Formation of coated platelets is regulated by the dense granule secretion of adenosine 5′diphosphate acting via the P2Y12 receptor

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Dual-agonist stimulation of platelets with thrombin and collagen (or convulxin, collagen receptor glycoprotein VI agonist) results in the formation of platelet subpopulation called coated platelets, which is characterized by retention of α-granule proteins on the cell surface and phosphatidylserine expression [1–3]. High procoagulant activity of coated platelets [4,5] suggests their importance for thrombosis and hemostasis, although their exact function and the mechanisms regulating their production are not clear [3,6–8]. The size of this subpopulation depends on the type and degree of activation [1], suggesting that the fraction of platelets to become coated is determined by some regulating mechanisms during activation. The objective of this study was to elucidate the role of secretion in this regulation.

Gel-filtered platelets were prepared, activated and assayed by flow cytometry essentially as described [9]. A detailed description of the experimental procedures is available as online supplementary material. Activation with either thrombin or thrombin plus convulxin resulted in the formation of a platelet subpopulation double-positive for phosphatidylserine and α-granule proteins (Fig. S1). Control experiments demonstrated inability of PAC-1 antibody and echistatin to displace fibrinogen from these platelets, and their analysis by fluorescent microscopy confirmed lack of significant aggregation (data not shown). These data confirmed that this subpopulation consisted of coated platelets for both types of activation.

To test whether platelet segregation into subpopulations is regulated by platelet-derived substances, platelets were activated at different concentrations. The fraction of coated platelets rapidly increased with the increase of platelet concentration from 1000 to 16 000 μL^{-1} (Figs. 1A and 1B). At higher platelet concentrations, it slowly increased for thrombin-stimulated platelets (Fig. 1A); for potent thrombin-plus-convulxin-induced activation, there already was saturation.

It has been previously reported that an increase of platelet count leads to an increase of phosphatidylserine exposure by thrombin-stimulated platelets; this was ascribed to the stimulation by platelet–platelet contacts [10]. However, in contrast to Dormann *et al*. [10], our experiments were performed in the absence of stirring or shaking, making this explanation unlikely. Fluorescence microscopy experiments revealed ~10% aggregate formation in our experiments at the highest platelet count used (100 000 μL^{-1}), and only 1–2% of coated platelets were in aggregates, which also does not favor the aggregation-dependent mechanism. The remaining mechanism to explain this dependence was secretion.

To identify the roles of specific platelet-derived substances, we tested contributions of two major platelet-derived stimulators, thromboxane A2 and adenosine 5'-diphosphate (ADP). Neither inhibition of thromboxane synthesis by aspirin nor addition of thromboxane analog U46619 had any effect on coated platelets (Fig. S2). This agrees with the report of Prodan *et al*. [11] that only chronic, and not intermittent, aspirin use inhibits coated platelet production (probably because of aspirin effects on megakaryocyte development). In contrast, apyrase dose-dependently decreased the coated platelet subpopulation, while ADP potently increased it (Figs. S3A and S3B). The ADP dose response (Fig. S3B) in the presence of thrombin and convulxin was also saturated at lower concentrations than in the presence of thrombin alone. The dependence of coated platelets on platelet concentration, shown in Figs. 1A and 1B, disappeared in the presence of apyrase (Figs. S3C and S3D).

These data indicated that dense granule secretion of ADP is a major regulator of coated platelet formation upon either thrombin- or thrombin-plus-collagen stimulation. To monitor the time course of dense granule release directly, platelets were dual-labeled with mepacrine and annexin V, and subjected to flow cytometry analysis. All platelets secreted their dense granule content by ~30 s after stimulation, which was followed by their segregation into subpopulations several minutes later (Fig. S4).

