Two Types of Procoagulant Platelets Are Formed Upon Physiological Activation and Are Controlled by Integrin \( \alpha_{\text{IIb}}\beta_3 \)

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**Objective**—Phosphatidylserine (PS) externalization by platelets upon activation is a key event in hemostasis and thrombosis. It is currently believed that strong stimulation of platelets forms 2 subpopulations, only 1 of which expresses PS.

**Methods and Results**—Here, we demonstrate that physiological stimulation leads to the formation of not 1 but 2 types of PS-expressing activated platelets, with dramatically different properties. One subpopulation sustained increased calcium level after activation, whereas another returned to the basal low-calcium state. High-calcium PS-positive platelets had smaller size, high surface density of fibrin(ogen), no active integrin \( \alpha_{\text{IIb}}\beta_3 \), depolarized mitochondrial membranes, gradually lost cytoplasmic membrane integrity, and were poorly aggregated. In contrast, the low-calcium PS-positive platelets had normal size, retained mitochondrial membrane potential and cytoplasmic membrane integrity, and combined retention of fibrin(ogen) with active \( \alpha_{\text{IIb}}\beta_3 \) and high proaggregatory function. Formation of low-calcium PS-positive platelets was promoted by platelet concentration increase or shaking and was decreased by integrin \( \alpha_{\text{IIb}}\beta_3 \) antagonists, platelet dilution, or in platelets from kindlin-3-deficient and Glanzmann thrombasthenia patients.

**Conclusion**—Identification of a novel PS-expressing platelet subpopulation with low calcium regulated by integrin \( \alpha_{\text{IIb}}\beta_3 \) can be important for understanding the mechanisms of PS exposure and thrombus formation. (Arterioscler Thromb Vasc Biol. 2012;32:2475-2483.)

**Key Words:** adhesion molecules ■ phosphatidylserine ■ platelets ■ signal transduction
different from the previously reported PS-positive platelets by having an unusually low intracellular calcium level.

The aim of the current work was to investigate the properties of this new subpopulation and to elucidate the mechanisms responsible for its formation. By using pharmacological inhibitors or studying patients with well-defined inherited disorders of platelets, we found that formation of these low-calcium procoagulant platelets is facilitated by physiological platelet concentration and by shaking and is integrin α₃β₃-dependent.

Methods

Materials

The sources of the following materials are as follows: thrombin (Haemalogic Technologies, Essex Junction, VT); convulxin (Pentapharm, Basel, Switzerland); prostaglandin E1 (MP Biochemicals, Irvine, CA); phycocerythrin-conjugated annexin V, calcium-sensitive cell-permeable fluorescent dye Fura Red, cell-permeable mitochondrial inner membrane potential sensor calcein, acetylxythel ester (Molecular Probes, Eugene, OR); fluorescein-conjugated annexin V (Biovision, Mountain View, CA); Alexa Fluor 647-conjugated annexin V (Biologend, San Diego, CA); fluorescein isothiocyanate–labeled polyclonal antifibrinogen antibody (Lab Vision, Fremont, CA); Z-VAD-FMK, AYPGKF (Toeris Bioscience, Ellisville, MO); Phe-Pro-Arg-chloromethylketone (EMD Chemicals, Gibbstown, NJ); S2238 (Chromogenix, Milano, Italy); SFLRNN (AnaSpec, San Jose, CA); HEPES, bovine serum albumin, Sepharose CL-2B, apyrase grade VII (Sigma-Aldrich, St Louis, MO). Integrin α₃β₃ antagonist Monafram, an (P(ab′)₅ fragment, of a monoclonal antibody that blocks this receptor, was a generous gift of Prof A.V. Mazurov (Russian Cardiology Research and Production Center, Moscow, Russia). Collagen-related peptide was kindly provided by Prof R.W. Farnade (University of Cambridge, Cambridge, UK). Active site titration of thrombin was performed using Phe-Pro-Arg-chloromethylketone and S2238 essentially as described.

Patients

The first Glanzmann thrombasthenia (GT) patient (GT-1) lacked integrin α₃β₃ expression as determined by flow cytometry. The platelets failed to aggregate with physiological agonists. The patient had a relatively mild bleeding syndrome and did not routinely require treatment except for iron supplementation. The second patient (GT-2) also lacked integrin α₃β₃ and the platelets failed to aggregate or support clot retraction. GT-2 had a more severe bleeding syndrome, with frequent hematomas and purpuras, and had a history of blood transfusion with platelet concentrates. GT-1 shows double heterozygosity for its formation. By using pharmacological inhibitors or studying patients with well-defined inherited disorders of platelets, we found that formation of these low-calcium procoagulant platelets is facilitated by physiological platelet concentration and by shaking and is integrin α₃β₃-dependent.

