Rapid Procoagulant Phosphatidylserine Exposure Relies on High Cytosolic Calcium Rather Than on Mitochondrial Depolarization

Amal Arachiche, Danièle Kerbiriou-Nabias, Isabelle Garcin, Thierry Letellier, Jeanne Dachary-Prigent

Objective — Relationships between intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{cyt}\)] and apoptotic events, such as mitochondrial depolarization (\(\Delta \Psi m\) loss) and Bcl-2 and Bad phosphorylation, were analyzed in platelets and Jurkat cells in relation to rapid procoagulant phosphatidylserine (PS) exposure.

Methods and Results — Platelets were stimulated with A23187, thapsigargin (TG) and thrombin plus convulxin (Thr/Cvx), and Jurkat cells with ionomycin, in the presence or absence of cyclosporin A (CsA), a mitochondrial permeability transition pore inhibitor. \(\Delta \Psi m\) loss occurred when platelets were stimulated in Ca\(^{2+}\) medium in conditions exposing PS, but also in EGTA medium. CsA inhibited PS exposure, \([Ca^{2+}]_{cyt}\) increase, and \(\Delta \Psi m\) loss in platelets stimulated with TG and Thr/Cvx, but had no inhibitory effect on A23187 stimulation. CsA reduced TG-induced Ca\(^{2+}\) release from the endoplasmic reticulum and, consequently, external Ca\(^{2+}\) influx. In ionomycin-stimulated Jurkat cells, rapid PS exposure was evidenced but not \(\Delta \Psi m\) loss, and CsA did not inhibit the process. The status of phosphorylated Bad and Bcl-2 in both cell types remained unchanged on stimulation.

Conclusions — Whether \(\Delta \Psi m\) loss occurs or not, PS exposure is triggered by a high \([Ca^{2+}]_{cyt}\) increase. Data further demonstrate that CsA prevents membrane scrambling by inhibiting the high \([Ca^{2+}]_{cyt}\) increase, independently of its effect on mitochondrial permeability transition pore.

Key Words: platelets ■ Jurkat ■ apoptosis ■ procoagulant phosphatidylserine ■ thrombin plus convulxin

Phospholipid scrambling, leading to phosphatidylserine (PS) exposure to the external leaflet of cell membranes, characterizes blood platelet procoagulant activation. The essential role of phospholipid reorganization on the surface of stimulated blood cells is illustrated by the existence of Scott syndrome, a rare bleeding disorder caused by defective

mitochondrial permeability transition pore (mPTP), and requires an increase in mitochondrial Ca\(^{2+}\).6 The mPTP is composed of several proteins, including the adenine nucleotide translocator (ANT), the voltage-dependent anion channel, and cyclophilin D (CypD).7 mPTP opening is blocked by cyclosporin A (CsA), which binds to CypD and dissociates it from the translocator.8 In platelets, the physiological procoagulant activity is triggered by dual agonist stimulation with thrombin (Thr) plus convulxin (Cvx), the latter stimulating GPVI, a platelet collagen receptor.9 This leads a subpopulation of platelets, named coated-platelets, to express PS and high levels of several procoagulant proteins on their surface.10 Studies have shown that both \(\Delta \Psi m\) loss and PS exposure were prevented by CsA in Thr/Cvx-stimulated platelets, leading to the conclusion that mPTP plays a role in PS exposure.4 Moreover, platelets from CypD\(^{−/−}\) mice failed to support prothrombinase activity induced by Thr/Cvx.11 Altogether, these results suggest that procoagulant PS exposure in platelets is an apoptotic process involving mPTP pore opening.

However, no causal link has been firmly established between \(\Delta \Psi m\) loss and PS exposure, particularly because when activated with Thr/Cvx, platelets from Scott syndrome dogs which cannot expose PS exhibit normal mitochondrial depolarization in a platelet subpopulation.12

Apoptosis also implicates proteins of the Bcl-2 family, many of which were identified in platelets.2 Studies showed that: (1) Bak/Bcl-2 ratio determined platelet life span,13 (2) increased expression of proapoptotic Bax and Bak proteins occurred in platelets activated with Ca\(^{2+}\) ionophores or Thr,14 (3) Bax activators increased the proportion of PS-exposed platelets by Thr/Cvx stimulation,15 and (4) agonist-induced platelet procoagulant activity was independent of Bak and Bax.16 Taken together, the data suggest that proteins of the Bcl-2 family may participate in the procoagulant activity of platelets.
Importantly, the function of Bcl-2 proteins is regulated by phosphorylation (reviewed in 15), and dephosphorylation is mediated by calcineurin, a Ca$^{2+}$-activated protein phosphatase, inhibited by a complex formed by CsA and a cytosolic cyclophilin.18 This raises the question as to whether the inhibitory activity of CsA on PS exposure4 may alternatively act via inhibition of calcineurin-mediated dephosphorylation of Bcl-2 proteins, as calcineurin and its partner cyclophilin, are ubiquitously expressed in cells.