To identify receptors mediating the ADP effect, we activated platelets in the presence of specific antagonists of ADP receptors P2Y1 and P2Y12, MRS2179 and 2-MeS-5′-AMP,

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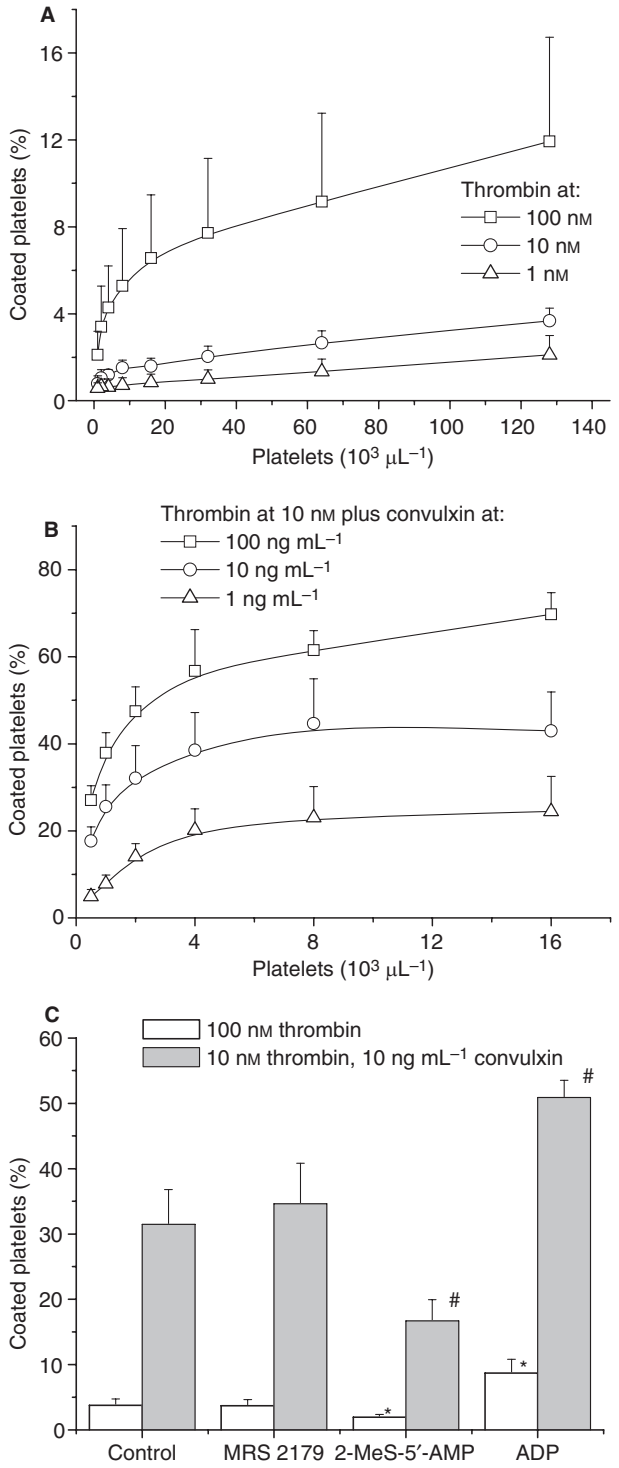

Fig. 1. Role of platelet secretion in the formation of coated platelets. Platelets were stimulated as indicated in the figure and analyzed by flow cytometry. (A,B) Effect of platelet concentration on coated platelet formation. The number of coated platelets is shown as a function of platelet concentration. (C) Contribution of adenosine 5′diphosphate (ADP) receptors subtypes in coated platelet formation. Platelets at 4000 μL^{-1} were stimulated in the absence (labeled as 'Control' in the figure) or in the presence of specific purinoreceptor antagonists MRS2179 (10 μM) or 2-MeS-5'-AMP (100 μM), or in the presence of saturating ADP (10 μM). Mean ± SEM for $n = 4$ experiments using platelets from different donors are shown. *$P < 0.05$; $P < 0.01$.

respectively. While MRS2179 had no effect, 2-MeS-5′-AMP inhibited coated platelet formation 2-fold (Fig. 1C). When compared with the positive control, where ADP was added, the difference was 3- to 4-fold suggesting a predominant role of the P2Y12 receptor in the ADP effect. This observation is in line with prior reports that the P2Y12 receptor plays an important role in the development of thrombin-induced platelet procoagulant activity [12–16]. Here we observed the specific mechanism of this activity development, which is regulation of a special platelet subpopulation with large amounts of phosphatidylserine and α-granule proteins retained on their surface.

