest the calcium sensitivity of mPTP as an important determinant of Thr/Cvx-initiated PS exposure.

Reactive Oxygen Species Positively Modulate PS Exposure Independent of the Magnitude of $\text{Ca}^{2+}_{\text{cyt}}$ Elevation

In addition to $\text{Ca}^{2+}_{\text{cyt}}$, another key determinant of mPTP formation is ROS.¹⁷ The effects of ROS on mPTP formation are calcium independent and are mediated by thiol oxidation of mPTP regulatory components.³³ 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 $\text{Ca}^{2+}_{\text{cyt}}$ were examined.

In platelets treated with phenylarsine oxide, an oxidant with potent effects on mPTP formation,³⁴ platelet sensitivity to ionomycin stimulation was markedly increased (Figure 3A). Similarly, increasing amounts of the physiological oxidant H_2O_2 potentiated thrombin-initiated PS exposure²¹ (Figure 3B).

Whether the effects of these oxidants are mediated through $\text{Ca}^{2+}_{\text{cyt}}$ elevation or mPTP formation has not previously been examined. mPTP formation was potentiated by phenylarsine oxide (Figure 3C) or H_2O_2 (Figure 3D) treatment, and loss of $\Delta\psi_m$ and PS exposure were closely associated (compare Figures 3A and 3B with Figures 3C and 3D). In contrast, when $\text{Ca}^{2+}_{\text{cyt}}$ levels were examined, no significant difference was noted between phenylarsine oxide-treated and untreated platelets (Figure 3E). Similarly, in Thr/ H_2O_2 -stimulated platelets (compare Figure 3B and Figure 3F), $\text{Ca}^{2+}_{\text{cyt}}$ elevation and PS exposure were poorly correlated although here the presence of H_2O_2 positively impacted thrombin-initiated $\text{Ca}^{2+}_{\text{cyt}}$ at a concentration as low as 50 $\mu\text{mol/L}$. These results demonstrate that ROS can increase the platelet's potential for PS exposure independent of its effects on $\text{Ca}^{2+}_{\text{cyt}}$. The close association of mPTP formation, but not $\text{Ca}^{2+}_{\text{cyt}}$ elevation, with PS exposure induced in the presence of ROS provides further evidence that $\text{Ca}^{2+}_{\text{cyt}}$ 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

Because ROS profoundly impacted platelet mPTP formation and PS exposure (Figure 3), their production in strongly