This report examines whether Ca$^{2+}$-induced PS exposure is a rapid apoptotic process involving mPTP pore formation leading to mitochondrial depolarization, and regulation of Bcl-2 and Bad phosphorylation. Experiments were performed with platelets and Jurkat T lymphocytes stimulated with Ca$^{2+}$ ionophores, as lymphocytic cell lines are relevant models for studying the PS translocation of hematopoietic cells.19–21 Platelets were also stimulated by Thr/Cvx and thapsigargin (TG), a sarco-endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA) inhibitor. TG triggers Ca$^{2+}$ release from the endoplasmic reticulum (ER) and induces store-operated Ca$^{2+}$ entry and rapid PS exposure in platelets, by-passing the ligand-receptor interaction.22

We show that ΔΨ$m$ loss occurred in platelets activated in the presence or absence of Ca$^{2+}$, as shown with the PS exposure process, which is dependent on high [Ca$^{2+}$]$_{cyt}$ increase. CsA inhibited PS exposure, ΔΨ$m$ loss, and the high [Ca$^{2+}$]$_{cyt}$ in the bulk of TG-stimulated platelets, as well as in a subpopulation in Thr/Cvx-stimulated platelets, and did not inhibit these events in A23187-stimulated platelets. Jurkat cells did not demonstrate an immediate ΔΨ$m$ loss under ionomycin stimulation. The basal phosphorylated status of Bcl-2 and Bad did not change in platelets and Jurkat cells after ionomycin stimulation. Further details are provided as supplementary data.

Western Blotting

Platelets or Jurkat cells were activated in the absence or presence of CsA and proceeded as usual for Western blotting. Proteins of interest were immunoocepted with specific antibodies or their phospho-specific counterparts.

Results

ΔΨ$m$ Loss in Ca$^{2+}$- or EGTA-Containing Medium in Stimulated Platelets

ΔΨ$m$ loss results from dissipation of the proton gradient across the inner mitochondrial membrane, after mPTP opening mediated by Ca$^{2+}$ entry. We therefore studied variations of ΔΨ$m$ in a medium containing 2 mmol/L Ca$^{2+}$, condition in which PS exposure occurs, using JC-1 as a mitochondrial potential sensitive probe (Figure 1A).

Addition of A23187 to platelets induced ΔΨ$m$ loss, as shown by the rapid decline in the JC-1 fluorescence ratio (black line in Figure 1A-a). In a control experiment, the lipid-soluble protonophore CCCP, which disrupts the proton gradient by carrying protons across the mitochondrial membrane and therefore induces ΔΨ$m$ loss independently of mPTP,23 triggered an instantaneous drop in the JC-1 fluorescence ratio (hatched line in Figure 1A-a). Pretreating the platelets with 5 μmol/L CsA for 3 minutes did not significantly change the kinetics of ΔΨ$m$ loss induced by A23187 (gray line in Figure 1A-a). This instantaneous loss of ΔΨ$m$ was probably attributable to insertion of Ca$^{2+}$-ionophore in the mitochondrial membrane, transporting Ca$^{2+}$ ions down their electrochemical gradient via H$^+$ exchange,24 thus dissipating the transmembrane proton gradient similarly to CCCP.
ΔΨm loss also occurred when TG was added to platelets (black line in Figure 1A-b). CsA drastically blocked ΔΨm loss, which collapsed further after addition of CCCP (gray lines in Figure 1A-b).

Thr/Cvx also caused a partial ΔΨm loss in platelets, which was inhibited by CsA (Figure 1A-c), in agreement with previous results showing that the platelet subpopulation exposing PS and undergoing ΔΨm loss was inhibited by CsA.4

To assess the role of Ca\(^{2+}\) in ΔΨm loss under conditions where PS exposure cannot occur, platelets were stimulated in a Ca\(^{2+}\)-free medium (containing 0.1 mmol/L EGTA). Results showed that A23187, TG, and Thr/Cvx induced ΔΨm loss in Ca\(^{2+}\)-free medium (black lines in Figure 1B-a through 1B-c), which was inhibited by CsA, excepted for A23187 (gray lines in Figure 1B-a through 1B-c). Addition of Ca\(^{2+}\) after Thr/Cvx stimulation induced a further decrease in the JC-1 fluorescence ratio, to approximately the level observed in the presence of Ca\(^{2+}\) in Thr/Cvx-stimulated platelets (black line in Figure 1A-c). Indeed, it has been demonstrated that thrombin induces ΔΨm loss in an EGTA-medium, presumably as a consequence of Ca\(^{2+}\)-store depletion, as it was not observed in BAPTA-loaded platelets.25

Altogether, these results showed that ΔΨm loss occurred in activated platelets, with and without Ca\(^{2+}\) in the medium, and was inhibited by CsA, except in A23187-stimulated platelets. As ΔΨm loss is a consequence of mitochondrial Ca\(^{2+}\) uptake, the results suggest that Ca\(^{2+}\) released from intracellular stores in an EGTA-containing medium is sufficient to induce mitochondrial depolarization, probably because of direct Ca\(^{2+}\) tunneling to mitochondria, as described in many cell types.26

**Simultaneous [Ca\(^{2+}\)]\(_{cyt}\) and PS Exposure Measurements in Platelets by Flow Cytometry**

A key element in the mechanism governing PS exposure is an elevated [Ca\(^{2+}\)]\(_{cyt}\). We analyzed by flow cytometry simultaneous [Ca\(^{2+}\)]\(_{cyt}\) changes and PS exposure in Fura-Red-loaded platelets activated in the presence of FITC-annexin A5.

In the absence of CsA, annexin A5 versus Fura-Red fluorescence dot-plots revealed that the majority of A23187- and TG-stimulated platelets (R1 in Figure 2B and 2C, left) bound annexin A5 and exhibited low Fura-Red fluorescence, indicating high [Ca\(^{2+}\)]\(_{cyt}\). Results are representative of at least 4 independent experiments.