Flow Cytometry

Gel-filtered platelets at different concentrations were activated by incubation with indicated agonists either alone or in combinations (see Results section), for selected time periods (usually 15 minutes) in buffer A with 2.5 mmol/L CaCl₂.

Calcium Signaling

Intracellular calcium levels were determined by flow cytometry essentially as described. Briefly, before gel filtration, resuspended platelets were incubated with 10 μmol/L Fura Red/AM for 30 minutes at room temperature in the presence of apyrase (0.1 U/mL) and prostaglandin E1 (1 μmol/L). The concentration of the calcium-sensitive dye solvent, dimethylsulfoxide, did not exceed 0.1%; additional controls confirmed that the vehicle did not affect the results and that the loading procedure did not have any significant effect on the PS-positive platelet formation.

Analysis of the Proaggregatory Abilities of the Subpopulations

Platelets at 1×10⁸ mL⁻¹ were activated for 15 minutes as described above in the presence of fluorescein-annexin V and PerCP-CD61, supplemented with 1 μmol/L Phe-Pro-Arg-chloromethylketone and 1 mg/mL fibrinogen, and analyzed by flow cytometry. The same sample was prepared, shaken at 600 rpm on a microshaker for 3 minutes after 15 minutes of stimulation, diluted ≥10-fold, and analyzed by flow cytometry. Platelet subpopulations were gated by annexin V fluorescence and scatter characteristics. The number of events in the corresponding regions with and without shaking was compared with the estimated proaggregatory abilities of subpopulations.

Statistics

Comparisons between 2 paired sets of values were carried out with the paired Student t test. Statistical significance was set as P<0.05. Experiments were reproduced 3x with platelets from different donors unless indicated otherwise, and values are reported as mean±SEM unless specified otherwise.

Results

Two Distinct PS-Positive Subpopulations Are Formed Upon Platelet Stimulation at a Physiological Concentration

Figure 1A illustrates annexin V binding for Fura Red–loaded platelets after activation at their physiological concentration.

Platelet Isolation

Blood was collected into sodium citrate and supplemented by apyrase (0.1 U/mL) and prostaglandin E1 (1 μmol/L). Investigations were performed in accordance with the Declaration of Helsinki, and written informed consent was obtained from all patients and control donors or from their parents. Platelets were purified by centrifugation and gel filtration as described. Briefly, platelet-rich plasma was obtained by centrifugation at 100 to 170g for 8 minutes (for giant Bernard-Soulier syndrome platelets, we used 80g). Platelet-rich plasma was centrifuged at 400g for 5 minutes, and platelets were resuspended in buffer A (150 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L MgCl₂, 0.4 mmol/L NaH₂PO₄, 20 mmol/L HEPES, 5 mmol/L glucose, 0.5% bovine serum albumin, pH 7.4) and gel filtered on Sepharose CL-2B.
of 2×10⁸ mL⁻¹ with 100 nmol/L thrombin. In contrast to previous reports,²,¹⁰,¹¹,¹⁷ not all of the PS-positive platelets sustained a high level of intracellular calcium after an initial increase. After several minutes from the beginning of activation, we could observe formation of 3 distinct subpopulations: (1) PS-negative platelets with low intracellular calcium level (PS–/Ca–), (2) PS-positive platelets with sustained high calcium level (PS+/Ca+) similar to those described previously,²,¹⁰,¹¹,¹⁷ and (3) a new subpopulation of platelets that had high PS together with unexpectedly low calcium (PS+/Ca–).

This segregation was clearly observed for the platelet concentration of 2×10⁸ mL⁻¹ (Figure 1B–1D), whereas at
0.2×10⁸ mL⁻¹ the new PS+/Ca⁻ platelets were present in much smaller numbers (Figure 1E). Besides thrombin stimulation, the same phenomenon was observed for platelet stimulation with collagen-related peptide or protease-activated receptor-1/ protease-activated receptor-4 agonist peptides, and there were no qualitative differences when varying the agonist concentration (Figure I in the online-only Data Supplement). So, strong platelet activation with all commonly used activators at high (physiological) platelet concentration led to the formation of 2 different types of PS-expressing platelets.

