Subpopulations in purified platelets adhering on glass

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Understanding how platelet activation is regulated is important in the context of cardiovascular disorders and their management with antiplatelet therapy. Recent evidence points to different platelet subpopulations performing different functions. In particular, procoagulant and aggregating subpopulations have been reported in the literature in platelets treated with the GPVI agonists. How the formation of platelet subpopulations upon activation is regulated remains unclear. Here, it is shown that procoagulant and aggregating platelet subpopulations arise spontaneously upon adhesion of purified platelets on clean glass surfaces. Calcium ionophore treatment of the adhering platelets resulted in one platelet population expressing both the procoagulant and the adherent population markers phosphatidylserine and the activated form of GPIIb/IIIa, while all of the platelets expressed CD62P independently of the ionophore treatment. Therefore, all platelets have the capacity to express all three activation markers. It is concluded that platelet subpopulations observed in various studies reflect the dynamics of the platelet activation process. © *2016 American Vacuum Society*. [http://dx.doi.org/10.1116/1.4953866]

I. INTRODUCTION

Platelets are best known for their crucial role in hemostasis,[1,2] but they also play a significant role in cardiovascular disorders: from the inflammation of the vessel wall that leads to atherosclerosis and plaque formation to the growth of the thrombi at the site of the arterial stenosis.[3–5]

Platelet activity is tightly regulated. They are activated at the site of vascular injury where their agonists such as collagen and collagen-adsorbed von Willebrand factor are exposed, and thrombin is generated at the tissue factor exposed on the damaged endothelium. Activated platelets catalyse clot formation and subsequently orchestrate inflammatory response and wound healing cascade reactions.[6–9]

Platelet activation is characterized by events such as translocation of phosphatidylserine (PS) to the outer platelet membrane surface, where it serves as the site for the coagulation factor assembly catalyzing clot formation; activation of the major integrin GPIIb/IIIa responsible for platelet aggregation through fibrinogen binding; secretion of α- and dense granules associated with the expression of granule-specific proteins such as CD62P (P-selectin), responsible for the interactions between platelets and other cells: endothelial cells and leukocytes.[10,11] α-granules contain various coagulation factors, growth factors, and cytokines responsible for the subsequent inflammatory and wound healing reactions,[6–9] while dense granules contain small molecules such as serotonin, calcium, and ADP.[1]

Several authors reported that stimulation with GPVI agonists (collagen and its analogues), alone or in combination with thrombin, induces distinct platelet subpopulations with respect to the expression of PS in purified platelets.[12–14] Both PS– and PS+ platelets were found to express CD62P, while calcium ionophore (CaIoP) treatment led to one PS+ population.[12] It was also recently reported that GPIIb/IIIa on the PS+ platelet subpopulation was not activated;[13] we will refer to these as aGPIIb/IIIa− platelets. The same study reported a time-dependent increase in the fraction of PS+ platelets and a transient GPIIb/IIIa activation after stimulation. Combined with the intravital microscopy results, these observations lead Heemskerk *et al*. to postulate that there are at least two distinct platelet subpopulations: procoagulant (PS+/aGPIIb/IIIa–) and aggregating (PS–/aGPIIb/IIIa+).[15] Two other subpopulations have also been reported: the so-called "coated" PS+ platelets expressing fibrinogen and coagulation factors such as FV,[15,16] and platelets expressing both PS and aGPIIb/IIIa.[17,18] Finally, intravital microscopy following vascular injury revealed hierarchical thrombus organization in which a core of P-selectin expressing platelets was surrounded by a shell of platelets that do not express CD62P.[19] The authors attributed the differential CD62P expression to the agonist gradients that arise during thrombus formation. α-granule secretion heterogeneity was also observed in the adhering platelets.[20,21] These observations

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lead to an emerging hypothesis that different platelet subpopulations participate in these different processes.[15] The significance of this hypothesis to the clinicians is highlighted by the fact that whole-blood assays for detecting platelet subpopulations are beginning to emerge.[22] The relationship between these differently activated platelet types remains to be clarified. Here, we report observations that shed some light on this issue.