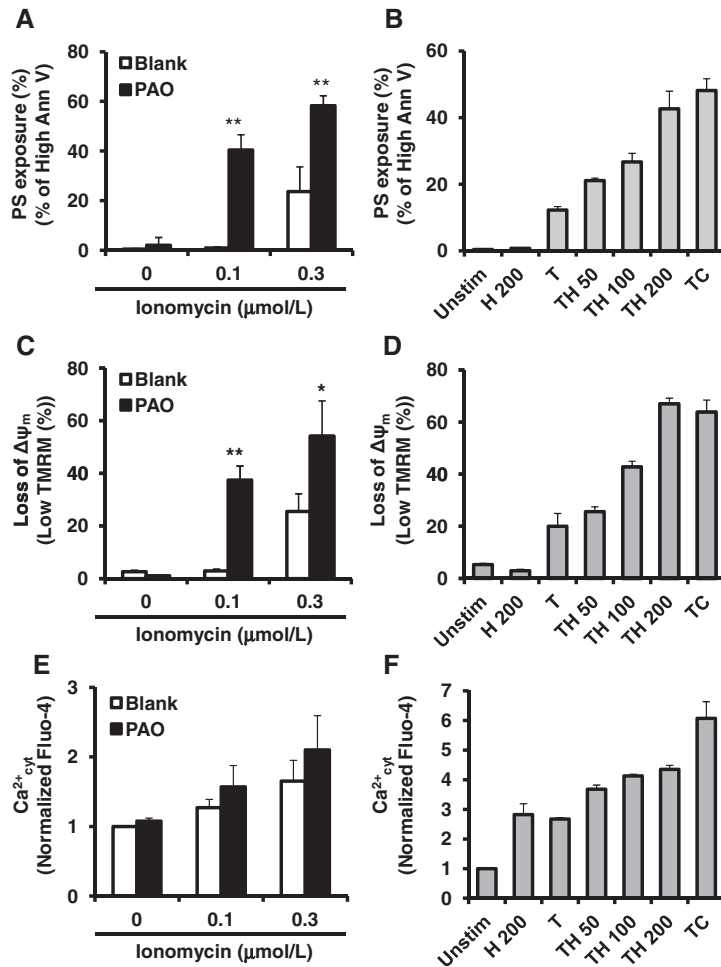


Figure 3. Reactive oxygen species modulate agonist-initiated phosphatidylserine (PS) exposure independent of the magnitude of cytoplasmic calcium (Ca^{2+}_{cyt}) elevation. PS exposure, mitochondrial transmembrane potential ($\Delta\psi_m$), or Ca^{2+}_{cyt} were analyzed by flow cytometry. **A** and **B**, Murine platelets were stimulated with the indicated doses of (A) ionomycin in the presence or absence of 25 μ M phenylarsine oxide (PAO) or with (B) thrombin (T) \pm indicated dose of H_2O_2 (μ M/L, H) or convulxin (100 ng/mL, C) and labeled with-Ann V. **C** and **D**, Using agonists as indicated, $\Delta\psi_m$ was analyzed using tetramethylrhodamine methyl ester (TMRM). **E** and **F**, Using agonists as indicated, Ca^{2+}_{cyt} was analyzed using Fluo-4-AM. $n=4-5$. * $P<0.05$, ** $P<0.01$.

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.²⁶ ROS formation was investigated in platelets activated with either single or dual agonists using the ROS-sensitive dye dichlorodihydrofluorescein diacetate. Five minutes after stimulation a pronounced increase in ROS production was noted in platelets stimulated with Thr/Cvx together relative to platelets stimulated with either agonist alone (Figure 4A).

Key sources of ROS production within the platelet include nicotinamide adenine dinucleotide phosphate (NADPH) oxidases²⁶ and mPTP formation.³⁵ Addition of the nicotinamide adenine dinucleotide phosphate oxidase inhibitor diphenyleneiodonium blunted Thr/Cvx-initiated ROS production, whereas 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 after stimulation with either convulxin, Thr/Cvx or Thr/ H_2O_2 (Figure 4C). However, this increase in mitochondrial superoxide production, unlike the generalized ROS production measured by dichlorodihydrofluorescein diacetate, 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 dichlorodihydrofluorescein diacetate.

Because sustained elevation of Ca^{2+}_{cyt} is prominent in strongly stimulated platelets (Figure 1), the importance of extracellular Ca^{2+} in the regulation of ROS production in strongly stimulated platelets was investigated. ROS production required the presence of extracellular Ca^{2+} , and addition of the calcium chelators EGTA or 1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA) prevented ROS elevation (Figure 4D). The importance of SOCE and noncapacitative calcium entry in the ROS burst was examined. Addition of either SKF96365, an inhibitor of both SOCE and noncapacitative calcium entry,¹² or 2-aminoethoxydiphenyl borate (2-APB), an inhibitor of SOCE,³⁶ 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^{2+}_{mit}) Regulates Agonist-Initiated PS Exposure

The close communication between Ca^{2+}_{cyt} and Ca^{2+}_{mit} led us to examine the possibility that sustained Ca^{2+}_{cyt} elevations may affect mPTP formation and PS exposure by increasing Ca^{2+}_{mit} .³²