**PS+/Ca⁻ Platelets Have Activated α_{IIb}β₃, Retain High Levels of Surface Fibrin(ogen), and Are Proaggregatory**

To characterize properties of the platelet subpopulations formed at 2×10⁸ mL⁻¹, we incubated Fura Red–loaded platelets with annexin V and either PAC-1 or antifibrinogen antibody and analyzed them by 3-color flow cytometry. This was done 15 minutes after activation, when all subpopulations essentially finished their formation and were stable. Figure 2 shows typical dot plots of annexin V versus Fura Red fluorescence.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Properties of the 2 phosphatidylserine (PS)-positive subpopulations. A to G, Three-color analysis of the activated platelets. Fura Red–loaded platelets were stimulated at 1×10⁸ mL⁻¹ with 100 nmol/L thrombin in the presence of allophycocyanin-annexin V for 15 minutes, labeled with either fluorescein isothiocyanate (FITC)-antifibrinogen (D and E) or FITC-PAC-1 (F and G) and analyzed by flow cytometry. Dot plots of annexin V binding versus Fura Red fluorescence identified the subpopulations as indicated by the arrows (A). B, D, and F show the PS-negative subpopulation, whereas C, E, and G show the PS-positive platelets. The PS+/Ca⁻ platelets (C, top) appear distinctly larger than the PS+/Ca⁺ (C, bottom) or PS⁻ (B) ones. Both PS⁺ subpopulations have high levels of surface fibrinogen (E). Intriguingly, PS⁻/Ca⁻ platelets (G, top) have high PAC-1 binding similar to the PS-negative platelets (F) and in contrast to the more traditional PAC-1-negative PS+/Ca⁻ subpopulation (G, bottom). Typical experiments performed with platelets from different donors are shown (n=3). H, Averaged ratios±SEM of the forward light scatter (FSC) and fluorescence intensities for the subpopulations in the experiments of A to G. I and J, Proaggregatory abilities of the procoagulant subpopulations. Platelets were stimulated at 1×10⁸ mL⁻¹ with 50 nmol/L thrombin and 2 ng/mL convulxin for 15 minutes, labeled with FITC-annexin V, shaken at 600 rpm for 3 minutes, and analyzed by flow cytometry. The platelet subpopulations were identified by their annexin V binding and scatter properties (l). Both PS−/Ca− and PS+/Ca− platelets contribute to the aggregates, whereas the proaggregatory ability of PS+/Ca⁺ platelets is lower (l). Means±SEM are shown for n=3 experiments with different donors. Symbols indicate statistical significance of differences between the respective bars, P<0.05.
(A); Fura Red fluorescence of the selected events was further plotted versus forward scatter (B, C), or antifibrinogen antibody (D, E), or PAC-1 (F, G); the left column contains the PS− events, whereas the right one represents the PS+ ones. Averaged data are shown in Figure 2H. Forward scatter of the PS+/Ca− platelets was similar to that of the PS− platelets and ≈5-fold higher than that of PS+/Ca+ platelets, indicating differences in size and morphology between these 2 PS-positive subpopulations (Figure 2B and 2C). The PS+/Ca− platelets retain a high fibrin(ogen) expression that compares well with the well-characterized PS+/Ca+ coated platelet,17,18 whereas the PS− ones do not retain fibrin(ogen) (Figure 2D and 2E). In contrast to the PS+/Ca+ platelets and similar to the PS−/Ca− ones, the PS+/Ca− subpopulation binds PAC-1 (Figure 2F and 2G) and thus has integrin αIIbβ3 in the active state. This circumstance is of special interest in view of the previous report demonstrating different integrin expression on platelets of different sizes.23 Analysis of the integrin activation kinetics (Figures II and III in the online-only Data Supplement) revealed that most platelets originally had activated integrin in agreement with a previous report7 but that all Ca-positive cells lost PAC-1 binding as soon as they became PS positive.