II. RESULTS AND DISCUSSION

Freshly purified platelets were allowed to adhere to the freshly cleaned, uncoated glass surfaces. Surface characterization of the glass slides by x-ray photoelectron spectroscopy (XPS) before and after cleaning is shown in Fig. 1. It indicates that the freshly cleaned glass surface has minimal adventitious contamination, organic or inorganic.

The expression of activation markers on the adhering platelets was examined by immunofluorescence microscopy [Fig. 2(a)]. We used the platelet-specific marker CD41a to visualize the platelets, CD62P to reveal the secretion of α-granules, as well as activation of GPIIb/IIIa and expression

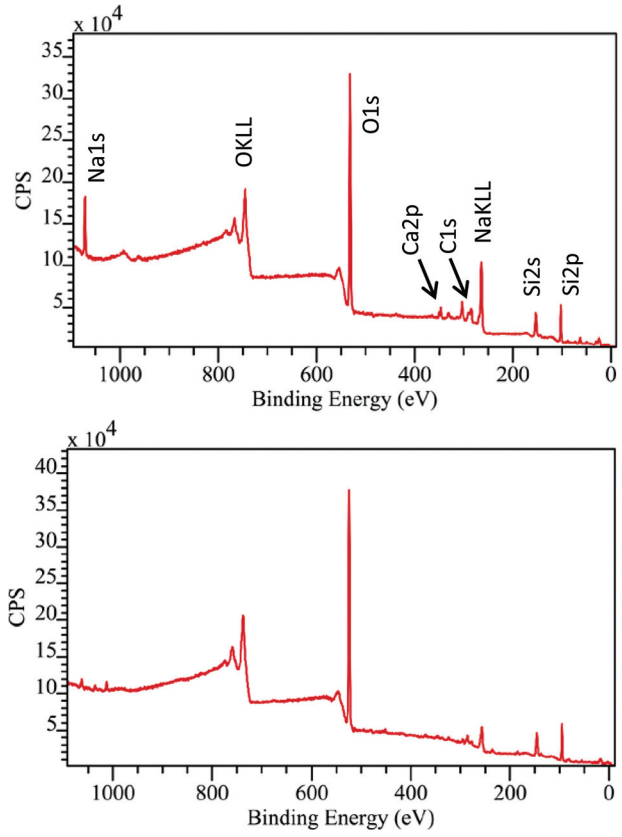

FIG. 1. XPS analysis of glass surfaces before and after cleaning. Top: an XPS spectrum of the glass surface before cleaning. Bottom: an XPS spectrum of the glass surface subjected to the SDS-UV/ozone cleaning procedure. Organic contaminants (represented by the C1s peak) were reduced from 17 at. % of carbon before cleaning to <4% after cleaning. No nitrogen was observed on our surfaces, also ruling out protein contamination. For comparison, XPS spectra of glasses and silicon wafers can be found in Refs. 34 and 35. Surface-adsorbed proteins contain significantly greater amounts of carbon as well as nitrogen (Ref. 36).