Our results reveal that the number of coated platelets appearing upon activation is regulated by a complex mechanism involving a positive feedback of platelet secretion. Because of this, coated platelets are dynamically formed in quantities determined by the degree of external activation and their own concentration so that their number can be easily regulated from 1% to 70% and higher. Our experiments do not exclude the possibility that there are some pre-existing parameters, which determine platelet segregation into the coated platelet subpopulation (e.g. there is evidence that young platelets become coated more readily [1]). However, the existence of a single predetermined, predestined subpopulation of 'platelets-to-become-coated' appears unlikely in view of our result that almost any platelet can become coated given sufficient activation. It can be speculated that the formation of coated platelets is a special mechanism, whose function is to regulate the degree of platelet phosphatidylserine exposure (and other forms of procoagulant activity) in response to different degrees of hemostatic challenge. From the point of view of the organism, it might be more convenient to have a trigger determining which part of platelets should become 'maximally procoagulant' than to regulate the degree of phosphatidylserine exposure by an individual cell.

It can also be speculated that the function of the ADP-dependent mechanism of coated platelet regulation observed in this work is to increase coated platelet formation within the platelet plug, thus stimulating fibrin clotting, strengthening and stabilization of the plug. Although, in our purified system, this secretion-dependent effect is saturated at platelet concentrations below physiological ones (Figs. 1A and 1B), much more ADP is likely to be required in order to stimulate coated platelet formation *in vivo*, where ADP can be degraded by blood ectonucleotidases or removed by flow and diffusion.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Platelets double-positive for α-granule protein retention and phosphatidylserine externalization are produced with either single- or dual-agonist stimulation. Typical contour plots of thrombospondin (FL1 in A and B) or fibrinogen (FL1 in C and D) vs. annexin V (FL2) binding for platelets (50 000 μL^{-1}) stimulated with (A, C) thrombin alone (100 nM) or (B, D) thrombin (10 nM) with convulxin (10 ng mL^{-1}). For all plots, in the upper right region of the plot, a subpopulation of double-positive platelets (henceforth identified as coated platelets) is observed. This subpopulation is marked as a rectangular region in the panels. The contour plots are logarithmic with a 15% interval between contours.

Fig. S2. Thromboxane A2 signaling is not involved in coated platelet production. Platelets (4000 μL^{-1}) were incubated for 1 h with or without aspirin (100 μM). They were stimulated with either thrombin or thrombin-plus-convulxin in the presence or in the absence of stable thromboxane analog U46619 (5 μM). Mean values ± SEM for $n = 5$ experiments using platelets from different donors are shown. There was no statistically significant difference in coated platelet formation between control experiments, pre-incubation with aspirin, and U46619 addition ($P > 0.5$).

Fig. S3. Adenosine 5′diphosphate (ADP) regulation of coated platelet formation. (A) Coated platelet formation as a function of apyrase concentration. (B) Coated platelet formation as a function of ADP concentration. (A, B) Platelets at 4000 μL^{-1} were activated with either thrombin or thrombin and convulxin at indicated concentrations in the presence of either apyrase or ADP, and analyzed by flow cytometry. Mean values ± SEM for $n = 4$ experiments using platelets from different donors are shown. (C, D) Dependence of coated platelet formation on platelet concentration disappears in the presence of apyrase. Platelets were activated with either 100 nM thrombin (C) or 10 nM thrombin with 10 ng mL^{-1} convulxin (D) under the conditions of Fig. 1 in the presence or in the absence of apyrase. Typical experiments are shown.

Fig. S4. Secretion of dense granules during coated platelets formation. The contour plots show typical time course of mepacrine secretion upon platelet (20 000 μL^{-1}) stimulation with thrombin (10 nM) and convulxin (10 ng mL^{-1}). The plots are for mepacrine fluorescence (FL1) vs. annexin V fluorescence (FL2). (A)–(F) 0, 0.5, 1, 2, 4, and 8 min after stimulations, respectively. The coated platelet subpopulation is marked as a rectangular region in (F). The contour plots are logarithmic with a 15% interval between contours.

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