To evaluate the proaggregatory activities of the subpopulations, platelets were activated by thrombin plus convulxin for 15 minutes to obtain a sufficient quantity of platelets from all subpopulations, incubated with or without shaking at 600 rpm, supplemented with Phe-Pro-Arg-chloromethylketone and fibrinogen, and analyzed by flow cytometry. Platelet subpopulations were gated using fluorochrome-labeled anti-CD61 (antibody against integrin β3 subunit) or annexin V and light scatter parameters. The ability of the platelets from different subpopulations to form aggregates was estimated by comparing the number of platelets in the corresponding regions before and after shaking. Only those platelets not incorporated into the aggregates were, therefore, assessed. Figure 2I shows that the 3 regions lose events differently. Specifically, PS− platelets and PS+/Ca− platelets were preferentially decreased, implying that PS+/Ca+ platelets have a much lower aggregatory capacity. Figure 2J shows a histogram plot of the events remaining in the corresponding regions after shaking and confirms that PS+/Ca+ platelets aggregate poorly in comparison with PS− and PS+/Ca− platelets. In control experiments performed at time point of 5 minutes, the PS+/Ca− subpopulation tended to go into aggregates as well (Figure IV in the online-only Data Supplement), likely because of initial integrin activation in all platelets that preceded their PS exposure (Figure III in the online-only Data Supplement).

**Cell Death–Related Phenomena in the Platelet Subpopulations**

High calcium and loss of PAC-1 binding were associated with mitochondrial membrane depolarization, whereas PS+/Ca− platelets retained their mitochondrial membrane potential as judged by tetramethylrhodamine fluorescence (Figure III in the online-only Data Supplement). Evaluation of the cytoplasmic cell membrane integrity using platelets loaded with calcein (Figure V in the online-only Data Supplement), a fluorescent molecule similar in size to Fura Red, showed that all subpopulations retained intact membranes and showed no leakage over the time intervals usually studied (0–20 minutes after activation). However, the PS+/Ca+ platelets began to lose their membrane integrity at 30 minutes, and they released calcein completely by 90 minutes. This leakage is important for correct interpretation of the data obtained with any cell-loaded dyes upon prolonged activation.

To test whether the PS-positive subpopulations are caspase-dependent,35 we carried out experiments on platelet activation using a pan-caspase inhibitor Z-VA-D-FMK. No effect of this inhibitor on the formation of the PS-positive subpopulations was detected (Figure VI in the online-only Data Supplement).

### Formation of the PS+/Ca− Platelets Is Regulated by Integrin αIIbβ3

Given that the novel PS+/Ca− subpopulation is formed at a relatively high platelet concentration, we tried to unravel the mechanisms responsible for its formation. As ADP-dependent signaling is known to regulate the PS-positive subpopulation,18 we performed experiments at different platelet concentrations and in the presence of ADP, GTP, serotonin, or apyrase (Figure VIIA–VIIID in the online-only Data Supplement). This revealed that none of the platelet-secreted substances influenced the proportion between the PS+/Ca+ and the PS+/Ca− subpopulations. Stimulation of platelets at a low concentration by the supernatant from platelets activated at a high concentration, which contained both the initial agonist and the platelet releasate, could not do this either (Figure VIIIE in the online-only Data Supplement).

As platelet-dense granule secretion during activation could not explain PS+/Ca− platelet formation at high platelet concentrations, an alternative hypothesis is that a high platelet concentration leads to more platelet–platelet interactions. A major surface component in platelet surface contact interactions is integrin αIIbβ3, which is able to regulate platelet activation via outside-in signaling.26 To investigate the role of αIIbβ3, we activated platelets at 0.2×10^9 mL^{-1} or 2×10^9 mL^{-1} with thrombin in the presence or absence of F(ab′)2 fragments of a monoclonal antibody that blocks this receptor (monafram). Experiments were performed in the presence of ADP added to mimic, respectively, the major effect of platelet secretion. Figure 3 shows the formation of PS+/Ca+ and PS+/Ca− platelet subpopulations under the different conditions. Shaking of platelets at 0.2×10^9 mL^{-1} led to a decrease in the number of PS+/Ca+ platelets and a small, nonproportional increase in the number of PS+/Ca− ones. This effect was completely prevented by monafram addition; in a control experiment without shaking, monafram had no effect at 0.2×10^9 mL^{-1}. Shaking of platelets at 0.2×10^9 mL^{-1} in the presence of ADP led to the formation of both PS+/Ca+ and PS+/Ca− subpopulations in quantities similar to those observed at 2×10^9 mL^{-1} platelets.