![Figure 2](image-url)
whereas the remaining platelets (R2) did not bind annexin A5 but demonstrated an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ (MFI 100 in R2, supplemental Table I). The increase, however, was not as high as that observed in A23187 and TG-stimulated platelets (see above), indicating an intermediate $[\text{Ca}^{2+}]_{\text{cyt}}$.

In the presence of CsA, results for A23187-stimulated platelets were unchanged (dot-plots in Figure 2B, right), whereas the majority of platelets stimulated with TG (R2 in dot-plots in Figure 2C, right) did not bind annexin A5, and exhibited a lower intermediate $[\text{Ca}^{2+}]_{\text{cyt}}$ (MFI 94 compared to 35 in the absence of CsA, supplemental Table I). The platelet population responding to Thr/Cvx stimulation with annexin A5 binding and high $[\text{Ca}^{2+}]_{\text{cyt}}$ was clearly inhibited (R1 in Figure 2D, right), whereas the $[\text{Ca}^{2+}]_{\text{cyt}}$ of the bulk of platelets (R2 region) was similar to that without CsA (supplemental Table I).

Therefore, dual fluorescence flow cytometry data revealed that PS-exposing platelets were those with high $[\text{Ca}^{2+}]_{\text{cyt}}$. They further showed that in platelets stimulated with TG and Thr/Cvx, CsA inhibited PS exposure, concurrently with a reduction in $[\text{Ca}^{2+}]_{\text{cyt}}$ to the level of that of the bulk of Thr/Cvx-stimulated platelets, which do not expose PS.

### Analysis of Cytosolic and Mitochondrial Ca²⁺ Concentrations in Platelets

We next examined the interrelationship between cytosolic and mitochondrial Ca²⁺, as mitochondria are involved in cellular Ca²⁺ homeostasis.

In Ca²⁺-free medium, A23187 induced an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Figure 3A-a), because of internal Ca²⁺ store depletion, and in $[\text{Ca}^{2+}]_{\text{mit}}$ (Figure 3B-a), because of the buffering activity of mitochondria. The $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{Ca}^{2+}]_{\text{mit}}$ increases were both transient. The decreases after the $[\text{Ca}^{2+}]_{\text{cyt}}$ peaks resulted from active cytosolic Ca²⁺ extrusion and refilling of the Ca²⁺ stores, as well as Ca²⁺ efflux from mitochondria. Adding Ca²⁺ to the medium provoked a rapid sustained increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ and in $[\text{Ca}^{2+}]_{\text{mit}}$ (Figure 3A-a and 3B-a), attributable to A23187-induced Ca²⁺ entry, and subsequent Ca²⁺ uptake by mitochondria. CsA had no effect on Ca²⁺ mobilization from internal pools or mitochondrial Ca²⁺ uptake but delayed the increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ on addition of extracellular Ca²⁺. Consequently, mitochondrial Ca²⁺ uptake was also delayed (gray lines in Figure 3A-a and 3B-a).

In the absence of CsA, similar Ca²⁺ movements were observed in TG-stimulated platelets (black lines in Figure 3A-b and 3B-b). CsA partially inhibited Ca²⁺ release from the ER, capacitative Ca²⁺ influx, and mitochondrial Ca²⁺ uptake (gray lines in Figure 3A-b and 3B-b). The decrease in $[\text{Ca}^{2+}]_{\text{cyt}}$ is in agreement with that seen in flow cytometry experiments, showing an intermediate MFI for Fura-Red loaded platelets (supplemental Table I; Figure 2).

In Ca²⁺-free medium, Thr/Cvx induced a transient store depletion leading to an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{Ca}^{2+}]_{\text{mit}}$ (Figure 3A-c and 3B-c). Adding Ca²⁺ to the medium induced a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$, attributable to Ca²⁺ influx (Figure 3A-c) and a sustained increase in $[\text{Ca}^{2+}]_{\text{mit}}$ (Figure 3B-c). CsA had no major impact on these Ca²⁺ movements. This contrasted with results from the flow cytometry experiments showing that CsA inhibited the increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in the annexin A5-positive platelet population (R1 in Figure 2). The reason is that 2-color flow cytometry analyzing 2 responses simultaneously is
more efficient at sorting and identifying a small population with different properties than spectrofluorimetry, which analyses the whole platelet suspension. Therefore Figure 3A-c reflects the Ca\(^{2+}\) response of the majority of platelets, which do not expose PS, and where the increase in cytosolic Ca\(^{2+}\) is controlled by the combined action of Thr and Cvx on their respective receptor, leading to Ca\(^{2+}\) release from the ER through inositol 1,4,5-trisphosphate formation.\(^27\)

**Relationship Between [Ca\(^{2+}\)]\(_{cyt}\) and Membrane Remodeling Inducing PS Exposure**

CsA inhibits PS exposure concurrently with a decrease in [Ca\(^{2+}\)]\(_{cyt}\). We confirmed a direct link between PS exposure and [Ca\(^{2+}\)]\(_{cyt}\) rise in experiments where [Ca\(^{2+}\)]\(_{cyt}\) was progressively increased by stepwise addition of extracellular Ca\(^{2+}\) (supplemental Figure I).

To prove the requirement of [Ca\(^{2+}\)]\(_{cyt}\) increase for PS exposure, experiments were further performed with BAPTA-loaded platelets (supplemental Figure II). Results demonstrated that a platelet population failed to respond to A23187 and TG stimulation, whereas no response was obtained with Thr/Cvx. The latter result indicates that a high increase in [Ca\(^{2+}\)]\(_{cyt}\) is required for PS exposure in Thr/Cvx stimulated platelets, irrespective of the signaling events induced by these agonists.

