Supramaximal calcium signaling triggers procoagulant platelet formation

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Key Points
• Procoagulant platelets exhibit a distinct pattern of Ca2+ signaling that we term “supramaximal” Ca2+ signaling.
• Our model provides a new framework to interpret the role of different platelet signaling systems in the generation of procoagulant platelets.

Procoagulant platelets promote thrombin generation during thrombosis. Platelets become procoagulant in an all-or-nothing manner. We investigated how distinct Ca2+ signaling between platelet subpopulations commits some platelets to become procoagulant, using the high-affinity Ca2+ indicator Fluo-4, which may become saturated during platelet stimulation, or low-affinity Fluo-5N, which reports only very high cytosolic Ca2+ concentrations. All activated platelets had high Fluo-4 fluorescence. However, in Fluo-5N–loaded platelets, only the procoagulant platelets had high fluorescence, indicating very high cytosolic Ca2+. This finding indicates a novel, “supramaximal” Ca2+ signal in procoagulant platelets (ie, much higher than normally considered maximal). Supramaximal Ca2+ signaling and the percentage of procoagulant platelets were inhibited by cyclosporin A, a mitochondrial permeability transition pore blocker, and Ru360, an inhibitor of the mitochondrial Ca2+ uniporter, with no effect on Fluo-4 fluorescence. In contrast, Synta-66, an Orai1 blocker, reduced Fluo-4 fluorescence but did not directly inhibit generation of the supramaximal Ca2+ signal. Our findings show a distinct pattern of Ca2+ signaling in procoagulant platelets and provide a new framework to interpret the role of platelet signaling pathways in procoagulant platelets. This requires reassessment of the role of different Ca2+ channels and may provide new targets to prevent formation of procoagulant platelets and limit thrombosis.

Introduction

Procoagulant platelets are a subpopulation of activated platelets that expose phosphatidylserine (PS), allowing a burst of thrombin generation that is responsible for producing an occlusive thrombus.1-3 Selective inhibition of procoagulant platelets is a potential antithrombotic strategy.3

Procoagulant platelets form in an all-or-nothing manner: procoagulant platelets expose PS, whereas activated but noncoagulant platelets do not.4-6 However, almost all platelets can become procoagulant if treated with a Ca2+ ionophore, and almost all platelets become activated but noncoagulant if stimulated with some platelet activators, such as the protease-activated receptor 1 agonist SFLLRN-amide.7 Individual platelets are therefore capable of forming either subpopulation, depending on the activating stimulus. During activation, differences in intracellular signaling between activated platelets may lead platelets to commit to becoming procoagulant or noncoagulant. Increased cytosolic Ca2+ concentration ([Ca2+]cyt) is required for procoagulant and noncoagulant platelet activation, but higher or more sustained increases in [Ca2+]cyt may commit some platelets to becoming procoagulant.1,8,9,11 However, it is currently unclear how variation in [Ca2+]cyt between platelets leads to an all-or-nothing response.

Mitochondrial permeability transition pore (mPTP) opening is also required for platelets to become procoagulant.6 Ca2+ enters mitochondria from the cytosol through the mitochondrial Ca2+ uniporter (MCU), leading to mPTP opening above a threshold of high mitochondrial Ca2+ concentration ([Ca2+]mitochondria).
mito.6 Cyclophilin D (CypD) reduces the threshold of [Ca^{2+}]_{mito} for mPTP opening.12 CypD-deficient or MCU-deficient mouse platelets generate significantly fewer procoagulant platelets than wild-type platelets.6,13,14 Cyclosporin A (CsA), which inhibits CypD, and Ru360, which inhibits the MCU, also inhibit the procoagulant platelet formation.5,5,15

Two models have been proposed to explain how mPTP opening and cytosolic Ca^{2+} signaling interact to commit platelets to become procoagulant. Choo et al16 reported that because [Ca^{2+}]_{cyt} signaling was not obviously different in CypD-deficient mouse platelets, mPTP opening causes stimulated platelets to become procoagulant without further altering [Ca^{2+}]_{cyt}. In contrast, Panteleev et al5,17 reported that stochastic variation in [Ca^{2+}]_{mito} and [Ca^{2+}]_{cyt} between activated platelets leads to mPTP opening in some platelets, changing [Ca^{2+}]_{cyt} spikes from Ca^{2+} signals to Ca^{2+} signals. The goal of the current study was to resolve these differences and propose a new model for how platelets commit to become procoagulant in an all-or-nothing manner.