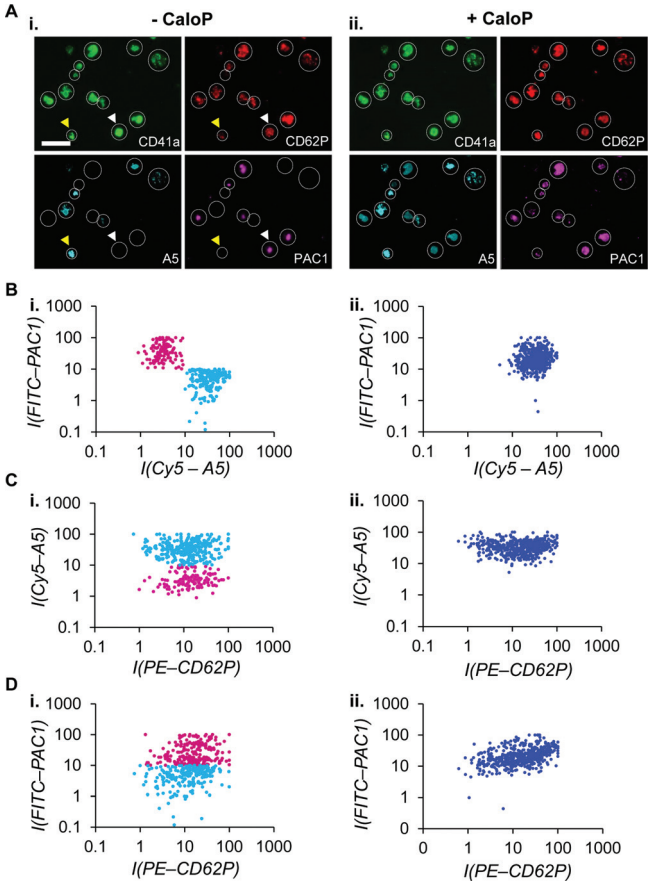

FIG. 2. Distinct platelet populations in purified platelets adhering on glass: fluorescence images and statistical analysis. (a) Platelets adhering on glass for 10 min were stained with anti-CD41a (green), anti-CD62P (red), A5 to reveal PS (cyan) and PAC1 to reveal aGPIIb/IIIa (magenta). (i) Before CaIoP treatment. Adhering platelets (green) uniformly express CD62P (red). Some of the platelets express PS (cyan) and some of the platelets express aGPIIb/IIIa (magenta). (ii) After treatment with 5 μM CaIoP all platelets express all three markers. Dashed circles highlight the platelets for ease of identification. The images shown are representative of one experiment. Scale bar: 10 μm. Note, how platelets that express PS do not express aGPIIb/IIIa and vice a versa (white and yellow arrowheads, respectively). (b)–(d) Dot plots representing statistical analysis of fluorescence images, such as those shown in (a), from 15 experiments performed with blood from ten different donors. (b) Mean fluorescence intensities of Cy5-A5 (PS exposure) are plotted vs those of the FITC-PAC1 (GPIIb/IIIa activation). Before CaIoP treatment (i), two platelet subpopulations are visible: PS+/aGPIIb/IIIa– (cyan) and PS–/aGPIIb/IIIa+ (magenta). The colors reflect the identity of the fluorescence markers and are identical to the colors used in (a), while the existence of the subpopulations is inferred from the localization of the fluorescence intensities to the different quadrants of the plot. After CaIoP treatment (ii), only one population (PS+/aGPIIb/IIIa+) is observed. It is colored in blue. (c) and (d) Mean fluorescence intensities of PE-anti-CD62P are plotted vs those of Cy5-A5 (C) and FITC-PAC1 (D). It is evident that CD62P is expressed on all of the platelets both before and after CaIoP treatment (cf. panels i and ii, where the fluorescence intensities span the left and the right quadrants of the plots in both cases). On the contrary, differences in the distributions of fluorescence intensities of A5 and FITC-PAC1 are visible before and after CaIoP treatment: while in the latter case, the intensities appear in the upper quadrants of the plots, in the former case, they appear both in the lower and in the upper quadrants. Once again, the color assignment reflects the identity of the fluorescence markers used in (a).

of PS detected with PAC1 and annexin A5, respectively. Figure 2(a) shows that all of the adhering platelets (CD41a+, green) expressed CD62P (red) upon contact with glass. On the contrary, not all of the platelets expressed PS

(cyan) or aGPIIb/IIIa (magenta). Moreover, the expression of these two markers was anticorrelated: the platelets expressing PS do not stain positive for aGPIIb/IIIa and vice versa [white and yellow arrowheads in Fig. 2(a)]. Treating these adhering platelets with 5 μM CaIoP elicited a uniform expression of both markers on all of the platelets. The treatment with CaIoP has no further effects on the expression of CD62P [Fig. 2(a-ii)].