**Ionomycin-Induced [Ca\(^{2+}\)]\(_{cyt}\) Increase Does not Correlate With \(\Delta \Psi_m\) Loss in Jurkat Cells**

To determine whether the [Ca\(^{2+}\)]\(_{cyt}\) increase induced by ionomycin was associated with \(\Delta \Psi_m\) loss in Jurkat cells, time-dependent fluorescence changes in cells loaded with Fluo-4 Ca\(^{2+}\) indicator and TMRM mitochondrial potential dye were simultaneously monitored by confocal video-microscopy (Figure 4).

Control experiments with CCCP showed a collapse of \(\Delta \Psi_m\), detected by the instantaneous sharp increase in TMRM fluorescence, coinciding with efflux of this dye from the mitochondria to cytoplasm, followed by a gradual egress from cells\(^28\) (Figure 4A and 4C), whereas the baseline fluorescence level of Fluo-4 was not modified (Figure 4D).

Conversely, TMRM fluorescence staining of mitochondria did not vary after addition of 2 \(\mu\)mol/L ionomycin to the cells (Figure 4B and 4E), whereas [Ca\(^{2+}\)]\(_{cyt}\) instantaneously increased, as indicated by the changes in Fluo-4 fluorescence (Figure 4F). In other cell types stimulated with Ca\(^{2+}\) ionophores, any mitochondrial depolarisation that occurred was weak and independent of CsA, as shown in HepG2 and in primary hippocampal neurons.\(^29\) However, loss of \(\Delta \Psi_m\) in Jurkat cells has been shown to occur under prolonged proapoptotic incubation with 2 \(\mu\)mol/L Ca\(^{2+}\) ionophore, as demonstrated after 10-hour treatment.\(^30\)

We also established that neither membrane phospholipid scrambling nor [Ca\(^{2+}\)]\(_{cyt}\) increase in Jurkat cells was blocked in the presence of 20 \(\mu\)mol/L CsA (supplemental Figure III) and that membrane remodeling induced by ionomycin still occurred in Jurkat cells where the mitochondria had been depolarized by prior incubation with CCCP (supplemental Figure IV).

**Bcl-2 and Bad Phosphorylation in Platelets and Jurkat Cells**

Both Bcl-2 and Bad are regulated by phosphorylation, and dephosphorylation has been shown to be mediated by calcineurin, which is inhibited by CsA. Western blot analysis showed that Bcl-2 and Bad were expressed in both platelets and Jurkat cells (Figure 5). The use of a specific phosphoSer70–Bcl-2 antibody revealed a strong labeling of this species in Jurkat cells, unchanged on ionomycin stimulation. This phosphorylation characterizes normally cycling Jurkat cells in the G2/M growth phase, resulting in stimulation of the antiapoptotic effect of Bcl-2.\(^31\) Platelets did not contain this phosphorylated form, either at rest or on stimulation. Thr56 residue in Bcl-2 was not phosphorylated in any cell type (Figure 5).

Platelets and Jurkat cells did not express phosphoSer155–Bad but contained phosphoSer112-Bad and phosphoSer136–Bad in untreated cells. In platelets, the labeling intensity did not change on stimulation, with or without CsA. In Jurkat cells, ionomycin stimulation induced a small increase in the labeling intensity of phosphoSer112-Bad. Consequently, the
phosphorylation status of Bad and Bcl-2 in platelets and Jurkat cells treated for Ca\(^{2+}\)-dependent rapid membrane scrambling did not undergo any of the calcineurin-dependent regulation change associated with apoptosis.

**Discussion**

The data showed that PS exposure and \(\Delta \Psi \text{m} \) loss occur concurrently in platelets stimulated with TG or Thr/Cvx in the presence of Ca\(^{2+}\), and are inhibited by CsA, whereas neither response is inhibited in A23187-stimulated platelets. In contrast, ionomycin-stimulated lymphocytes exhibit membrane remodeling in conditions showing no evidence of \(\Delta \Psi \text{m} \) loss, and the remodeling also occurs when mitochondria are depolarized by prior addition of CCCP. Importantly, we show that \(\Delta \Psi \text{m} \) loss occurs in platelets stimulated in the absence of Ca\(^{2+}\), when PS exposure cannot happen. Additionally, the data showed that depolarizing mitochondria with CCCP does not trigger PS exposure in platelets and Jurkat cells. The findings also exclude calcineurin-dependent dephosphorylation of Bad and Bcl-2 proteins in Ca\(^{2+}\)-induced rapid membrane scrambling. The data highlight the fact that PS exposure depends on a high level of [Ca\(^{2+}\)]\text{cyt}. Therefore inhibition of PS exposure by CsA is related to a drastic reduction of [Ca\(^{2+}\)]\text{cyt}. Finally, this article provides experimental evidence inferring a new effect of CsA in regulating Ca\(^{2+}\) movements in platelets.

The exhaustive study of mitochondrial polarization changes, using different experimental conditions and several agonists including Thr/Cvx, demonstrated that PS exposure occurs independently of \(\Delta \Psi \text{m} \) loss, and vice versa. The data confirm previous results analyzing apoptosis in platelets, and showing that PS can be exposed without \(\Delta \Psi \text{m} \) loss,\(^5\) as in Jurkat cells, and that \(\Delta \Psi \text{m} \) loss can occur in aged platelets without PS exposure,\(^32\) as in platelets stimulated in an EGTA medium.