To further test the hypothesis that signaling through αIIbβ3 regulates the formation of PS+/Ca− platelets, patients with either GT (genetic αIIbβ3 deficiency) or a deficiency of kindlin-3 (a critically important molecule involved in activation of αIIbβ3) in a patient with leukocyte adhesion deficiency disease type III were examined for their ability to form PS+/Ca− platelets at different concentrations (Figure 4).
A Bernard-Soulier syndrome patient (deficiency in glycoprotein Ib/IX/V) was also included. Although platelets from healthy donors formed the PS+/Ca– platelets at physiological platelet concentrations, platelets from 2 patients with GT and a third with kindlin-3 deficiency had much smaller ratio of the PS+/Ca– to the PS+/Ca+ subpopulations (Figure 4B). It should be noted, however, that the significant variability in the PS+/Ca– platelet formation in normal donors does not allow to reliably judge whether this is completely because of the significant decrease in PS+/Ca– subpopulation only (Figure VIIIA in the online-only Data Supplement) or the possible increase in PS+/Ca+ subpopulation as well (Figure VIIIB in the online-only Data Supplement). Platelets from the Bernard-Soulier patient formed the PS+/Ca– platelets normally. Furthermore, a decrease in the ratio of PS+/Ca– platelets to PS+/Ca+ platelets with either monafram or tirofiban (a synthetic inhibitor of α\textsubscript{IIb}β\textsubscript{3}) (Figure 4C) was observed for platelets from healthy donors and the patient with Bernard-Soulier syndrome but not for those from patients with GT or kindlin-3 deficiency. Unexpectedly, though, monafram and tirofiban had some effects on the absolute numbers of platelets in the PS+/Ca+ and PS+/Ca– subpopulations in the GT-2 patient, suggesting nonspecific interactions; there is also some effect of tirofiban on the PS+/Ca– subpopulation in the leukocyte adhesion deficiency disease type III patient (Figure VIII in the online-only Data Supplement). These data strongly suggest that the formation of PS+/Ca– platelets is regulated by α\textsubscript{IIb}β\textsubscript{3} signaling during activation. Finally, in view of the revealed role of α\textsubscript{IIb}β\textsubscript{3} and platelet interactions, we evaluated the formation of PS+/Ca– platelets in a more physiological medium with naturally present concentrations of α\textsubscript{IIb}β\textsubscript{3} ligands by performing experiments on platelet activation with collagen-related peptide in platelet-rich plasma, where they were produced in quantities similar to those with platelets analyzed after gel filtration (Figure VIII in the online-only Data Supplement).

**Discussion**

The formation of a highly procoagulant necrotic PS-positive subpopulation upon strong platelet activation (reviewed in Refs 3, 4, and 7) has been a puzzle for almost 20 years.\(^6\) These platelets are highly effective in binding coagulation factors and accelerating membrane-dependent coagulation reactions,\(^8,11\) but their integrin α\textsubscript{IIb}β\textsubscript{3} is inactivated,\(^12,17\) which reduces their chances of being accumulated into thrombi or hemostatic plugs. Their significance for hemostatic and thrombotic events is not well understood: their deficient production caused by inhibition of the cyclophilin D–dependent necrotic pathways is not associated with bleeding but rather with increased thrombosis.\(^14\) On the other hand, the deficiency of PS expression in Scott syndrome in humans\(^27\) and dogs\(^16\) is associated with bleeding.

In the present study, we found that platelet stimulation under more physiological conditions than is usually used (specifically, at a platelet concentration of 2×10\textsuperscript{8} mL\textsuperscript{-1}) leads to the formation of a so far unknown PS-positive subpopulation. These platelets seem to possess a unique function...
in hemostasis and thrombosis, as they are unusually both procoagulant and proaggregatory. The data indicate that their formation is promoted by cell–cell contacts and is dependent on integrin αIIbβ3, probably on the outside-in signaling (Figure 5). These new procoagulant platelets have low intracellular calcium but express integrin αIIbβ3 in an activated state. Several lines of evidence suggest a critical role of integrins in their formation: these include the positive effects of platelet concentration and of platelet–platelet contact, the prevention of their formation by αIIbβ3 antagonists, and their absence in the case of αIIbβ3 and kindlin-3 deficiency. The phenomenon was not prevented by caspase inhibitors. The increased formation of the new subpopulation upon physiological platelet concentrations was similar in buffer and platelet-rich plasma. This strongly suggests the possible formation of the procoagulant/proaggregatory platelets in vivo.

The physiological role of this new subpopulation remains to be established. If we assume that specific targeting of necrosis-regulating pathways predominantly affects only the poorly aggregating PS+/Ca+ platelets, whereas general prevention of the PS exposure affects both the PS+/Ca+ and the PS+/Ca– platelets, this could explain why the former model is prothrombotic with no effect of bleeding, whereas the latter has a strong bleeding phenotype.