Statistical analysis of the adhering platelets from different donors (a total of 15 experiments performed with blood from ten different donors) confirmed that the observation of PS+/aGPIIb/IIIa– (procoagulant) and one PS–/aGPIIb/IIIa+ (aggregating) platelet subpopulations was reproducible. The level of activation of GPIIb/IIIa versus the level of expression of PS on the adhering platelets is plotted in Fig. 2(b-i). Platelets that express PS (colored cyan) are clearly separated from the platelets that express aGPIIb/IIIa (magenta); the two populations appear in the diagonally opposing quadrants of the plot. This separation disappears upon CaIoP treatment [Fig. 2(b-ii)].

It should be underscored that the color scheme in Fig. 2(b) was chosen to reflect the labels (PAC1 and annexin A5) used in Fig. 2(a). Indeed, the separation between the populations is visible in the plots of the raw (background-subtracted) mean fluorescence intensities shown in Fig. 3(a-i, ii). The histograms exhibit bimodal distributions. They become unimodal when platelets are treated with CaIoP [Fig. 3(a-iii)], reflecting what is seen in the dot plot shown in Fig. 2(b-ii).

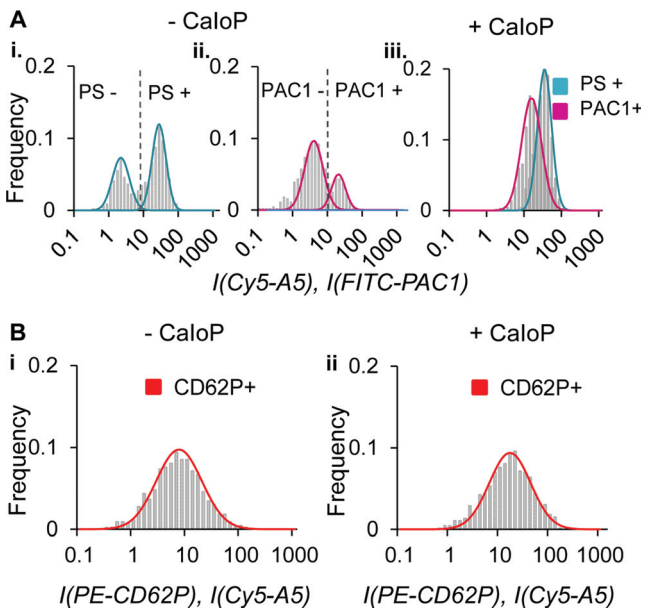

FIG. 3. Fluorescence intensity distributions in purified platelets adhering on glass. (a) Distributions of mean fluorescence intensities reflecting A5 and PAC1 binding to the adhering platelets (i and ii) are bimodal, reflecting the existence of the two subpopulations of adhering platelets with respect to the expression of these markers. CaIoP treatment leads to unimodal distributions, indicating that all of the adhering platelets express these markers (iii). (b) Distributions of mean fluorescence intensity of PE-anti-CD62P on the platelets adhering on glass before (i) and after (ii) CaIoP treatment. The distributions are unimodal in both cases. Frequency indicates the fraction of activated platelets.

CD62P was expressed on all of the adhering platelets. Therefore, the distributions of mean fluorescence intensities reflecting CD62P expression were unimodal both before and after CaIoP treatment [Fig. 3(b)]. Consequently, there is no separation into diagonally opposing quadrants in the dot plots shown in Figs. 2(c) and 2(d). Instead, the PS+ platelets (cyan) appear above the aGPIIb/IIIa+ platelets (magenta) in Fig. 2(c), and below - in Fig. 2(d), indicating that the two subpopulations we observe in platelets adhering on glass are CD62P+/PS+/aGPIIb/IIIa– and CD62P+/PS–/aGPIIb/IIIa+. Once again, the colors in Figs. 2(c-i) and 2(d-i), were only used to indicate which marker was expressed on which platelets. The grouping of the data according to the set of markers expressed that is visible in Fig. 2 is very distinct. It is also consistent with the histograms shown in Fig. 3.

Treatment with 5 μM CaIoP resulted in one population expressing all three markers: CD62P+/PS+/aGPIIb/IIIa+, reflected by one cloud of points visible in Figs. 2(c-ii) and 2(d-ii). On the contrary, not all of the platelets treated with 5 μM CaIoP in solution expressed aGPIIb/IIIa [Fig. 4(b)], implying that there is a synergy between surface adhesion and CaIoP treatment.