Other mPTP inhibitors, such as coenzyme Q and bongkrekic acid, have been shown to inhibit \(\Delta \Psi \text{m} \) loss and annexin A5 binding to platelets stimulated with Thr/Cvx.\(^4\) However, their mechanism of action has not been thoroughly defined. They may be able to modify Ca\(^{2+}\) homeostasis, as shown here for CsA. They have additional effects: inhibition of ATP production for bongkrekic acid,\(^33\) and antioxidant properties for coenzyme Q.\(^34\) Indeed ATP is required for active maintenance of membrane phospholipid asymmetry, and oxidative stress has been involved in PS exposure.\(^35\) Therefore, these inhibitors can prevent PS exposure by unknown effects, as also suggested by Remenyi et al.\(^4\)

Because CsA inhibited PS exposure in platelets stimulated by TG and Thr/Cvx, the impact of CsA on Ca\(^{2+}\) movements was analyzed in detail. Results showed that CsA decreases Ca\(^{2+}\) depletion from the ER induced by TG inhibition of SERCA (Figure 3A-b). These data are in line with a study showing that CsA inhibits the Ca\(^{2+}\)-leak through the SERCA. Using vesicles of ER isolated from platelets treated with diacylglycerol analogues which uncouple the Ca\(^{2+}\)-ATPases, the Ca\(^{2+}\)-leak through the SERCAs, which then behaved as Ca\(^{2+}\) channels, was inhibited by CsA. The authors have postulated that a cyclolipin located in ER was involved in the process.\(^36\) Our results support this hypothesis. One likely cyclolipin protein candidate is s-CyP detected in platelet ER.\(^37\) As PS exposure in platelets depends on [Ca\(^{2+}\)]\text{cyt} (supplemental Figure I), the lack of PS exposure response of TG-stimulated platelets in the presence of CsA is related to the fact that CsA strongly decreases [Ca\(^{2+}\)]\text{cyt} (Figures 2C and 3A-b).

For Thr/Cvx-stimulated platelets, simultaneous analysis of [Ca\(^{2+}\)]\text{cyt} and PS exposure by flow cytometry revealed that the PS-exposing platelets are also those with high [Ca\(^{2+}\)]\text{cyt} (Figure 2D), reflecting previously reported results on platelets stimulated with thrombin plus collagen.\(^38-40\) Further evidence that this elevated [Ca\(^{2+}\)]\text{cyt} is involved in coated-platelet formation was provided by demonstrating their virtual absence in BAPTA-loaded platelets (supplemental Figure II).

A new finding of this work is that CsA inhibited [Ca\(^{2+}\)]\text{cyt} rise and annexin A5 binding in the platelet population responding to Thr/Cvx and in the majority of platelets stimulated with TG (Figure 2C and supplemental Table I). Therefore, CsA may inhibit Ca\(^{2+}\) release from ER in coated-platelets.

However, despite similar high [Ca\(^{2+}\)]\text{cyt} and PS exposure, Thr/Cvx-stimulated coated-platelets and platelets stimulated by A23187 (and probably by TG) are not physiologically identical, because the former exhibited higher level of surface expressed factor V and higher prothrombinase activity than the latter, suggesting that coated proteins are closely associated with procoagulant activity.\(^10,44\)

In conclusion, the data showed that rapid Ca\(^{2+}\)-induced PS exposure was not dependent on the apoptotic events studied here: mitochondrial depolarization and regulation of Bcl-2 and Bad phosphorylation. The results also indicate that rapid procoagulant phospholipid scrambling, which requires a sustained high [Ca\(^{2+}\)]\text{cyt} after Ca\(^{2+}\) influx, could be downmodulated by decreasing [Ca\(^{2+}\)]\text{cyt}.

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Disclosures
None.

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Rapid procoagulant phosphatidylserine exposure relies on high cytosolic calcium rather than on mitochondrial depolarization

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Materials

JC-1, carbonyl cyanide m-chlorophenylhydrazone (CCCP), CsA and bovine thrombin (T7513) were purchased from Sigma Chemical Co (St Louis, MO, USA). Convulxin was supplied by Alexis (San Diego, CA, USA). Fluo-3 AM, Fura-2 AM, Rhod-2 AM and FM1-43 were purchased from Molecular Probes (Eugene, OR, USA). FM1-43, TG, 4-bromo-A23187 (A23187), BAPTA-AM, and ionomycin were supplied by Calbiochem (La Jolla, CA, USA), and annexin VFluo (FITC-annexin A5) by Roche. X-VIVO 15 culture medium for lymphocytes, containing 1.8 mM Ca$^{2+}$, was purchased from BioWhittaker (Walkersville, MD, USA), RPMI 1640 glutamax-1 culture medium, non-essential amino-acids, Na$^+$ pyruvate, gentamycin and phosphate buffered saline (PBS) were supplied by Gibco; Fetal calf serum (FCS) was purchased from PAA; the kit for quantifying protein concentrations in lymphocyte cell lysates from Sigma; and the lysis buffer in the phospho-MAPK array kit from R and D Systems. The pro-survival Bcl-2 family antibody sampler kit (containing phosphoSer70-Bcl-2, phosphoThr56-Bcl-2, and Bcl-2 antibodies), the phospho-Bad antibody sampler kit (containing phosphoSer112-Bad, phosphoSer136-Bad, phosphoSer155-Bad, and Bad antibodies) were supplied by Cell Signaling. Peroxidase-labeled affinity-purified second antibodies specific to the F(ab')2 fragment of mouse and rabbit IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA). The chemiluminescence-
based Western blot detection system ECL Plus was obtained from GE-Healthcare (Buckinghamshire, UK).