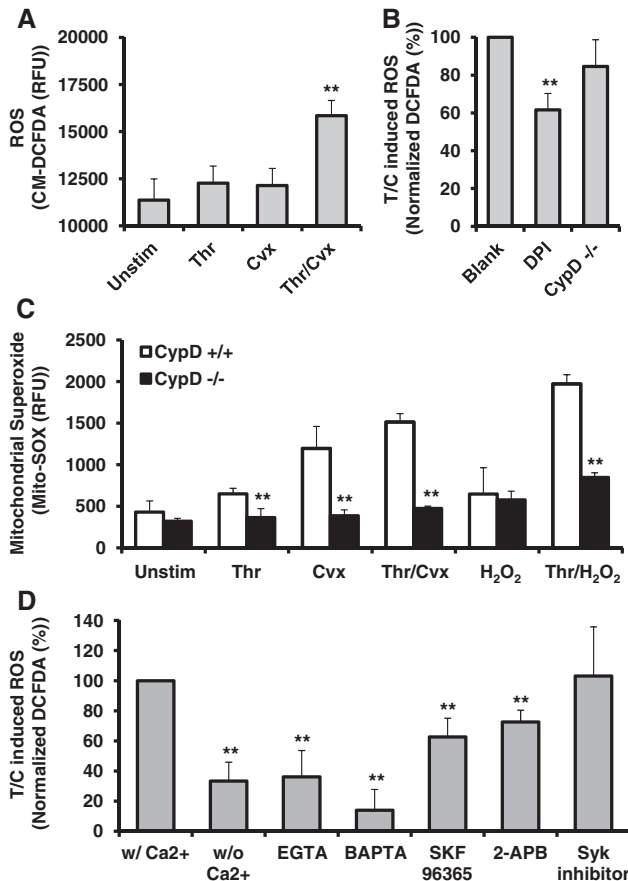


Figure 4. Increased reactive oxygen species (ROS) production in strongly stimulated platelets is dependent on extracellular calcium, but not mitochondrial permeability transition pore (mPTP) formation. **A**, Murine platelets were stained with 5-(and 6-) chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate ethyl ester (CM-DCFDA, 5 μ mol/L) for 30 minutes and stimulated with the indicated agonists for 7 minutes and analyzed by fluorimetry. $n=4$. **B**, Untreated or diphenyleneiodonium (DPI, 10 μ mol/L) treated platelets or cyclophilin D (CypD)^{-/-} platelets were stained with H₂-DCFDA (50 μ mol/L) for 30 minutes, stimulated with the thrombin and convulxin (Thr/Cvx), and analyzed by fluorimetry at 5 minutes. $n=4$. **C**, CypD^{+/+} and CypD^{-/-} platelets were stimulated with the indicated agonists for 5 minutes, stained with Mito-SOX (5 μ mol/L) for 5 minutes, and analyzed by flow cytometry. $n=4$. **D**, Murine platelets were stained with H₂-DCFDA (50 μ mol/L) for 30 minutes in the presence of EGTA (5 mmol/L), BAPTA-AM (10 μ mol/L), SKF96365 (25 μ mol/L), 2-APB (10 μ mol/L), Syk inhibitor (2 μ mol/L). Platelets were then stimulated with Thr/Cvx and analyzed by fluorimetry after 5 minutes. $n=4$. ** $P<0.01$.

Ca²⁺_{mit} levels were examined in adherent strongly stimulated platelets using the mitochondrial-specific calcium indicator Rhod-2 (Figure II in the online-only Data Supplement). Thr/Cvx stimulation caused a rapid increase in Ca²⁺_{mit} (Figure 5A and 5B) with higher Ca²⁺_{mit} levels observed in PS⁺ platelets. Interestingly, when Ca²⁺_{mit} was examined in Thr/H₂O₂-stimulated platelets, Ca²⁺_{mit}, unlike Ca²⁺_{cyt}, was closely correlated with PS exposure (Figure 5B). The relationship of Ca²⁺_{mit} elevation to mPTP formation and PS exposure was studied in strongly stimulated CypD^{+/+} and CypD^{-/-} platelets. As with Ca²⁺_{cyt},²¹ Ca²⁺_{mit} levels were similar between CypD^{+/+} and CypD^{-/-} platelets consistent with mPTP formation acting downstream of Ca²⁺_{mit} elevation to regulate agonist-initiated PS exposure (Figure 5C). Together, these results suggest that increased Ca²⁺_{mit} levels, influenced either by Ca²⁺_{cyt} 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 occurs as the result of passive flow of Ca²⁺_{cyt} down its electrical and chemical gradient through the mitochondrial calcium uniporter (MCU).³⁷⁻³⁹ The mitochondrial electrical gradient ($\Delta\psi_m$) is generated by the proton-motive force of the mitochondrial respiratory complexes. The importance of Ca²⁺_{mit} levels in PS exposure was tested in 2 ways, by using Ru360, a specific MCU inhibitor, and by disruption of the proton-motive force using mitochondrial depolarizing agents.³⁸ Because of its relative membrane impermeability, the effects of Ru360 have typically

been ascertained after incubation with the agent for 6 to 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 Ca²⁺_{mit} entry. To rule out potential off-target effects of Ru360 on other calcium entry pathways, Ca²⁺_{cyt} and Ca²⁺_{mit} were both assessed. Treatment of platelets with Ru360 effectively blocked the agonist-initiated increase in Ca²⁺_{mit}, and this decreased Ca²⁺_{mit} elevation was associated with a significant decrease in platelet PS exposure (Figure 5D). Consistent with a specific effect of Ru360 on Ca²⁺_{mit}, Ca²⁺_{cyt} levels were not substantially affected in Ru360-treated platelets. To further assess the importance of Ca²⁺_{mit}, the effects of mitochondrial depolarizing agents were examined. Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone is a protonophore, whereas metformin blocks complex I of the mitochondrial respiratory chain.⁴⁰ 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 Ca²⁺_{mit} (Figure 5E). Ca²⁺_{cyt} transients were unaffected by either depolarizing agent, and it was confirmed that pretreatment of platelets with either of these agents caused mitochondrial depolarization (Figure 5F). Together, these results indicate the importance of Ca²⁺_{mit} elevation in regulating platelet PS exposure and identify the MCU and the mitochondrial electrical gradient as key mediators regulating mitochondrial calcium entry in the activated platelet.

