

Platelet phosphatidylserine exposure and procoagulant activity in clotting whole blood – different effects of collagen, TRAP and calcium ionophore A23187

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Summary

We have studied the effects of different platelet agonists on phosphatidylserine (PS) exposure and clotting times in blood without anticoagulants. Similar reductions in clotting time were obtained for collagen, TRAP-6 or calcium ionophore A23187 (50 μmol/L), in spite of huge differences in PS expression [6.7 ± 2.4%, 2.3 ± 0.5% and 99.9 ± 0.1%, respectively (mean ± SD, n = 5)]. Furthermore, the clotting times were much longer for samples with A23187 exposing the same amounts of PS as samples with collagen or TRAP-6. Annexin V reversed the clotting time reduction, but could not prevent coagulation. Addition of phospholipid vesicles containing 20% PS neither affected the clotting times nor induced clotting in recalcified, platelet-free plasma.

We conclude that platelet PS exposure is necessary, but not sufficient, for the coagulation amplification observed when platelets are stimulated via physiological receptors in a whole blood environment.

Keywords

Hemostasis, blood platelets, platelet activation, platelet procoagulant activity, platelet factor 3

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Introduction

Platelets are known to participate in all phases of haemostasis *in vivo*. Besides the obvious contribution in formation of the platelet plug, they provide the surface necessary for the assembly of the tenase and prothrombinase complexes, a process leading to the formation of thrombin, and ultimately the fibrin clot. The complex assembly involves the translocation of negatively charged phosphatidylserine (PS) to the platelet surface. This translocation is thought to be due to a protein called scramblase, which in the presence of elevated cellular calcium levels arranges a rapid, non-selective bidirectional movement of phospholipids over the membrane in activated platelets (1). The γ-carboxylated factors II, VII, IX and X all show a high affinity for calcium ions, and the conformational change induced by the calcium binding exposes hydrophobic residues thought to interact with the platelet surface (2, 3).

Platelet activation also induces a conformational change in the GPIIb/IIIa complex, making it able to bind vWf and fibrinogen, thereby strengthening the clot and enabling platelet aggregation (4) and clot retraction (5). Studies with GPIIb/IIIa antagonists also implicate that this receptor as important for PS exposure and factor V binding to activated platelets (6).

We have earlier reported that activation of platelets in unanticoagulated freshly drawn whole blood (subsequently termed native blood) shortens the coagulation time (7). In this work we have studied the exposure of phosphatidylserine on the surface

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of platelets in coagulating blood with flow cytometry, and compared it to the coagulation time reduction obtained by the addition of different types of platelet agonists. The effect of the inhibition of exposed PS by addition of annexin V was also studied. Altogether, this study supports the view that additional features besides the exposure of negatively charged surface must be involved in the platelet propagation of coagulation in native whole blood.

Materials and methods

Instruments and reagents

Four channel-free oscillating rheometers (FOR), ReoRox4® (8, 9), disposable polypropylene sample cups, as well as software (ReoRox4 v2.00 and ReoRox4Viewer v2.0 or 2.01) were purchased from GHI (Global Hemostasis Institute MGR AB), Linköping, Sweden. In this instrument, oscillation is initiated by a forced turn of the sample cup every 2.5 s. After a brief hold time, the sample cup is released, allowing rotational oscillation with very low friction around the longitudinal axis. An optic angular sensor records the frequency (Fq) and damping (D) of the oscillation as a function of time. This is plotted as a curve, from which the time for different curve features might be determined. Clot detection in the present study was based on Pythagorean summation of changes in Fq and D, reaching a predefined level C:

$$\sqrt{(\Delta Fq^2 + \Delta D^2)} \geq C$$

The "high sensitivity state detector" (where C = 0.01) was used, and the related endpoint is referred to as "clotting time". We have shown that this point correlates extremely well with the manual reference method of visual clot detection (r^2 = 0.97, submitted for publication).

Flow cytometry was performed on an Ortho Cytoron Absolute Flow Cytometer with the software ImmunoCount II (v. 2.00) (Ortho, Raritan, NY, USA). The exposure of PS on the platelet surface was examined using fluorescein (FITC)-conjugated annexin V (Actiplate®, Tau Technologies BV, Kattendijke, Netherlands), known to bind to PS in the cell membrane. In these experiments native whole blood was used and the coagulation was inhibited with the Gly-Pro-Arg-Pro amide (GPRP) (Sigma Chemical Company, St. Louis, MO, USA), which inhibits the polymerisation of fibrin monomers (10, 11).