**Platelet isolation and stimulation**

Freshly drawn blood was obtained from control subjects by venepuncture using acid-citrate-dextrose formula A as an anticoagulant and platelets were washed three times in 145 mM NaCl, 4 KCl, 0.5 mM NaH_2PO_4_, 0.5 mM MgCl_2_, 5 mM PIPES pH 6.8 and 5.5 mM glucose, in the presence of 0.1 µg/ml PGE1 and 5 µg/ml apyrase (washing buffer). Washed platelets were resuspended at 1 x 10^8 ml in 137 mM NaCl, 4 mM KCl, 0.5 mM NaH_2PO_4_, 0.5 mM MgCl_2_, 10 mM Hepes pH 7.4, and 5.5 mM glucose, (reaction buffer). Platelets were pre-incubated or not with 5 µM CsA at 37 °C for 3 min before stimulation with 3 µM 4- Bromo-A23187 or TG, or 0.5U/500 ng/ml Thr/Cvx.

**Ca^{2+} ionophores activation conditions**

In the literature, µmolar concentration of either Ca^{2+} ionophore A23187 or of ionomycin was used to stimulate membrane scrambling in platelets, erythrocytes or lymphocytes (see references in the main section). Several parameters including ionophore and Ca^{2+} concentration in the buffer (see supplementary Fig II), are essential for the process. It has been demonstrated that ionomycin, A23186 and bromo-A23186 all exhibit similar Ca^{2+} transport properties.\(^1\) Our experimental conditions were based on previously published studies by our laboratories which have determined that efficient membrane scrambling and PS exposure occurs for the whole population of platelets with 3 µM A23187,\(^2\) and for the whole population of lymphocytes with 2 µM ionomycin.\(^3\)

**Analysis of platelet mitochondrial membrane potential using JC-1**
The fluorescent probe JC-1 was used to determine the mitochondrial membrane potential. JC-1 fluoresces at 590 nm in polarized and 527 nm in depolarized mitochondria. Therefore, changes in ΔΨ were monitored using the JC-1 590/527 emission fluorescence ratio, with excitation at 480 nm. Platelets in plasma-rich platelets (PRP) were incubated with 10 µM JC-1 at 37 °C for 15 min and washed as detailed above. Fluorescence was measured in a Spex FluoroMax fluorimeter (Spex Industries Inc., Edison, NJ), equipped with a thermostatic jacket and a stirring device.

**Flow cytometry assays measuring both [Ca\(^{2+}\)]\(_{cyt}\) and PS exposure**

Platelets in PRP were loaded with the Ca\(^{2+}\) indicator Fura-Red AM (20 µM) at 37 °C for 45 min, then processed as described above. Fura-Red fluorescence decreases when the indicator binds Ca\(^{2+}\). Fura-Red loaded platelets were stimulated in the presence of FITC-annexin A5, as previously described.\(^4\) The presence of annexin A5 during stimulation inhibits microvesiculation and the decrease in platelet size that follows the loss of microparticles.\(^2\) In this way, Ca\(^{2+}\) variations and PS exposure are analyzed in platelets of constant size. Flow cytometry experiments used a Beckton Dickinson FASC-flow cytometer. Gating was as described\(^2\) and 10 000 particles were acquired from each sample according to forward and side light scatter, and analyzed for Fura-Red fluorescence (FL3, 670 long pass filter) and FITC-annexin A5 (FL1, 530 ± 30 nm bandpass filter), with appropriate compensations. In other studies, 100 µM BAPTA-AM was co-incubated with Fura-Red to evaluate the effect of cytosolic Ca\(^{2+}\) chelation on PS exposure.

**Cytosolic and mitochondrial Ca\(^{2+}\) measurements in platelets**

Platelets in PRP were incubated with 10 µM Fura-2 AM or 5 µM Rhod-2 AM at 37 °C for 45 min, and washed as detailed above. [Ca\(^{2+}\)]\(_{cyt}\) changes were monitored by measuring the Fura-2
fluorescence ratio at 340/380 nm with emission at 510 nm. $[\text{Ca}^{2+}]_{\text{mit}}$ was calculated as already described$^5$ with Rhod-2 excitation/emission wavelengths of 556/576 nm, using the expression $[\text{Ca}^{2+}] = K_d \times (F-F_{\text{min}}) / (F_{\text{max}}-F)$ where $K_d = 500$ nM, and $F =$ the fluorescence of the sample.

The calibration procedure consisted of obtaining $F_{\text{max}}$ by lysing platelets with 0.05% Triton X-100 in the presence of 2 mM Ca$^{2+}$, and $F_{\text{min}}$ by adding 4 mM EGTA from a 0.5 M EGTA stock solution in 3 M Tris at pH 8.3, 0.15 M NaCl. Ca$^{2+}$ was measured in reaction buffer containing 0.1 mM EGTA before adding 2 mM extracellular Ca$^{2+}$.