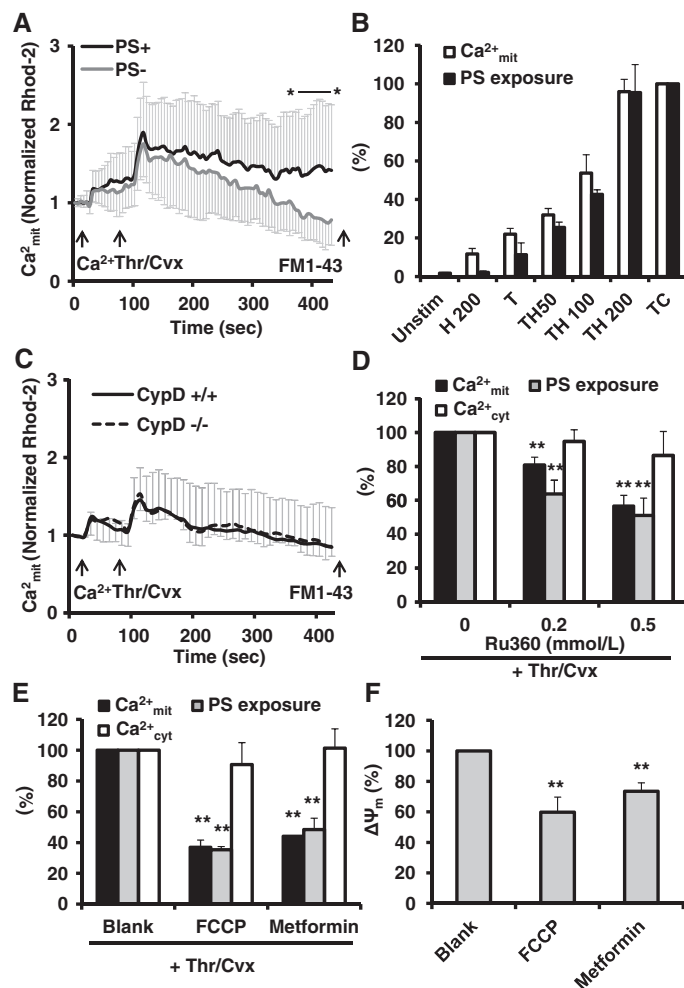


Figure 5. Mitochondrial calcium regulates agonist-initiated phosphatidylserine (PS) exposure. **A–C**, Mitochondrial calcium ($\text{Ca}^{2+}_{\text{mit}}$) in stimulated platelets. **A**, Fibrinogen-adherent, Rhod-2-AM-stained, murine platelets were analyzed by confocal microscopy. Calcium, thrombin and convulxin (Thr/Cvx), and FM1-43 were added at the indicated time points. **B**, $\text{Ca}^{2+}_{\text{mit}}$ elevations and PS exposure in stimulated platelets. Murine platelets were stimulated with the indicated agonists (0.5 U/mL Thr± indicated dose of H_2O_2 ($\mu\text{mol/L}$) or 100 ng/mL convulxin), labeled with 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 cyclophilin D (CypD)^{+/+} and CypD^{-/-} 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**), Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; 2 $\mu\text{mol/L}$, 5 minutes; **E**) or metformin (10 mmol/L, 30 minutes; **E**), and stained with Rhod-2-AM and Ann V or Fluo-4-AM. Labeled platelets were then stimulated with Thr/Cvx and analyzed by flow cytometry after 7 minutes. $n=3$. Fluo-4-AM-labeled platelets were stimulated with Thr/Cvx and analyzed by fluorometry after 5 minutes. Data were normalized to the intensity of Thr/Cvx-stimulated platelets. $n=3$. **F**, Human platelets were pretreated with FCCP (2 $\mu\text{mol/L}$) or metformin (10 mmol/L) for 30 minutes and stained with tetramethylrhodamine methyl ester (TMRM) to determine platelet mitochondrial transmembrane potential ($\Delta\psi_m$) before stimulation. Data were normalized to the TMRM intensity of unstimulated platelets. $n=3$. * $P<0.05$, ** $P<0.01$.

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.^{12–14} 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 used 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. Whereas each of the agents effectively inhibited PS exposure (Figure 6A), none affected either granule release, as measured by PAC-1 (Figure 6B), or the initial activation of integrin $\alpha_{\text{IIb}}\beta_3$ (Figure 6C), as measured by procaspase-activating compound 1. A similar effect of these pharmacological agents on platelet activation was observed using murine platelets (Figure III in the online-only Data Supplement).