No influence of the degree of platelet spreading on the fluorescence intensities was observed (not shown).

To the best of our knowledge, this is the first observation that adhesion to glass induces the formation of the procoagulant and aggregating subpopulations in a similar fashion as the treatment with the GPIV ligands (collagen, convulxin) alone or in combination with thrombin.[13,14] It adds to the growing body of evidence that artificial surfaces exhibit properties that are similar to those of classical platelet agonists, and that responses of platelets to different surfaces are different.[20,21,23–26] Most likely, the surface–platelet signaling is mediated by the adsorbed proteins.[27,28] Properly studied, these phenomena offer a possibility to improve the control over the response of platelets to artificial surfaces.

Our results furthermore support the notion, advanced in the literature, that platelets have the capability to independently regulate PS expression and GPIIb/IIIa activation.[29] The ability to independently regulate α-granule secretion must further be supposed to reconcile observations of CD62P expression heterogeneity in thrombi[19] and in the adhering platelets.[20,21] Where does this ability originate?

In several studies, different platelet populations were observed after exposing platelet suspensions to one stimulus. For example, treatment with convulxin led to the appearance of aGPIIb/IIIa+ and aGPIIb/IIIa– platelets,[13] while treatment with collagen led to the appearance of three populations (PS+/aGPIIb/IIIa–, PS–/aGPIIb/IIIa+, PS+/aGPIIb/IIIa+).[17] This points to differences in the signaling machinery between the platelets that should be detectable at the level of the proteome. Indeed, functionally significant platelet receptor polymorphisms have been reported,[30] but the field of platelet proteomics does not yet appear to be