The "Lipo-so-fast" extruder used for preparation of phospholipid vesicles was from Avestin, Inc. (Ottawa, Canada).

Chemicals for the HEPES buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L $MgCl_2$, 5.6 mmol/L glucose, 1 g/L bovine serum albumin and 20 mmol/L HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), pH 7.40), the calcium ionophore A23187, the bovine thrombin and the phospholipid suspensions in chloroform (phosphatidylcholine (PC) from egg yolk and phosphatidylserine (PS) from bovine brain) were from Sigma Chemical Company (St. Louis, MO, USA). Celite was a kind gift from Dr. Jerry Steiner. All chemicals were of reagent grade. The PAR-1 thrombin receptor agonist peptide TRAP-6 (amino acid sequence SFLLRN) (12) was purchased from the Biotechnology Centre of Oslo, Oslo University, Oslo, Norway and was dissolved in dimethyl sulfoxide (DMSO) to 30 mg/mL and thereafter diluted to 1.5 mg/mL in 0.5 mol/L HEPES buffer, pH 7.4. The methylated collagen type I (13), kindly provided by Dr. Beate Kehrel, Münster, Germany, was dissolved in 0.05% HAc to 3.3 or 66 μg/mL. The recombinant chicken annexin V (430 μg/mL in PBS buffer, lot no. X01B0100) was from november AG, Erlangen, Germany, by courtesy of Dr. Wolf Bertling. The peptide-derived GPIIb/IIIa antagonist MK-852 was generously provided by Merck, Sharp & Dohme (West Point, PA, USA). The antibody derived GPIIb/IIIa antagonist abciximab (Reopro®) was from Eli Lilly (Indianapolis, IN, USA). Phycoerythrin-conjugated mouse antibodies against GPIb (CD42b) and FITC-conjugated IgG_1 were purchased from Dako AS (Glostrup, Denmark).

Blood collection

Venous blood was collected with minimum trauma and stasis via a 21-gauge needle (0.8 × 40 mm) into plain 4.5 mL plastic vacuum tubes (S-Monovette®, Sarstedt, Nümbrecht, Germany). All blood donors gave their informed consent. The study protocol was approved by the Ethics Committee at Linköping University Hospital.

In experiments with platelet-free plasma, 500 μL of trisodium citrate (130 mmol/L) was added to the tubes before blood collection (yielding a final citrate concentration of 13 mmol/L). The blood was centrifuged 15 min at 2500 × g, and the plasma fraction was filtered through a 0.2 μm sterile filter before use. Flow cytometry confirmed that this plasma did not contain any particles exposing the platelet specific antigen GPIIb/IIIa.

Rheometer measurements

Native whole blood was collected in empty plastic vacuum tubes. Within 5 min of collection, 285 μL blood was added to cups containing 15 μL of TRAP-6 or collagen, or the same amount of corresponding solvent. Final concentration for TRAP was 33.5 μg/mL and for collagen 3.3 μg/mL. Duplicate samples from five different individuals were analysed.

In other experiments 250 μL of blood was transferred to sample cups containing 25 μL of collagen and 25 μL of HEPES buffer or a GPIIb/IIIa antagonist, MK-852 or abciximab. Final concentrations were for collagen 0.3 μg/mL, for MK-852 12 μg/mL and for abciximab 20 μg/mL. The lower concentration of collagen in these experiments was chosen to assure that the eventual effect of the inhibitors on the clotting time would be discovered, since we have experienced in other experiments

that a too strong stimulation could obscure the effects of platelet inhibition.

A dilution experiment with the calcium ionophore A23187 was also performed, where 295 μL of native blood was added to cups containing 1.5 μL of A23187 (10, 1, 0.75 or 0.5 mmol/L dissolved in DMSO) or 1.5 μL of DMSO. Duplicate samples from five different individuals were analysed. Since the calcium ionophore also binds to other blood cells, the concentrations of this substance had to be ten times higher in the clotting experiments where the blood was not diluted 1:10 as in the flow cytometry experiments.

For inhibition studies, native whole blood was added to rheometer cups containing annexin V (final concentration ranging from 0 to 40 μg/mL) alone or in combination with a platelet agonist (final concentrations: TRAP-6, 33.5 μg/mL; collagen, 3.3 μg/mL and A23187, 50 μmol/L). The dilution was kept below 10% for 0 to 20 μg/mL and below 15% for 30 and 40 μg/mL annexin V.