Discussion

In strongly stimulating environments, platelet activation evokes a procoagulant response in a subpopulation of platelets.^{6–8} 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 $\Delta\psi_m$ in several experimental conditions and in CypD^{-/-} 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 to 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^{+/+} and CypD^{-/-} 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 the absence of CypD inhibition of mitochondrial calcium uptake, inhibited PS exposure, whereas agents that accentuated mPTP formation, namely phenylarsine oxide, 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 downstream 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,¹ 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.^{9,11} 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,^{12,14} 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 an mPTP-initiated signal to regulate PS exposure, remains to be determined.

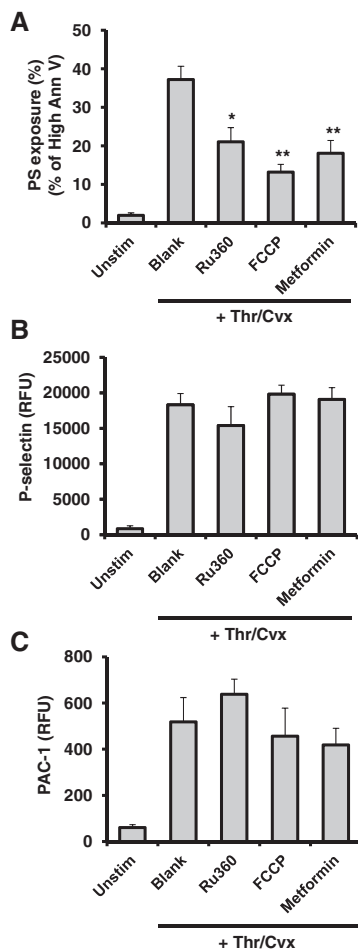


Figure 6. Inhibition of mitochondrial calcium ($\text{Ca}^{2+}_{\text{mit}}$) uptake blocks phosphatidylserine (PS) exposure, but not integrin $\alpha_{\text{IIb}}\beta_3$ activation or granule release. **A**, Human platelets were pretreated with Ru360 (0.5 mmol/L, 30 minutes), Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone [FCCP, 2 $\mu\text{mol/L}$, 5 minutes] or metformin (10 mmol/L, 30 minutes), labeled with Ann V, stimulated with thrombin and convulxin (Thr/Cvx), and analyzed by flow cytometry after 7 minutes. $n=3$. **B** and **C**, Human platelets were pretreated with Ru360 (0.5 mmol/L, 30 minutes), FCCP (2 $\mu\text{mol/L}$, 5 minutes), or metformin (10 mmol/L, 30 minutes), labeled with PE-P-selectin and FITC-PAC-1, stimulated with Thr and Cvx, and analyzed by flow cytometry after 1 minute. $n=3$. * $P<0.05$, ** $P<0.01$. Unstim indicates unstimulated platelets.

Our results contradict a previous study that concluded that mitochondrial events have only a minimal role in agonist-initiated PS exposure.⁴¹ However, this study was limited by the investigators use of cyclosporine to investigate the role of mPTP 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 sarcoplasmic calcium adenosine triphosphatase 2b and stromal interaction molecule 1 (STIM1).^{42,43} Here, by using CypD null platelets, these key limitations are overcome.

Because 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,^{25,44} 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 Thr/Cvx, consistent with the recent findings of Arthur et al.⁴⁵ The ROS burst was independent of mPTP formation and required extracellular calcium entry. Thus 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,³² and there is close communication of the mitochondria with the calcium-rich endoplasmic reticulum.⁴⁶ 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, 2 complementary approaches were used, 1 focused on blockade of the MCU,^{38,39} 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.⁴⁷ These respiratory inhibitors, because of 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 antidiabetic 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.⁴⁸ It is tempting to postulate that metformin's effectiveness in the prevention of cardiovascular disease in diabetes mellitus, above and beyond its antihyperglycemic actions,⁴⁹ can be attributed in part to its antiprocoagulant 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,⁴⁷ may provide an attractive therapeutic target in the treatment and prevention of thrombosis.

Acknowledgments

Hyo-Jung Choo designed and performed the experiments, analyzed the data, and wrote the manuscript; Talib B. Saafir performed and assisted in the design of confocal microscopy experiments; Laura Mkumba performed experiments; Mary B. Wagner assisted in the design and analysis of confocal microscopy experiments; and Shawn M. Jobe participated in experimental design and data analysis and wrote the manuscript.

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Disclosures

None.