Methods

Reagents

Synta-66, thapsigargin, thrombin, and fibrinogen were from MilliporeSigma. MitoTracker Deep Red FM, annexin V (AnV)–allophycocyanin (APC) conjugate, and tandem PE-Cy7–conjugated anti-CD41 antibody, Fluo-4 acetoxymethyl ester (AM), and Fluo-5N AM were from Thermo Fisher Scientific. MitoView Green was from Biotium. CsA was from Cambridge Bioscience. Ru360 was from VWR. Cross-linked collagen-related peptide (CRP-XL) was synthesized by one of the authors (J.D.M.) according to previously published methods.17

Platelet preparation

Blood from healthy drug-free volunteers was drawn into sodium citrate (3.2% vol/vol) with approval from the Human Biology Research Ethics Committee, University of Cambridge. Volunteers had given written informed consent in accordance with the Declaration of Helsinki. Washed platelets were prepared as previously described. Acid citrate dextrose (85 mM tri-sodium citrate, 71 mM citric acid, and 111 mM D-glucose) was added (1:7 vol/vol) and platelet-rich plasma was separated by centrifugation (200 g, 10 minutes, ambient temperature, and without any brake). Platelets were pelleted by centrifugation (600g, 10 minutes, ambient temperature) in the presence of prostaglandin E1 (100 nM) and apyrase (0.02 U/mL). Pelleted platelets were resuspended in N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)–buffered saline supplemented with D-glucose (135 mM NaCl, 10 mM HEPES, 3 mM KCl, 1 mM MgCl_2, 0.34 mM NaH_2PO_4, 5 mM D-glucose, pH 7.4,) and apyrase (0.02 U/mL). Platelets were rested for 30 minutes at 30°C before treatment protocols were initiated. Where indicated, platelets were loaded with Fluo-4-AM or Fluo-5N-AM (1 μM, 10 minutes, 30°C) immediately before stimulation. Under these conditions, the dyes reported cytosolic increases in Ca^{2+} (supplemental Figure 1). CaCl_2 was added at the final concentration of 2 mM immediately before stimulation.

Confocal imaging

Platelets (10^9/mL) were allowed to adhere to a fibrinogen (0.1 mg/mL)-coated coverslip for 30 minutes in the copresence of 1 μM Fluo-4-AM and 20 nM MitoTracker Deep Red FM, or 1 μM Fluo-5N-AM and 20 nM MitoTracker Deep Red FM. Nonadherent platelets (and unloaded dye) were gently washed away and HEPES-buffered saline supplemented with D-glucose replaced on the adherent platelets. Fluorescence was recorded by using a Leica SP5 confocal microscope. Platelets were then stimulated with thrombin (1 U/mL) and CRP-XL (1 μg/mL) in the presence of extracellular CaCl_2 (2 mM), and samples were recorded for an additional 10 minutes.

Flow cytometry

Fluo-4– or Fluo-5N–loaded platelets were pretreated with either inhibitors or their vehicle controls as indicated in the main text followed by stimulation with thrombin (1 U/mL) and CRP-XL (1 μg/mL). AnV-APC was used to detect surface PS exposure, and PE-Cy7–conjugated anti-CD41 antibody was used to positively identify platelets. Fluorescence was detected by flow cytometry (BD Accuri C6). CD41+ events were identified as platelets based on their forward and side-scattered profile, using 1 μm silica beads to define (and exclude) microparticles. For end point analysis, 10,000 platelets were collected. For real-time flow cytometry, events were collected at a slow flow rate for 10 minutes after stimulation.

Plate reader

Fluo-4– and Fluo-5N–loaded platelets were stimulated in black 96-well plates (FLUOSTar Plate Reader; excitation, 485 nm; emission, 520 nm).

Estimation of [Ca^{2+}]_{cyt}

Platelets were loaded with Fluo-5N, as noted. After stimulation with A23187 (10 μM) with 2 mM extracellular CaCl_2, Fluo-5N fluorescence was detected by using flow cytometry. The median fluorescence intensity (MFI) was used to estimate \( F_{\text{max}} \). Separately, Fluo-5N–loaded platelets were also treated with BAPTA-AM to chelate intracellular Ca^{2+}. The MFI of unstimulated, BAPTA-AM–treated platelets was used to estimate \( F_{\text{min}} \). These were used with the in vitro dissociation constant (\( K_d \)) of Fluo-5N (90 μM) to estimate [Ca^{2+}]_{cyt} from the MFI of Thr/CRP-XL–stimulated platelets, using the equation:

\[
[Ca^{2+}]_{\text{cyt}} = K_d \times \frac{(F - F_{\text{min}})}{(F_{\text{max}} - F)}
\]

Data analysis

Data presented are mean ± standard error of the mean from at least 4 independent platelet preparations. Statistical tests used are described in the figure legends.