**Dual measurement of $\Delta \Psi_m$ variations and $[\text{Ca}^{2+}]_{\text{cyt}}$ in Jurkat cells by fluorescence imaging confocal video microscopy**

Jurkat cells were cultured in suspension at $2 \times 10^5$ cells mL$^{-1}$ and used 24 or 48 hours later. Before experiments, the cells were centrifuged at 220 g for 10 min and suspended at $10^6$ cells mL$^{-1}$ in X-VIVO 15 culture medium containing fluorescent dyes: Fluo-4 (4 μM), a $[\text{Ca}^{2+}]_{\text{cyt}}$ indicator, and tetramethylrhodamine methyl ester (TMRM, 1 μM), which is taken up by polarized mitochondria.$^6$ Verapamil (20 μM), which inhibits the multidrug resistance transporter (MDR), was added to the medium, as TMRM may be transported out of the cell by this pathway,$^7$ but did not modify $[\text{Ca}^{2+}]_{\text{cyt}}$ variations or membrane remodeling in our video-microscopy experiments (see below) with added ionomycin. Samples of the cell suspension (500 μL) were layered onto poly-L-lysine-coated round glass slides at 37°C for 40 min. The attached cells were washed three times with lymphocyte reaction buffer containing 116 mM NaCl, 1.8 mM CaCl$_2$, 5.6 mM KCl, 1.2 mM MgCl$_2$, 5 mM NaHCO$_3$, 1 mM NaH$_2$PO$_4$, and 20 mM Hepes, pH 7.3.$^3$ The slides were then set between adapted metal circles to form an observation chamber, and 500 μL reaction buffer containing 20 μM verapamil were added to the cells. The chamber was positioned under a confocal microscope (Nikon, EZT1). Fluo-4 ($\lambda_{\text{ex}}=488\text{nm}; \lambda_{\text{em}}=500 \text{ to } 530$) and TMRM ($\lambda_{\text{ex}}=543\text{nm}; \lambda_{\text{em}}=560 \text{ to } 610\text{nm}$)
fluorescence was simultaneously recorded every 6 seconds for all individual cells. At the indicated times, 500 μL of the same medium containing either 4 μM (final concentration: 2 μM) Ca\(^{2+}\) ionophore ionomycin or 10 μM (final concentration 5 μM) CCCP, a protonophore that depolarizes mitochondria, were added. Circular regions of interests were drawn around the cells and the fluorescence values recorded with time were transferred to an excel table to draw kinetic curves. Three independent experiments were performed in each condition. Representative data are given in Fig 4.

**Dual measurement of [Ca\(^{2+}\)]\(_{\text{cyt}}\) and plasma membrane remodeling in Jurkat cells by fluorescence imaging video microscopy**

Jurkat cells were loaded with the Ca\(^{2+}\) probe, Fura-2 (4 μM), while attaching on the glass slides, and washed with the lymphocyte reaction buffer described above. Experiments were performed as shown previously.\(^8\) The slides were then set to form an observation chamber as described above. 500 μl reaction buffer containing 5 μM FM1-43 were added to the cells. FM1-43 is a styryl dye which fluoresces on interaction with anionic PL at the external membrane layer. It is used to monitor membrane remodeling. The chamber, maintained at 37°C, was positioned under the microscope equipped with a video camera. Fura-2 and FM1-43 fluorescence were recorded simultaneously every 4 seconds and images were analyzed by drawing regions of interest over single cells. Emitted light was collected with a 515 nm dichroic mirror and a 520 nm longpass filter. Excitation at 450-490 nm was used to assess increases in FM1-43 fluorescence. Increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\) were indicated by the ratio of Fura-2 fluorescence on excitation at 340 nm versus 380 nm. Two independent experiments were performed in each condition with CCCP or CsA just after control experiments respectively. Representative data are given in supplementary Fig. III and IV.
Parallel studies of $[\text{Ca}^{2+}]_{\text{cyt}}$ increase and PS exposure measured as a function of extracellular $\text{Ca}^{2+}$ concentration

Fura-2 loaded platelets were stimulated with 3 µM A23187 in an EGTA-medium before addition of different concentrations of extracellular $\text{Ca}^{2+}$ from 0.1 to 2 mM, and $[\text{Ca}^{2+}]_{\text{cyt}}$ were analyzed as Fura-2 fluorescence ratio as explained above. With these platelets, a similar procedure was applied to study the kinetics of PS exposure as a function of extracellular $\text{Ca}^{2+}$, measured by analyzing fluorescence of FM1-43 (5µM), with excitation and emission wavelength 510 and 590 nm respectively, as already described.\(^8\)

Cell activation for Western blotting

Platelets were pre-incubated for 3 min with or without 5 µM CsA, and stimulated with 3 µM A23187 or TG at 37° C. After 10 min activation, the reaction was stopped by adding 5X Laemmli reducing buffer and samples were boiled for 2-3 min. Jurkat cells were suspended in culture medium at 2 X $10^5$ mL\(^{-1}\) 24 hours before the experiments. Immediately before the experiments, the cells were centrifuged, suspended at $10^7$cells mL\(^{-1}\) in lymphocyte reaction buffer, and maintained at 37° for 10 min. Samples (1 ml) of the cell suspension were then withdrawn before and 10 min after the addition of 2 µM ionomycin, and immediately centrifuged at 220 g for 7 min. The cells were then suspended in 500 µl lysis buffer, agitated at 4°C for 30 min, and centrifuged (14,000 g, 5 min at 4°C) to eliminate the cell debris. The protein content in the supernatant was determined by Micro-BC assay and 100-µg protein fractions were heated at 90°C for 5 min in 5X Laemmli buffer.