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Arteriosclerosis, Thrombosis, and Vascular Biology



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Mitochondrial Calcium and Reactive Oxygen Species Regulate Agonist-Initiated Platelet Phosphatidylserine Exposure

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Supplemental Material

Supplemental Materials and Methods

Supplemental Figure I

Supplemental Figure II

Supplemental Figure III

Supplemental Materials and Methods

Reagents and antibodies

Thrombin was obtained from Haematologic Technologies, and convulxin was obtained from Centerchem. Syk inhibitor and Ru360 were obtained from Calbiochem, and metformin was obtained from Enzo Lifesciences. Alexa Fluor 647 conjugated annexin V, Fluo-4-AM, Rhod-2-AM, CM-DCFDA, H₂-DCFDA, Mito-SOX, and tetramethylrhodamine methyl ester (TMRM) were obtained from Invitrogen. PE-conjugated P-selectin was obtained from ebioscience and FITC-conjugated-PAC-1 was obtained from BD. All other reagents were obtained from Sigma Aldrich.

Preparation of washed human platelets

Human whole blood was obtained from medication-free volunteers, and platelet-rich plasma (PRP) was isolated by centrifugation at 140g. 10ml of Pipes-saline buffer with prostaglandin E1 (1 µM) was then mixed with the PRP followed by centrifugation at 1,900g for eight minutes. The platelet pellet was resuspended in a modified Tyrode's buffer without calcium. Platelet counts were measured using a HemaTrue hematology analyzer (HESKA, Loveland, CO).

Flow cytometry

Washed platelets were suspended at a concentration of 10^7 / mL in Tyrode's buffer containing 2.1 mM CaCl₂. Platelets were either left unstimulated or stimulated with thrombin (0.5 U/mL), convulxin (100 ng/mL), thrombin plus convulxin, H₂O₂, thrombin plus H₂O₂ or ionomycin. In the indicated experiments platelets were preincubated with the following pharmacologic agents: including SKF96365 (25 µM, thirty minutes), 2-APB (10 µM, thirty minutes), Syk inhibitor (1 µM, thirty minutes), DPI (10 µM, thirth

minutes), phenylarsine oxide (25 μM), Ru360 (200, 500 μM , third minutes), FCCP (2 μM , 5 minutes), metformin (10 mM ²³, third minutes). Human platelets were labeled with Alexa Fluor 647 conjugated annexin V, PE-conjugated P-selectin or FITC-conjugated-PAC-1 following agonist(s) stimulation.

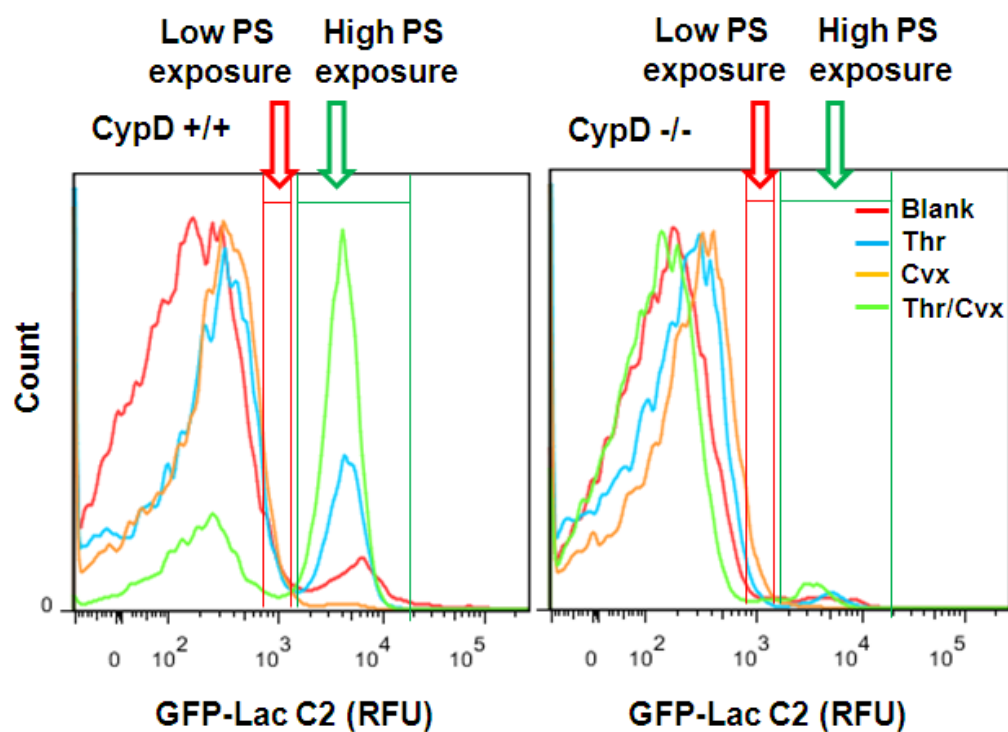
For experiments evaluating loss of $\Delta\psi_{\text{m}}$, platelets were incubated with 500 nM TMRM for 15 minutes prior to stimulation with the indicated agonist(s). For two-color flow cytometry with TMRM, the APC-labeled annexin V was added immediately after agonist stimulation. Labeled platelets were evaluated by flow cytometry with agonists for ten minutes. Labeled platelets were analyzed on a Becton Dickinson FACS Canto II (San Diego, CA). Platelets were gated by forward and side scatter. Appropriate compensation was performed for experiments using multi-color flow cytometry.