Results

Procoagulant platelets have a “supramaximal” [Ca^{2+}]_{cyt} signal

Although platelet Ca^{2+} signaling has been widely studied with high-affinity Ca^{2+}-sensitive dyes (eg, Fluo-4, Fura-2), these may be relatively saturated during the strong stimulation required to generate procoagulant platelets.19 To investigate this theory, platelets were loaded with two Ca^{2+}-sensitive fluorescent dyes, either Fluo-4 or Fluo-5N (in vitro \( K_d = 390 \text{ nM and 90 } \mu\text{M, respectively} \)). Confocal microscopy imaging of Fluo-4– and Fluo-5N–loaded platelets confirmed that these indicators reported [Ca^{2+}]_{cyt} in platelets...
under our conditions (supplemental Figure 1A-B). Platelets were then stimulated with Thr/CRP-XL and observed by using real-time flow cytometry. AnV-APC binding identified procoagulant platelets. A difference in Fluo-4 fluorescence between procoagulant (AnV<sup>+</sup>) and noncoagulant (AnV<sup>−</sup>) platelets was difficult to discern because there is considerable overlap between the Fluo-4 fluorescence distributions of these 2 subpopulations (Figure 1A-B).

In contrast, the difference in Fluo-5N fluorescence between the subpopulations was clear. Fluo-5N fluorescence was high in procoagulant platelets, whereas the noncoagulant platelets had fluorescence similar to unstimulated platelets (Figure 1F-G). The very low Ca<sup>2+</sup> affinity of Fluo-5N (90 μM) means that [Ca<sup>2+</sup>]<sub>cyt</sub> in procoagulant platelets must be very high compared with noncoagulant platelets whose fluorescence was too low to be accurately reported by Fluo-5N. We term this very high Ca<sup>2+</sup> signal in procoagulant platelets "supramaximal"; that is, much higher than normally considered maximal (1-2 μM).<sup>19</sup>

When platelets were stimulated with the Ca<sup>2+</sup> ionophore A23187, almost all platelets became AnV<sup>+</sup>, as expected.<sup>20</sup> These platelets also had high Fluo-4 or Fluo-5N fluorescence (supplemental Figure 2). To estimate [Ca<sup>2+</sup>]<sub>cyt</sub> in Fluo-5N–loaded procoagulant platelets, A23187 stimulation was used to estimate <i>F<sub>max</sub></i> and BAPTA-loaded, unstimulated platelets to estimate <i>F<sub>min</sub></i>. Another aliquot of the same preparation of Fluo-5N–loaded platelets was then stimulated with Thr/CRP-XL in the presence of AnV-APC. Using the median fluorescence intensity of procoagulant (AnV<sup>+</sup>) and noncoagulant (AnV<sup>−</sup>) platelets, [Ca<sup>2+</sup>]<sub>cyt</sub> in procoagulant platelets was estimated as 166 ± 56 μM (n = 4; see "Methods"). This is ~1.8 × the <i>K<sub>d</sub></i> of Fluo-5N and thus within the range that it is sensitive to changes in Ca<sup>2+</sup> concentration. In contrast, it is ~250 × the <i>K<sub>d</sub></i> of Fluo-4. Although the [Ca<sup>2+</sup>]<sub>cyt</sub> in noncoagulant platelets was estimated as 3.9 ± 0.7 μM (n = 4) using Fluo-5N, we do not consider the estimate in noncoagulant platelets to be reliable, as Ca<sup>2+</sup>–sensitive dyes are likely to be inaccurate beyond 0.1 to 10 × the <i>K<sub>d</sub></i> of the dye.<sup>21</sup> This finding shows the importance of using dyes whose Ca<sup>2+</sup> affinities are appropriate to the signal being measured.

One potential explanation for these observations is that procoagulant platelets have lost plasma membrane integrity and that Ca<sup>2+</sup> has passively entered through this leaky plasma membrane. To test this theory, platelets were loaded with calcein-AM before stimulation. Loss of calcein fluorescence would indicate loss of plasma membrane integrity. Under these conditions, only a small proportion of procoagulant platelets lost calcein fluorescence, whereas most procoagulant platelets retained calcein (supplemental Figure 3).

As a positive control, heat-killed platelets all lost calcein fluorescence. Together, these data indicate that procoagulant platelets with supramaximal Ca<sup>2+</sup> signal have an intact plasma membrane.