Flow cytometry

Immediately after the rheometer experiments had been started, 10 μL portions of blood from the vacuum tube were transferred to tubes containing GPRP peptide solution (final concentration 2 mmol/L) and mouse anti-GPIb-phycoerythrin antibody (final concentration 1.84 μg/mL). In experiments with GPIIb/IIIa antagonists, 8 μL of MK-852 (final concentration 12 μg/mL), abciximab (final concentration 20 μg/mL) or HEPES buffer was also added. Calcium-containing buffer ("Binding buffer", included in the Actiplate kit) was included to give a final volume of 100 μL after agonist addition. 0.5-8 μL of a platelet agonist (final concentrations: collagen, 0.3 or 3.3 μg/mL; thrombin, 2.5 U/mL; A23187, 5, 0.5, 0.375 or 0.25 μmol/L; TRAP-6, 33.5 or 75 μg/mL) or the same amount of corresponding solvent was then added to the tubes. After exactly 25 min at room temperature in the dark, 5 μL of annexin V-FITC (Actiplate, 25 μg/mL) was added, together with 365 μL of binding buffer. The samples were incubated for 15 min in the dark, according to the instructions in the Actiplate kit, and were then diluted 1:10 in a calcium-containing buffer (10 mmol/L HEPES buffer, pH 7.4, containing 140 mmol/L NaCl and 2.5 mmol/L $CaCl_2$) before flow cytometry analysis. In each sample 1000 cells were examined. A discrimination frame was set around the platelet cluster using forward light scatter and FL2 (GPIb-phycoerythrin). Analytical markers were set in the FL1 fluorescence channel (FITC) to divide gated particles in samples with buffer and an irrelevant fluorescent probe (FITC-conjugated IgG_1) into two fractions, one that contained 98.5-99.5% of the platelets and the other containing the brightest 0.5-1.5% of the platelets (14). Those platelets with fluorescence intensity higher than the marker were identified as annexin V-positive (PS-exposing) cells. The scattergram also contained a gate for the determination of the percentage of microparticles set with 1-2% of the GPIb-positive particles in a control sample below the set value for forward scatter. All samples were run in duplicate. The variability of this protocol was tested by repeated measurements on the same sample, yielding a CV of 12.7% for a sample with a mean of 4.3% positive platelets (n = 22) and a CV of 2.1% for a sample with 74.7% positive platelets (n = 21).

The flow cytometer was also used to measure the platelet concentration utilising the ImmunoCount II software together with the Ortho-Count Calibration Beads and Ortho-Count Verifier Beads (Ortho, Raritan, NY, USA). This procedure enables the determination of the cell concentration by counting the number of events in a specified time period, corresponding to a known volume of sample.

Preparation of PC/PS vesicles

Phospholipid vesicles containing phosphatidylcholine (PC) and phosphatidylserine (PS) in a 80:20 molar ratio were prepared by extrusion following the protocol proposed by JH Morrissey (15). The phospholipid suspensions (2.08 μmol PC, 0.52 μmol PS) were mixed in a glass tube, dried under nitrogen and centrifuged under vacuum for 60 min to remove all chloroform. To the tube was added 2.6 mL of a HEPES-containing buffer (100 mmol/L NaCl, 20 mmol/L HEPES, pH 7.5). After one hour at room temperature, the suspension was vortexed until a uniform milky suspension was obtained. The Lipo-so-fast device was cleaned with ethanol and dried. A membrane filter with 2 μm pore size was mounted and the device assembled. The suspension was collected in a glass syringe, passed through the filter 15 times and collected in the glass syringe at the rear end of the device. The final product (with a phospholipid concentration of 1 mmol/L) was stored at 4° C for no more than two weeks. Vesicles more than one week old were always tested in a plasma clotting assay with celite to assure unaltered activity before the performance of any other experiments.

Experiments with PC/PS vesicles

The PC/PS vesicles were added to blood or plasma samples and the clotting times determined. The vesicle suspension (yielding a phosholipid concentration of 0.003-100 μmol/L) or the HEPES buffer was added to platelet-free plasma. The sample was preincubated for 5 min at 37° C and then transferred to a ReoRox plastic sample cup containing 10 μL of calcium chloride (0.5 mol/L). The final volume was 300 μL.

To test the efficiency of the vesicle preparation, a modified APTT procedure was used. 30 μL of vesicle suspension or buffer was added to a plasma sample containing 33.3 μg/mL celite and incubated for 5 min before the transfer to a ReoRox plastic cup with 10 μL of calcium chloride (0.5 mol/L).

The vesicle suspension (final concentration 0.003-100 μmol/L) was also added to native whole blood samples. In some samples TRAP-6 was present in a final concentration of 50 μg/mL.