For experiments detecting mitochondrial calcium influx, platelets were incubated with 5 μM Rhod-2-AM for 30 minutes prior to stimulation with the indicated agonist(s). For experiments detecting cytosolic ROS generation, platelets were incubated with 5 μM CM-DCFDA for 30 minutes prior to stimulation with the indicated agonist(s). For experiments detecting mitochondrial superoxide generation, platelets were stimulated with the indicated agonist(s) and then incubated with 5 μM Mito-SOX for 5 min due to interference with agonist(s)-stimulation. Labeled platelets were evaluated by flow cytometry.

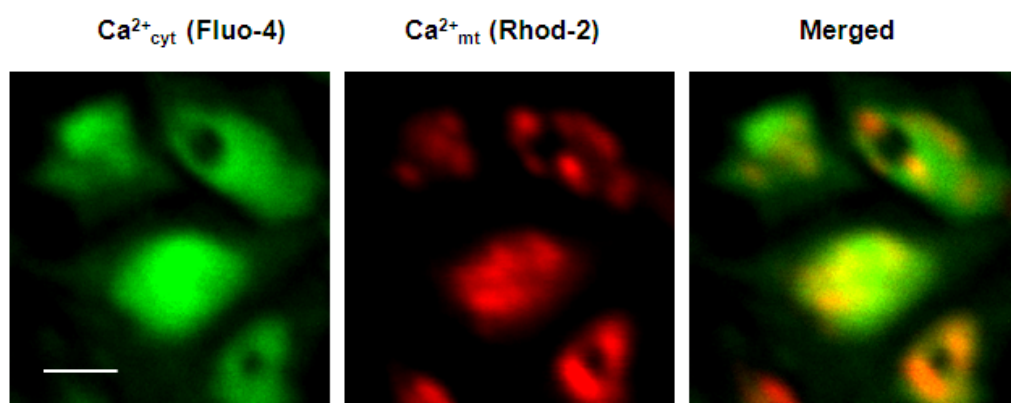
Confocal Microscopy

Washed platelets ($10^8/\text{mL}$ in Tyrode's buffer) were allowed to adhere to a fibrinogen (0.1mg/ml) coated coverglass for 30 minutes in the presence of 4 μM Fluo-4-AM and 5 μM Rhod-2-AM. To allow changes of buffer and agonist the coverglass with platelets attached was then placed on a flow system. Platelets were initially incubated with Tyrode's buffer. The buffer was then sequentially changed to Tyrode's buffer containing 2.1 mM Ca^{2+} at ten seconds after recording; and then to Tyrode's with Ca^{2+} , thrombin, and convulxin seventy seconds after recording. Epifluorescence was continually examined throughout this process using an Olympus Fluoview FV1000. Seven minutes subsequent to addition of thrombin and