**Supramaximal Ca<sup>2+</sup> signaling is dependent on mPTP opening**

CsA (2 μM) significantly reduced the proportion of platelets with high Fluo-5N fluorescence (Figure 1F-G), indicating that mPTP opening is required for the supramaximal Ca<sup>2+</sup> signal. Because the Fluo-5N fluorescence of the 2 subpopulations is very different, CsA reduced the Fluo-5N fluorescence of the whole population in flow cytometry and microplate experiments (Figure 1H-J). This outcome was not seen if Fluo-4 was used instead because the procoagulant and noncoagulant subpopulations have similar Fluo-4 fluorescence (Figure 1C-E).

To determine whether the likelihood of becoming procoagulant is related to the number of mitochondria in platelets, mitochondrial content was detected by using MitoGreen. However, there was no difference in MitoGreen fluorescence intensity between procoagulant and noncoagulant platelets (supplemental Figure 4).

**A model for all-or-nothing commitment to becoming procoagulant**

These data support a model in which mPTP opening commits a platelet to becoming procoagulant by triggering supramaximal [Ca<sup>2+</sup>]<sub>cyt</sub> signaling (Figure 2). Platelet activators trigger an initial increase in [Ca<sup>2+</sup>]<sub>cyt</sub> that varies between platelets, leading to interplatelet variation in [Ca<sup>2+</sup>]<sub>cyt</sub>, putting some above the threshold for mPTP opening (described elsewhere<sup>19</sup>). mPTP opening triggers supramaximal [Ca<sup>2+</sup>]<sub>cyt</sub> signaling in these platelets, committing them to becoming procoagulant. Variation in the initial [Ca<sup>2+</sup>]<sub>cyt</sub> signal is therefore converted into 2 subpopulations with distinct [Ca<sup>2+</sup>]<sub>cyt</sub> signals and an all-or-nothing commitment to becoming procoagulant.

**Reassessing the role of Ca<sup>2+</sup> channels in procoagulant platelets**

Subpopulation analysis of this all-or-nothing signaling requires reassessment of the role of different Ca<sup>2+</sup> channels in generating procoagulant platelets. Individual Ca<sup>2+</sup> channels could be involved in the initial [Ca<sup>2+</sup>]<sub>cyt</sub> signal, in regulation of [Ca<sup>2+</sup>]<sub>mito</sub>, or the generation of the supramaximal [Ca<sup>2+</sup>]<sub>cyt</sub> signal.

The store-operated channel, Orai1, contributes to PS exposure.<sup>22-25</sup> However, although the Orai1 blocker Synta-66 (supplemental Figure 5) reduced the percentage of procoagulant platelets and inhibited the median Fluo-4 fluorescence in the whole platelet population (consistent with previous cuvette or microplate-based experiments<sup>26</sup>) (Figure 3A-D), the Fluo-5N fluorescence in platelets that had become procoagulant was unaffected (Figure 3B,E). This finding suggests that Orai1 contributes to the initial [Ca<sup>2+</sup>]<sub>cyt</sub> signal, affecting the percentage of platelets that undergo mPTP opening and become procoagulant, but is not directly involved in the generation of the supramaximal Ca<sup>2+</sup> signal.

Similarly, Ru360, an inhibitor of the MCU, also reduced the percentage of procoagulant platelets (Figure 4A-C). However, Ru360 did not affect the median Fluo-4 fluorescence in the whole platelet population (Figure 4A,D), suggesting that it does not affect the initial [Ca<sup>2+</sup>]<sub>cyt</sub> signal. The Fluo-5N fluorescence of platelets that had become procoagulant was also unaffected (Figure 4B,E), suggesting that MCU is not directly involved in the generation of the supramaximal Ca<sup>2+</sup> signal.

This reassessment therefore distinguishes distinct contributions of the plasma membrane channel (Orai1) and the mitochondrial Ca<sup>2+</sup> transporters (MCU and mPTP opening) in generating procoagulant platelets.

**Discussion**

Procoagulant platelets form in an all-or-nothing manner, which suggests that during activation, platelets commit to becoming procoagulant or noncoagulant and that there are differences in
Figure 1.