The role of αIIbβ3 in platelet procoagulant activity formation has been a source of significant controversy for a decade. Interestingly, all researchers who reported inhibition of procoagulant activity by αIIbβ3 deficiency or antagonism used a physiological range of platelet concentrations with a single exception, and all those who reported no effect or stimulation by αIIbβ3 antagonists invariably used much lower platelet concentrations. This discrepancy may, therefore, be explained by the finding that αIIbβ3 antagonism has no effect on the PS+/Ca+ platelets but strongly inhibits the PS+/Ca– subpopulation (Figure 3B).

The findings of this work favor a hypothesis that there are probably 2 distinct pathways for PS exposure in platelets during activation. One of them leads to PS+/Ca+ platelet formation and is similar to necrosis, whereas another leads to PS+/Ca– platelet formation. This phenomenon is universal for all strong platelet agonists. Importantly, in contrast to previous experience, this new pathway seems not to require sustained high calcium levels: even after 40 minutes, when calcium in all platelets has reverted to the basal state, we still see formation of the PS+/Ca– platelets.

Lack of the PS+/Ca– platelets may be a contributing factor in the severe bleedings of patients with GT and kindling-3 deficiency and also contribute to the antithrombotic action of αIIbβ3 inhibitors, tirofiban and monafram, that were shown to inhibit PS+/Ca– platelets in the present study. Although the present study directly shows the importance of αIIbβ3 and its activation for PS+/Ca– platelet formation, it provides only indirect evidence that it is mediated by outside-in signaling. Therefore, additional experiments should be performed to test whether lack of the PS+/Ca– platelets can account for
defective hemostasis and formation of unstable thrombi associated with defective outside-in signaling.35–37

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Disclosures
None.

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Supplemental Material

Two types of procoagulant platelets are formed upon physiological activation and are controlled by integrin $\alpha_{IIb}\beta_3$.

![Figure S1](image)

**PS-positive platelet formation upon stimulation with different agonists.** (A-F) The typical dot plots show FITC-annexin V (abscissa axis) versus Fura Red fluorescence (ordinata axis) for gel-filtered platelets, either non-stimulated (A, D) or activated (B, C, E, F) with agonists as indicated. When platelets were activated with either thrombin or CRP at a physiological concentration of $2 \times 10^8$ ml$^{-1}$, there were two PS-expressing subpopulations with different intracellular calcium levels. (G-I) Formation of the subpopulations at different concentrations of thrombin or PAR agonists. Fura Red-loaded platelets were stimulated at $2 \times 10^8$ ml$^{-1}$ with indicated agonists in the presence of FITC-annexin V, and samples were subjected to flow cytometry after 15 min of activation. (G) Stimulation with thrombin. (H) Stimulation with PAR-1 agonist SFLLRN. (I) Stimulation with PAR-4 agonist AY-NH2. Means ± SEM for n=3 experiments with different donors are shown.
Kinetics of integrin activation in the platelet subpopulations. Fura Red-loaded gel-filtered platelets at $2 \times 10^8$ ml$^{-1}$ were stimulated with 100 nM thrombin and 10 µg/ml CRP in buffer A in the presence of 2.5 mM CaCl$_2$. Samples were collected at indicated time points, incubated with Alexa Fluor 647-conjugated annexin V and FITC-PAC-1 for 2 min, and immediately analyzed by flow cytometry. (A) Typical dot plots showing integrin activation versus procoagulant activity. The additional dot plot for t=10 min shows that PS+/Ca$^-$ platelets are PAC-1 positive, and PS+/Ca$^+$ platelets are PAC-1-negative. (B) Formation of the PAC-1 positive subpopulations as a function of time, means ± S.E.M. for n=3 experiments with platelets from different donors.
Kinetics of mitochondrial potential change in the platelet subpopulations. Gel-filtered platelets were preincubated at 4×10^8 ml^-1 with 10 µM tetramethylrhodamine for 15 min, followed by activation at 2×10^8 ml^-1 with 100 nM thrombin in buffer A in the presence of 2.5 mM CaCl_2 and Alexa Fluor 647-conjugated annexin V. Samples were collected at indicated time points, incubated with FITC-PAC-1 for 2 min, and immediately analyzed by flow cytometry. (A) Typical dot plots showing mitochondrial membrane potential development and PS externalization during activation. (B) Membrane potential versus PAC-1 fluorescence for the two PS-positive subpopulations from the same experiments as in panel A: it can be seen that the PS+/Ca+ platelets have low potential throughout the time of the experiment, while the PS+/Ca– platelets have high potential. (C) Membrane potential in the subpopulations as a function of time for the experiments in panel A. Means ± S.E.M. are shown, n=6 experiments with platelets from different donors.
Pro-aggregatory abilities of the procoagulant subpopulations at an early timepoint. Experiments were performed similarly to those in Fig. 2. Platelets were stimulated at $2 \times 10^8$ ml$^{-1}$ with 200 nM thrombin for 5 min, labeled with PE-annexin V and FITC-CD42b, shaken at 600 rpm for 3 min without any additions, and analyzed by flow cytometry. Platelet subpopulations were gated by annexin V fluorescence and scatter characteristics. The number of events in corresponding regions with and without shaking were compared to estimate pro-aggregatory abilities of the subpopulations. (A) Typical dot plots of annexin V fluorescence versus side scatter. (B) Fraction of platelets of each subpopulation that remained in the suspension, means ± S.E.M., n=3.
Kinetics of membrane permeability increase in the platelet subpopulations. Platelets were loaded with calcein by incubation for 20 min with 2 μM calcein ether; Fura Red was also loaded as described in the Methods, and double-loaded platelets were gel-filtered. They were stimulated at $2 \times 10^8 \text{ ml}^{-1}$ with 100 nM thrombin in buffer A in the presence of 2.5 mM CaCl$_2$ and Alexa Fluor 647-conjugated annexin V. Samples were taken at indicated time points and immediately analyzed by flow cytometry. (A) Typical dot plots of annexin V versus calcein fluorescence. The bottom dot plot shows that only high-calcium platelets have increased membrane permeability. (B) Kinetics of calcein loss for the three platelet subpopulations for the experiments shown in panel A. Shown are means ± S.E.M., n=3.
Effect of the pan-caspase inhibitor Z-VAD-FMK on the formation of the PS-positive subpopulations.