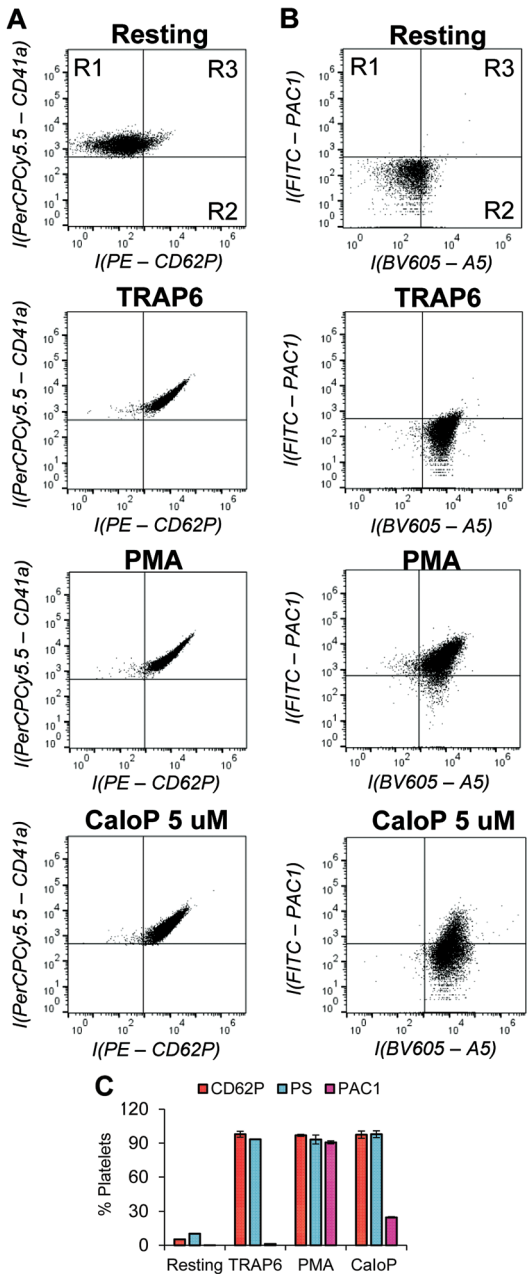

FIG. 4. Flow cytometry analysis of purified platelets. Before each surface experiment, platelets were tested for their purity, activation level and response to agonists. Platelets were used in further experiments only if their activation levels were below 5% for CD62P and GPIIb/IIIa, and below 10% for PS. (a) Expression of CD62P on the resting platelets and the agonist-treated platelets. Fluorescence intensities due to anti-CD41a binding vs those due to anti-CD62P binding are plotted. Maximal expression of CD62P [see panel (c)] is reached with all of the agonists used (TRAP6, PMA and CaIoP). Quadrant gates R1, R2, and R3 refer to events positive for anti-CD41a (R1), anti-CD62P (R2), or both markers (R3). (b) Same as (a), but for PAC1 (antibody against aGPIIb/IIIa) and A5 (binding to PS). TRAP6, PMA and CaIoP induce the expression of PS in all platelets, but different agonists have different effects on the activation of GPIIb/IIIa: no activation with TRAP6, complete activation with PMA, and incomplete with CaIoP. The scatter plots from one representative experiment are shown. (c) Activation level of resting and agonist-treated platelets with respect to CD62P expression, PS expression, and GPIIb/IIIa activation. The plot shows averaged results from five experiments performed with the blood from four different donors. Error bars are std. deviations.

sufficiently mature to detect differences in the signaling machinery.

On the other hand, we and others observed that all of the platelets are capable of expressing CD62P, PS, and aGPIIb/IIIa under appropriate conditions: platelets adhering on glass expressed all three markers after the treatment with CaIoP (Figure 2); the expression of all of these markers is routinely observed in PMA-treated platelets (an example is shown in Figure 4); and Bachelot-Loza *et al*. observed co-expression of PS and aGPIIb/IIIa on collagen and collagen + thrombin treated platelets,[17] as did Yakimenko *et al*. in thrombin and convulxin + thrombin-treated platelets.[18] Both these studies also report a three-population state (PS+/aGPIIb/IIIa−, PS−/aGPIIb/IIIa+, PS+/aGPIIb/IIIa+).[17,18] Therefore, the proteomic differences responsible for the appearance of platelet subpopulations do not extend to the level of the effectors. This makes the interconversion between platelet subpopulations possible upon appropriate stimulation. Typically, such interconversion requires stimuli that alter intracellular calcium dynamics, e.g., CaIoP (Fig. 2) or agonist combinations (collagen- or convulxin + thrombin).[13,15,17,31] In other words, the repertoire of the activation markers expressed on the platelet surface depends on the type and duration of the stimuli they are exposed to. Integrating all of this evidence, we suggest that platelet subpopulations that are observed under different conditions in various studies represent snapshots, transient stages, of a dynamic activation process. "Dynamic" in this case is taken to mean not only time-dependence, but dependence on the type stimulus.

III. CONCLUSION

Combined with the available literature data, our observations lead to the conclusion that platelet subpopulations observed in many of the studies represent snapshots of a dynamic activation process. This work also demonstrates the value of model studies performed with purified platelets.

IV. MATERIALS AND METHODS

A. Blood collection

Experiments reported here were conducted as a part of other studies at two different institutions and performed by two different investigators (A.D. and S.G.). Donors were healthy volunteers without exposure to medication such as aspirin in two weeks prior to donation who gave informed consent. Phlebotomy was performed by the qualified nurses of the medical service at KIT in Germany and of the Basque Biobank for Research in Galdakao, Spain. Study protocols were approved by the Ethics commission of Baden-Württemberg (Approval No. F-2014-077) and Comité de Etica de Investigaciones Clínicas (the Clinical Investigations Ethics Committee) of the Galdakao-Usansolo hospital in Spain, respectively. Blood was drawn through a 21 gauge needle into a BD vacutainer containing 0.129 M sodium citrate anticoagulant (Becton-Dickinson Biosciences, Heidelberg, Germany). First 2 ml of blood were discarded to avoid activation by residual thrombin and tissue factor.