Fluo-4

A

Time (s)

DMSO

CsA

B

AnV+

AnV-

C

Fluo-4 fluorescence (MFI)

D

All platelets - flow cytometer

E

All platelets – microplate reader

Fluo-5N

F

DMSO

CsA

G

AnV+

AnV-

H

I

All platelets - flow cytometer

J

All platelets – microplate reader

AnV+ AnV−

Fluo-4 fluorescence (MFI)

All platelets - flow cytometer

All platelets – microplate reader

DMSO CsA

DMSO CsA

DMSO CsA

DMSO CsA
intracellular signaling between these 2 subpopulations. Increased \([\text{Ca}^{2+}]_{\text{cyt}}\) is required for both procoagulant and noncoagulant platelet activation\(^1^1\); although there is considerable variation in the \([\text{Ca}^{2+}]_{\text{cyt}}\) signals between activated platelets, higher or more sustained \([\text{Ca}^{2+}]_{\text{cyt}}\) signals are associated with procoagulant platelets.\(^1,8,9,25\) However, how the variation in \([\text{Ca}^{2+}]_{\text{cyt}}\) signaling between activated platelets leads to an all-or-nothing phenotype is not clear.

In this study, we propose a new model to explain this. Our model (Figure 2) involves 3 different \([\text{Ca}^{2+}]_{\text{cyt}}\) signaling events. First, platelet activators, such as thrombin and collagen, trigger an increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) that varies between platelets. This initial \([\text{Ca}^{2+}]_{\text{cyt}}\) signal occurs through release of \([\text{Ca}^{2+}]\) from intracellular \([\text{Ca}^{2+}]\) stores and entry through plasma membrane \([\text{Ca}^{2+}]\) channels, such as Orai1 and TRPC6.\(^23,24\) Some of the cytosolic \([\text{Ca}^{2+}]\) is taken into mitochondria through MCU,\(^13\) increasing \([\text{Ca}^{2+}]_{\text{mito}}\). Because the initial \([\text{Ca}^{2+}]_{\text{cyt}}\) varies between platelets, in some platelets the mPTP opens, whereas in others it stays closed. A supramaximal \([\text{Ca}^{2+}]_{\text{cyt}}\) signal is triggered in these platelets, committing them to becoming procoagulant. Variation in the initial \([\text{Ca}^{2+}]_{\text{cyt}}\) is therefore converted into 2 distinct subpopulations with distinct \([\text{Ca}^{2+}]_{\text{cyt}}\) signals, leading to an all-or-nothing commitment to becoming procoagulant.

**Figure 1.** Procoagulant platelets have supramaximal \([\text{Ca}^{2+}]_{\text{cyt}}\) signaling that is detected by low-affinity Fluo-5N but not high-affinity Fluo-4. (A) Human platelets were loaded with Fluo-4 then treated with CsA (2 \(\mu\)M) or dimethyl sulfoxide (DMSO) (as control) before stimulation with Thr/CRP-XL (arrowhead) in the presence of AnV-APC. Samples were analyzed by using real-time flow cytometry. Platelets were gated on an FSC/SSC profile to exclude microparticles. Fluo-4 fluorescence is shown. Color represents event density from low (blue) to high (red). (B) A comparison of Fluo-4 fluorescence and AnV-APC fluorescence after 10 minutes of stimulation. The horizontal line defines platelets AnV\(^+\) (procoagulant) and AnV\(^-\) (noncoagulant) subpopulations. The vertical line indicates the Fluo-4 fluorescence of unstimulated platelets. All activated platelets exhibit high Fluo-4 fluorescence. (C) The Fluo-4 MFI in all platelets, AnV\(^+\) platelets, and AnV\(^-\) platelets. Data are mean \(\pm\) standard error of the mean (n = 5; 2-way analysis of variance with the Šidák multiple comparison test). (D) Mean Fluo-4 fluorescence (MFI normalized to initial MFI; F/F\(_0\)) of all platelets from real-time flow cytometry (n = 5). The dotted line is the standard error of the mean. (E) Mean Fluo-4 fluorescence (F/F\(_0\)) of platelets stimulated with Thr/CRP-XL measured in a microplate reader. (F-J) show the equivalent experiments in Fluo-5N–loaded platelets. ***\(P < .001\) for indicated comparison; ****\(P < .001\) for difference in MFI between AnV\(^+\) vs AnV\(^-\) for DMSO or CSA, as appropriate. n.s., not significantly different for the indicated comparison.