Fura Red-loaded platelets ($4 \times 10^8$ ml$^{-1}$) were incubated with a saturating concentration of Z-VAD-FMK (200 µM, 1% DMSO) for 1 hour, diluted 2-fold with buffer A containing thrombin (200 nM) and Alexa Fluor 647-conjugated annexin V. Samples were collected and analyzed by flow cytometry at indicated time intervals; for gating by PAC-1, they were additionally incubated with FITC-PAC-1 for 3 min. Typical experiments are shown out of n=3.
Platelet secretion does not explain formation of the PS+/Ca− platelets at high platelet concentrations. (A) The PS+/Ca− subpopulation formed upon platelet stimulation with 100 nM thrombin increases as the platelet concentration augments in contrast to the PS+/Ca+ one. Means ± S.E.M. for n=5 experiments with different donors are shown. (B) In contrast, both subpopulations are equally well inhibited by apyrase when stimulated at 2×10⁸ ml⁻¹ with 100 nM thrombin. Means ± S.E.M. for n=3 experiments with different donors are shown. (C) Effect of either ADP or apyrase on the formation of platelet subpopulations at different concentrations; a typical experiment. The data clearly show that the effects of secretion are not dependent on the platelet concentration change. (D) Presence of GTP and serotonin does not change the ratio between the PS+/Ca+ and the PS+/Ca− platelets. (E) Addition of supernatant from platelets activated with 100 nM thrombin at 2×10⁸ ml⁻¹ to platelets activated at 0.2×10⁸ ml⁻¹ does not change the ratio between the PS+/Ca− and PS+/Ca+ subpopulations. Means ± S.E.M. for n=3 experiments with different donors are shown.
Figure S8

Regulation of the PS+/Ca– and PS+/Ca+ platelets by outside-in signaling. The panels show absolute numbers of platelets in the Ca-negative procoagulant subpopulation (A, C) and the Ca-positive one (B, D) for experiments shown in Fig. 4B (panels A, B) and Fig. 4C (panels C, D).
Formation of the PS+/Ca− platelets in platelet-rich plasma. Platelets in recalcified platelet-rich plasma were activated with CRP in the presence of PPACK (2 µM) to prevent thrombin formation. Fluorescently labeled PAC-1 and annexin V were added to observe formation of the three platelet subpopulations.