B. Platelet isolation and characterization by flow cytometry

Platelets were purified from citrated whole blood by three centrifugation steps as described in our previous publications.[20,32] Briefly, platelet rich plasma (PRP) was prepared by centrifugation of whole blood at 40 × *g* for 20 min at 37 °C. PRP was then collected into 15 ml Falcon and incubated with acid-citrate-dextrose in a ratio of 1:6 for 10 min at 37 °C. To isolate platelets from the plasma, the samples were then centrifuged at 700 × *g* for 20 min at 22 °C. In order to get rid of the residual proteins, platelets were gently resuspended in citrate buffer (100 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 15 mM citrate, *p*H 6.5) and centrifuged at 700 × *g*, for 10 min at 22 °C. Finally, the pellet of platelets was resuspended in calcium-free-HEPES buffer (145 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 10 mM HEPES, *p*H 7.4) at a concentration of 1×10^8 platelet/ml.

All buffers were freshly prepared, autoclaved, and filtered through 200 nm sterile syringe filters (Whatman GE Healthcare Life Sciences, Freiburg, Germany).

Before use, purified platelets were characterized by flow cytometry to determine activation levels according to the expression of CD62P, PS, and the activation of GPIIb/IIIa and response to agonists: thrombin receptor activating peptide 6 (TRAP6) PMA and calcium ionophore (CaIoP) (Sigma Aldrich, Munich, Germany). In Spain, a FACS Calibur Flow Cytometer (Becton–Dickinson, Madrid, Spain) was used, and at KIT an Attune Acoustic Focusing Cytometer (Life Technology, Darmstadt, Germany) was used. Only minimally activated platelets that correctly responded to the agonists were used in further studies. See Fig. 4 for details. All experimental procedures were carried out in a sterile laminar flow cabinet to avoid contamination.

C. Surface cleaning

Glass was cleaned with 2% SDS followed by a UV-ozone treatment. This is a widely established cleaning protocol used in countless laboratories world-wide for removing organic contaminants reviewed in Ref. 33. See Fig. 1 for further details. Surfaces were characterized by XPS before and after cleaning. XPS analysis was performed with a SAGE HR100 spectrometer (Specs, Berlin, Germany) with a nonmonochromatic Mg Kα source operating at 300 W. Pass energy of 30 eV was used. Spectra were calibrated using the C 1s peak at 285.0 eV and analyzed using CASAXPS software (Casa Software, Ltd.).

D. Fluorescence experiments

Freshly purified platelets were allowed to adhere on freshly cleaned glass at the concentration of 5×10^7 platelet/ml for 10 min, 37 °C in calcium-free buffer. Adherent platelets were then gently washed with the same buffer, rinsed with the calcium-contained buffer and stained with PerCPCy5.5-anti-CD41a, PE-anti-CD62P, Cy5-Annexin AV (to reveal PS exposure), and FITC-PAC1 (to reveal the active form of GPIIb/IIIa) for 30 min, 37 °C. The antibodies were purchased from Becton-Dickinson Biosciences. To stimulate platelets with CaIoP, it was added to the buffer above the platelets for 30 min at 37 °C.

For the microscopy experiments, the glass slides were mounted in home-made cells designed to fit on the stages of the fluorescence microscopes. In Spain, a laser scanning confocal microscope (Carl Zeiss, Jena, Germany) was used. At KIT, a Zeiss Observed Z1 inverted microscope was used. All the experiments were conducted at 37 °C.

E. Image analysis

Fluorescence images were analyzed using the FIJI software. First, the multichannel images were split into separate channels for CD41a, CD62P, PS, and PAC1 fluorescence. The mean fluorescence intensities of individual platelets in each channel were calculated as follows.

The channel for CD41a fluorescence was thresholded in order to obtain a binary image. This image was used to define regions of interest (ROIs) representing individual platelets. The ROI data from the binary image were used as a mask, applied to the CD62P, PS, and PAC1 channels, to extract the fluorescence intensities reflecting the expression of these markers on the platelets. Background fluorescence was calculated manually using ROIs outside of the platelets in the same images and subtracted from the intensity for each platelet. The resulting background-subtracted values are indicative of the level of CD62P, PS, and aGPIIb/IIIa expression on the platelets. They are plotted in Figs. 2 and 3.

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