**Figure 2.** Proposed model for the role of \([\text{Ca}^{2+}]_{\text{cyt}}\) signaling in the commitment to becoming procoagulant. Platelet activators trigger an initial increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) that varies between platelets (1). This action will lead to variation in \([\text{Ca}^{2+}]_{\text{mito}}\) (2), putting some above the threshold for mPTP opening. A supramaximal \([\text{Ca}^{2+}]_{\text{cyt}}\) signal is triggered in these platelets (3), committing them to becoming procoagulant. Variation in the initial \([\text{Ca}^{2+}]_{\text{cyt}}\) is therefore converted into 2 distinct subpopulations with distinct \([\text{Ca}^{2+}]_{\text{cyt}}\) signals, leading to an all-or-nothing commitment to becoming procoagulant.
cyt signal varies between platelets, the subsequent [Ca\textsuperscript{2+}]mito signal also varies. The key commitment step is then opening of mPTP, which occurs above a threshold [Ca\textsuperscript{2+}]mito. This concept was first proposed by Panteleev et al in mathematical models and subsequent experiments. However, we further propose that mPTP opening triggers an additional increase in [Ca\textsuperscript{2+}]cyt that is of a different order of magnitude to the initial [Ca\textsuperscript{2+}]cyt signal, and much larger than any [Ca\textsuperscript{2+}]cyt signal previously reported in platelets. This supramaximal [Ca\textsuperscript{2+}]cyt signal occurs in platelets whose [Ca\textsuperscript{2+}]mito has increased above the threshold for mPTP opening and leads to these platelets rapidly becoming procoagulant. In platelets whose [Ca\textsuperscript{2+}]mito is below this threshold, mPTP remains closed, and the platelet remains noncoagulant. Graded variation in the initial [Ca\textsuperscript{2+}]cyt signal between platelets is therefore

Figure 3. Role of SOCE in procoagulant platelet generation. To test the role of SOCE, Fluo-4– or Fluo-5N–loaded platelets were treated with the Orai1 blocker Synta-66 (10 μM), stimulated with Thr/CRP-XL in the presence of AnV-APC, and analyzed by using flow cytometry. Representative density plots are shown for Fluo-4–loaded platelets (A) or Fluo-5N–loaded platelets (B). (C) The percentage of platelets that bound AnV (** P < .001; paired Student t test). The effect of Synta-66 on Fluo-4 (D) or Fluo-5N (E) fluorescence in all platelets, AnV\textsuperscript{+} platelets and AnV\textsuperscript{−} platelets. * P < .05 for dimethyl sulfoxide (DMSO) vs Synta-66–treated platelets; 2-way analysis of variance with the Šidák multiple comparison test; n = 4.
converted into an all-or-nothing signal and a commitment to either becoming procoagulant or to remaining noncoagulant.

The low affinity for Ca\(^{2+}\) of Fluo-5N is necessary to identify the supramaximal [Ca\(^{2+}\)]\(_{\text{cyt}}\) signal in procoagulant platelets. Using high-affinity Fluo-4, we could not detect a distinct difference in apparent [Ca\(^{2+}\)]\(_{\text{cyt}}\) between procoagulant and noncoagulant platelets. Although the median Fluo-4 fluorescence of procoagulant platelets was often higher than the median fluorescence of noncoagulant platelets, there was considerable overlap in their fluorescence distributions. Based on Fluo-4, we might conclude that although high [Ca\(^{2+}\)] is required to generate procoagulant platelets, there must be other signals that commit a platelet to becoming procoagulant. However, when we used the low-affinity Ca\(^{2+}\) dye, Fluo-5N, a clear difference in fluorescence was noted between procoagulant and noncoagulant platelets. Based on the

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**Figure 4. Role of MCU in procoagulant platelet generation.** To test the role of MCU, Fluo-4– or Fluo-5N–loaded platelets were treated with the MCU blocker Ru360 (10 μM), stimulated with Thr/CRP-XL in the presence of AnV-APC, and analyzed by using flow cytometry. Representative density plots are shown for Fluo-4–loaded platelets (A) or Fluo-5N–loaded platelets (B). (C) The percentage of platelets that bound AnV (**P < .01; paired Student t test). The effect of Ru360 on Fluo-4 (D) or Fluo-5N (E) fluorescence in all platelets, AnV\(^+\) platelets, and AnV\(^-\) platelets. **P < .01, ***P < .001 for dimethyl sulfoxide (DMSO) vs Ru360-treated platelets; 2-way analysis of variance with the Šidák multiple comparison test; n = 4.
in vitro $K_d$ of Fluo-5N, we estimate the $[Ca^{2+}]_{cyt}$ signal in procoagulant platelets as being $>100 \mu M$. This value is much larger than anything previously reported in platelets and much higher than 1 to 2 $\mu M$, the apparent maximal $[Ca^{2+}]_{cyt}$ often reported by using high-affinity dyes. It is also $>250 \times$ the $K_d$ of Fluo-4, far beyond the range that $Ca^{2+}$-sensitive dyes are likely to be reliable. $1\text{ Fluo-4 would be saturated before supramaximal }[Ca^{2+}]_{cyt}$ levels are reached. The same difficulty applies to Fura-red (used by Obydennyi et al$^5$), which has a similar high affinity to Fluo-4.