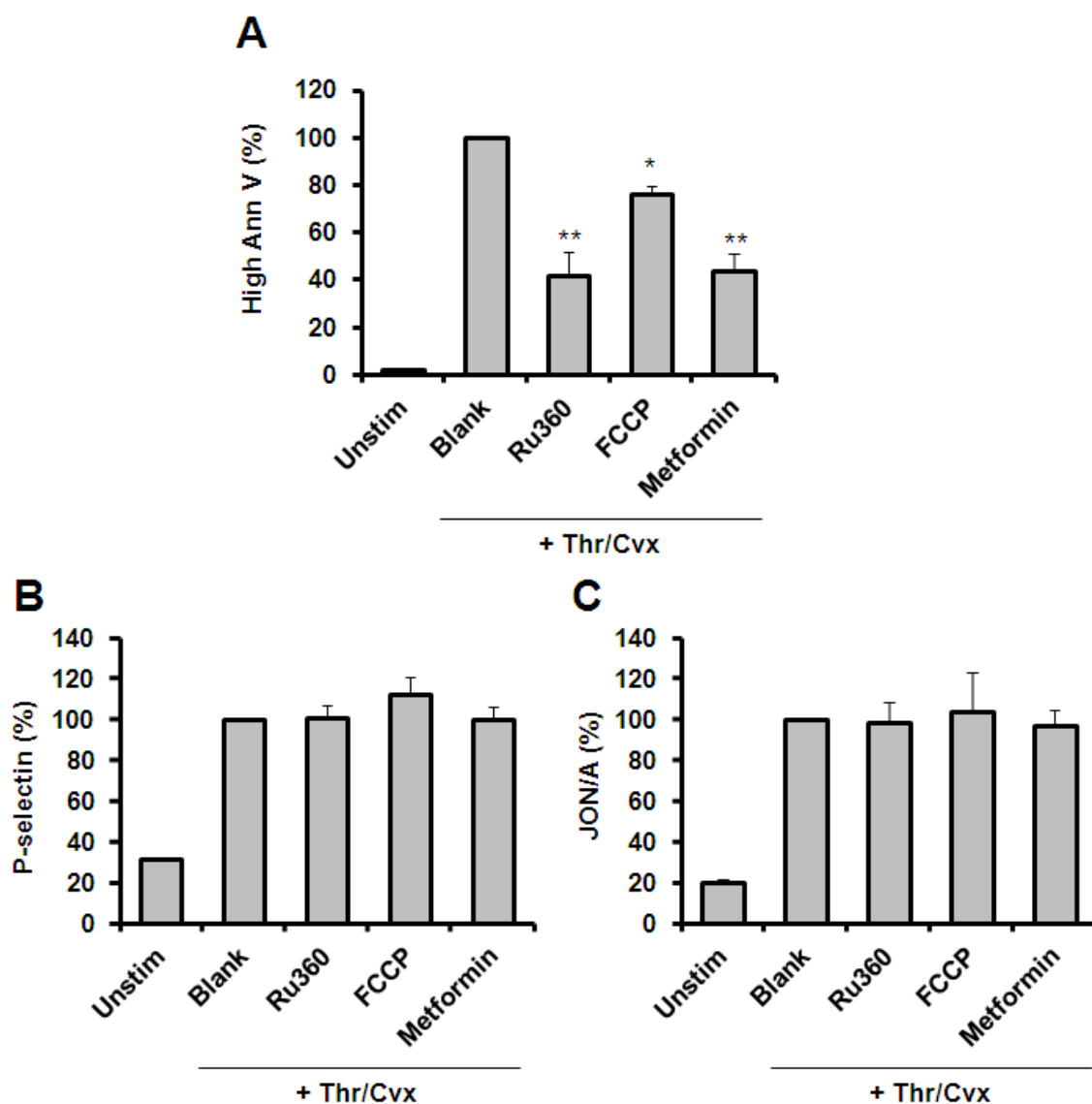
convulxin, 1.5 μ M FM1-43 was added to label PS-exposed platelets. Intensity of Fluo-4-AM and Rhod-2-AM was analyzed by Olympus Flowview software, and PS⁺ and PS⁻ platelets were sorted for analysis by positive or negative staining of FM1-43. For each experimental comparison over fifty platelets were selected for analysis of cytoplasmic or mitochondrial calcium transients.



Supplemental Figure I. Lactadherin staining of stimulated, murine platelets. Isolated CypD^{+/+} and CypD^{-/-} platelets were stimulated with 0.5 U/ml Thrombin, 100 ng/ml Convulxin or Thr/Cvx, labeled with a green-flourescent protein-lactadherin C2 domain fusion protein (GFP-Lac C2) and analyzed by flow cytometry after seven minutes. n=3.



Supplemental Figure II. Fibrinogen-adherent and Fluo-4-AM and Rhod-2-AM stained murine platelets were analyzed by confocal microscopy. White bar is 2 μm . The punctate pattern of staining with Rhod-2-AM is consistent with organellar (mitochondrial) localization.



Supplemental Figure III. (A) Isolated murine platelets were pre-treated with Ru360 (100 μ M, 30 minutes), FCCP (2 μ M, 5 minutes), or metformin (10 mM, 30 minutes), labeled with APC-Annexin V, stimulated with Thr and Cvx, and analyzed by flow cytometry after seven minutes. $n=4$. (B, C) Isolated murine platelets were pre-treated with Ru360 (100 μ M, 30 minutes), FCCP (2 μ 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. $n=4$. * $p<0.05$, ** $p<0.01$