The lysates were electrophoresed on SDS-polyacrylamide gels (10-12% (w/v)) in a Mini-PROTEAN II Electrophoresis Cell (BioRad). Proteins were electrophoretically transferred onto PVDF membrane at 100V for 1h, using the Trans-Blot Electrophoretic Transfer Cell, and the membranes were treated as usual.\(^9\)
References

Supplementary Table I. Mean fluorescence intensities (MFI) of Fura-Red fluorescence in R1 and R2 regions
nd: non determined

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Figure II: Parallel studies of $[\text{Ca}^{2+}]_{\text{cyt}}$ increase expressed as Fura-2 fluorescence ratio (left), and PS exposure measured by FM1-43 fluorescence increase (right) in platelets stimulated with 3 µM A23187 in the absence of Ca$^{2+}$, followed by addition of different concentrations of extracellular Ca$^{2+}$ from 0,1 to 2 mM. Results show that both $[\text{Ca}^{2+}]_{\text{cyt}}$ and PS exposure increase progressively with extracellular Ca$^{2+}$, and are maximal with 2 mM extracellular Ca$^{2+}$. The small FM1-43 fluorescence increase which occurs before addition of Ca$^{2+}$ could result from a small PS exposure due to A23187-induced increase in cytosolic Na$^{2+}$ concentration, as postulated by Bucki R et al, Biochim Biophys Acta 2006;1761:195-204. Alternatively, secretion which occurs in platelets stimulated in the absence of Ca$^{2+}$, could lead to increased plasma membrane surface by fusion of secretory granules with plasma membrane, thus increasing the background FM1-43 fluorescence. Results are representative of 3 independent experiments.
Supplementary Figure II

Figure II: Effect of intracellular Ca\textsuperscript{2+} chelation on PS exposure measured by dual color flow cytometry analysing both [Ca\textsuperscript{2+}]\textsubscript{cyt} and annexin A5 binding in platelets.

Platelets, pre-loaded with Fura-Red with or without 100 µM BAPTA-AM, were stimulated in a Ca\textsuperscript{2+} medium in the presence of FITC-annexin A5 for 5 min at 37°C, diluted ten fold and immediately analysed in the flow cytometer. In order to increase the proportion of PS exposing platelets, we stimulated platelets with 1UThr+500 ng Cvx /ml, instead of 0.5U/500 ng/ml in Fig. 2 in the main manuscript, since it has been shown that thrombin is responsible for the synergistic effect on collagen-induced procoagulant response, by triggering a sustained elevation in [Ca\textsuperscript{2+}]\textsubscript{cyt} over collagen (Keuren et al, Arterioscler Thromb Vasc Biol 2005;25:1499-505). We found that 29 ± 8 % (n=3) of platelets bind annexin A5 with high [Ca\textsuperscript{2+}]\textsubscript{cyt} (R1 in D -BAPTA). This population was drastically reduced in BAPTA-loaded platelets (R1 in D +BAPTA). Furthermore, the annexin A5 negative platelet population has a lower increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} in BAPTA-loaded platelets (see supplementary Table I).

The results also showed that BAPTA prevented the increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} in a platelet subpopulation which can no longer expose PS when activation was performed with A23187 and TG (R1 in B and C +BAPTA). The figure is representative of 3 independent experiments.
Figure III. CsA does not prevent [Ca\textsuperscript{2+}]\textsubscript{i} changes and PL scrambling in Jurkat cells. A: representative single-cell traces simultaneously recorded by video-microscopy (see Materials and methods) for [Ca\textsuperscript{2+}]\textsubscript{i} increase, as shown by Fura-2 fluorescence ratios at 340/380 nm (upper traces, arbitrary units), and B: membrane remodelling, revealed by FM1-43 fluorescence (lower traces, arbitrary units). Traces are colour coded: for an individual cell, Fura-2 fluorescence ratio (upper trace) and FM1-43 fluorescence (lower trace) are indicated by the same colour. Left side: In the absence of CsA, 2 μM ionomycin induces an increase in Fura-2 fluorescence ratio, and a rapid increase in FM1-43 fluorescence. Right side: In the continuous presence of 20 μM CsA, the addition of ionomycin provokes the onset of the increases in both fluorescences. This experiment is representative of two independent experiments. All cells on the observation field (20 cells) reacted similarly. Kinetics for 10 cells in a given experiment are shown.
Supplementary Figure IV

Figure IV. CCCP does not alter $[\text{Ca}^{2+}]_{\text{cyt}}$ changes and PL scrambling in Jurkat cells. A: representative single-cell traces simultaneously recorded by video-microscopy (see Materials and methods) for $[\text{Ca}^{2+}]_{\text{cyt}}$ increase, as shown by Fura-2 fluorescence ratios at 340/380 nm (upper traces, arbitrary units), and B: membrane remodelling, revealed by FM1-43 fluorescence (lower traces, arbitrary units). Traces are colour coded: for an individual cell, Fura-2 fluorescence ratio (upper trace) and FM1-43 fluorescence (lower trace) are indicated by the same colour. Left side: In the absence of CCCP, 2 μM ionomycin induces an increase in Fura-2 fluorescence ratio, rapidly followed by an increase in FM1-43 fluorescence. Right side: In the continuous presence of 2 μM CCCP, only the addition of ionomycin provokes the onset of the increases in both fluorescences. This experiment is representative of two independent experiments. All cells on the observed field (20 cells) reacted similarly. Kinetics for a dozen cells in a given experiment are shown.