One implication of such a high $[Ca^{2+}]_{cyt}$ signal in procoagulant platelets is that it may activate effectors with relatively low $Ca^{2+}$ affinity. In particular, supramaximal $[Ca^{2+}]_{cyt}$ signaling may be necessary to expose PS because the phospholipid scramblase is poorly $Ca^{2+}$ sensitive. The half-maximal $Ca^{2+}$ sensitivity of phospholipid scrambling, or of TMEM16F, the scramblase in platelets, red blood cells, and lymphocytes$^{26,27}$ has been estimated between 10 and 80 $\mu M$$^{28,29}$ With this low $Ca^{2+}$ sensitivity, it is unclear how it might be effectively activated by the low micromolar $[Ca^{2+}]_{cyt}$ previously suggested by high-affinity $Ca^{2+}$-dyes. However, the scramblase could be effectively activated by the concentrations reached during supramaximal $[Ca^{2+}]_{cyt}$ signaling.

Supramaximal $Ca^{2+}$ signaling has been observed in other cells. $[Ca^{2+}]_{cyt}$ $>100 \mu M$ was reported in adenosine triphosphate-depleted renal proximal tubular cells$^{32}$ and MDCK cells$^{33}$ by using low-affinity dyes. In excitorotic neurons, $[Ca^{2+}]_{cyt}$ was estimated as $>5 \mu M$ by using a low-affinity dye but only 0.3 to 0.4 $\mu M$ with high-affinity fura-2; cell death correlated with $[Ca^{2+}]_{cyt}$ reported by using the low-affinity dye$^{34}$. A consistent feature of these reports is that the cells were undergoing cell death, and procoagulant platelets are described as necrotic$^{35,36}$. Importantly, however, supramaximal $[Ca^{2+}]_{cyt}$ signaling is not simply a consequence of necrotic platelets losing plasma membrane integrity, as shown by their retention of calcein. Consistent with this finding, although procoagulant platelets stain with the cell-impermeable cell death marker 4-(N-(S-glutathionylacetyl)amino)phenylarsonous acid, entry of this cell death marker required organic anion transporters$^{35}$ also indicating that the plasma membrane remains intact during the early stages of procoagulant platelet formation.

Supramaximal $[Ca^{2+}]_{cyt}$ signaling requires mPTP opening, meaning that it only occurs in those platelets in which $[Ca^{2+}]_{mito}$ has exceeded the threshold. We could readily repeat the observation that the mPTP opening is required for procoagulant platelets, as CsA reduced the percentage of platelets that became procoagulant, consistent with previous reports with CsA and with CypD-deficient mouse platelets$^{5,6,30}$. In our study, CsA also reduced the percentage of platelets with high Fluo-5N fluorescence. This finding indicates that mPTP opening leads to supramaximal $[Ca^{2+}]_{cyt}$ signaling. In contrast, if supramaximal $[Ca^{2+}]_{cyt}$ signaling triggered mPTP opening, it would be expected to have no effect on the total percentage of platelets with high Fluo-5N fluorescence.

Although CsA reduced the percentage of platelets that became procoagulant, the inhibition was not complete. This is likely because CsA inhibits CypD, which normally reduces the threshold of $[Ca^{2+}]_{mito}$ required for mPTP opening. When platelets are treated with CsA, the threshold of $[Ca^{2+}]_{mito}$ required for mPTP opening will increase. Although this prevents most platelets from becoming procoagulant, a small percentage may have a sufficiently high initial $[Ca^{2+}]_{cyt}$ signal that the $[Ca^{2+}]_{mito}$ increases beyond the higher threshold.

Platelet $Ca^{2+}$ signaling is often measured in cuvettes or microplates, in which the fluorescence is the combined signal from all the platelets, both procoagulant and noncoagulant. The inhibitory effect of CsA can be readily seen by this approach when Fluo-5N was used. In contrast, CsA did not seem to inhibit $Ca^{2+}$ signaling when Fluo-4 was used. This is because there was considerable overlap in the Fluo-4 fluorescence distribution of procoagulant and noncoagulant platelets. These fluorescence data are consistent with those of Jobe et al$^5$, who reported that $[Ca^{2+}]_{cyt}$ signaling was not obviously different in CypD-deficient mouse platelets when using Fluo-4 as the $Ca^{2+}$ indicator. However, we suggest that $[Ca^{2+}]_{cyt}$ is, in fact, much higher in procoagulant platelets, which can be shown with a low-affinity $Ca^{2+}$ indicator.

Our model, and the observation that experiments with high-affinity indicators in cell populations do not readily report the effect of inhibition of supramaximal $[Ca^{2+}]_{cyt}$ signaling, prompted us to reassess the contribution of 2 different $Ca^{2+}$ channels to platelet procoagulant signaling. Synta-66 was used to assess the role of Orai1, which is responsible for store-operated $Ca^{2+}$ entry (SOCE) in platelets$^{24,37,38}$. Consistent with previous reports, inhibition of SOCE reduced the proportion of platelets that become procoagulant and inhibited the total fluorescence in Fluo-4-loaded platelets$^{22,29,37}$. Synta-66 also slightly inhibited the Fluo-4 fluorescence in noncoagulant platelets. It had no effect on the supramaximal $[Ca^{2+}]_{cyt}$ seen in those platelets that did become procoagulant. This finding suggests that SOCE through Orai1 contributes to the initial $[Ca^{2+}]_{mito}$ signal. Inhibition of SOCE leads to reduced $[Ca^{2+}]_{mito}$, fewer platelets exceeding the mPTP threshold, fewer platelets generating a supramaximal $[Ca^{2+}]_{cyt}$ signal, and therefore fewer platelets becoming procoagulant. Those platelets in which mPTP opens have a normal supramaximal $[Ca^{2+}]_{cyt}$ signal. This further suggests that SOCE does not play a direct role in generating the supramaximal $[Ca^{2+}]_{cyt}$ signal. SOCE is not a suitable target to selectively inhibit platelet procoagulant activity because it is involved in the initial $[Ca^{2+}]_{cyt}$ signal.

The MCU is required for $Ca^{2+}$ uptake into mitochondria. Consistent with previous reports$^{8,13}$, the MCU inhibitor Ru360 reduced the percentage of platelets that became procoagulant. However, unlike Synta-66, it had no effect on the Fluo-4 fluorescence in the total platelet population, indicating that MCU is not involved in the initial $[Ca^{2+}]_{cyt}$ signal; rather, by reducing mitochondrial $Ca^{2+}$ uptake, it reduces the proportion of platelets whose $[Ca^{2+}]_{mito}$ exceeds the mPTP threshold. Those platelets in which mPTP opens have a normal supramaximal $[Ca^{2+}]_{cyt}$ signal. This further suggests that MCU does not play a direct role in generating the supramaximal $[Ca^{2+}]_{cyt}$ signal. However, because MCU is also not involved in the initial $[Ca^{2+}]_{cyt}$ signal, it is a potential target to selectively reduce platelet procoagulant activity.

Many other signaling pathways have been proposed to regulate platelet procoagulant activity to some extent, and these may also be reinterpreted in terms of this model. These signaling pathways include other plasma membrane channels, such as TRPC6$^{23,38}$, $Cl^-$ channels$^{20}$, and aquaporins$^{39}$, plasma membrane receptors, including protease-activated receptor 1$^{1,40,41}$ protease-activated receptor 4$^{42,43}$ P2Y12$^{44}$ and integrin $\alphaIIb\beta3$ receptor$^{45-47}$, signaling platforms such as lipid rafts$^{18,48}$ and diverse intracellular signaling proteins such...
as Rac1; different protein kinase C isoforms; and nuclear receptors. We suggest that although many of these pathways are likely to regulate the initial [Ca\(^{2+}\)]\(_{\text{Cyt}}\) signal, it may be useful to reassess the contribution of these pathways because they may also reveal targets to selectively inhibit platelet procoagulant activity.

In conclusion, our findings illustrate supramaximal Ca\(^{2+}\) signaling in procoagulant platelets. Although it is not yet clear how mPTP opening leads to the supramaximal Ca\(^{2+}\) signal (and it may be release of Ca\(^{2+}\) from mitochondria themselves), our model provides a new framework to interpret the role of different platelet signaling systems in procoagulant platelet formation.

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**Authorship**

Contribution: N.A. designed and performed experiments, analyzed data, and wrote and edited the manuscript; S.L.M.-B. designed and performed experiments, analyzed data, and edited the manuscript; S.C. performed experiments and analyzed data; J.-D.M. synthesized CRP-XL; and M.T.H. designed experiments and wrote and edited the manuscript.

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