Statistics

Data analysis was performed using Statistica® for Windows (StatSoft Inc., Tulsa, OK, USA). Comparisons between groups were made with the non-parametric Wilcoxon Matched Pairs test.

Results

PS exposure vs. agonist incubation time

The change in PS exposure with different incubation times for the agonist was tested with 75 μg/mL of TRAP-6 or the same volume of HEPES buffer (Fig. 1). The number of annexin V-positive platelets did not seem to increase further after 15 min of incubation, but 25 min of incubation was chosen to ensure that coagulation would occur if the samples did not contain the GPRP peptide.

PS exposure with different incubation conditions

To check for differences in PS exposure due to the conditions during incubation with the agonists, blood samples from the same donor were incubated for 25 min in the dark, either standing at room temperature or in a water bath kept at 37° C, or shaken gently at room temperature. After 25 min, annexin V was added as described above, and all samples were incubated for 15 min standing still at room temperature before dilution and analysis. An absolute count of the number of platelets in the different tubes was also performed, to check if aggregation had occurred. Samples from three different individuals were analysed. The results are shown in Figures 2 and 3. Shaking of the samples reduced the number of single platelets for all agonists. When comparing the aggregation to the annexin V binding, however, only collagen showed an increase in annexin V binding in samples where aggregation had occurred. Collagen and A23187 reduced the number of single platelets with all incubation conditions, indicating a strong platelet aggregating effect. The collagen preparation used here was a very potent platelet activator without any stirring or shaking, at 0.3 μg/mL it induced fibrinogen binding to 77 ± 11% (mean ± SD, n = 4) of the platelets in citrate anticoagulated blood, and to 40 ± 24% of the platelets in hirudinized blood (n = 3), as measured by flow cytometry.

PS exposure vs. clotting time for different platelet agonists

Figure 4 shows PS exposure and clotting time for samples exposed for collagen or TRAP-6. No significant difference was found in clotting times or PS exposure for the different solvents alone, and they were therefore combined in the graph. Both agonists exhibited clotting times significantly shorter than for their solvent alone ($p < 0.05$). In these concentrations, TRAP-6 gave slightly shorter clotting times (8.7 ± 1.3 min, mean ± SD) than the collagen (10.0 ± 2.2 min), but the difference was not significant.

The samples with platelet agonists added showed significantly higher PS exposure than those to which only buffer was

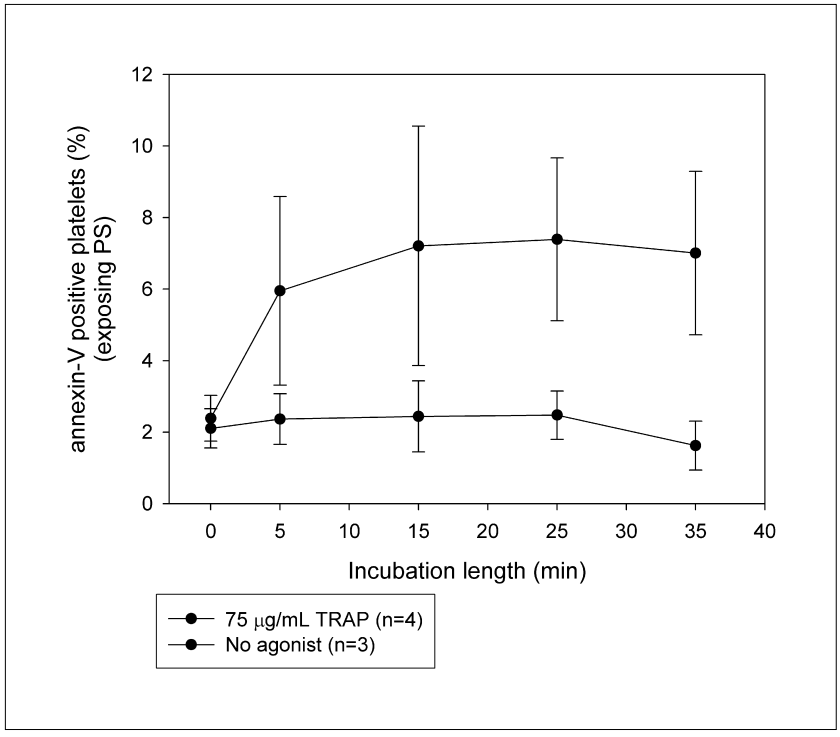

Figure 1: Changes in platelet phosphatidylserine (PS) exposure of native blood samples incubated with HEPES buffer or with 75 μg/mL TRAP-6 for up to 35 min. The points in the graph show mean values and the error bars show the standard deviation.

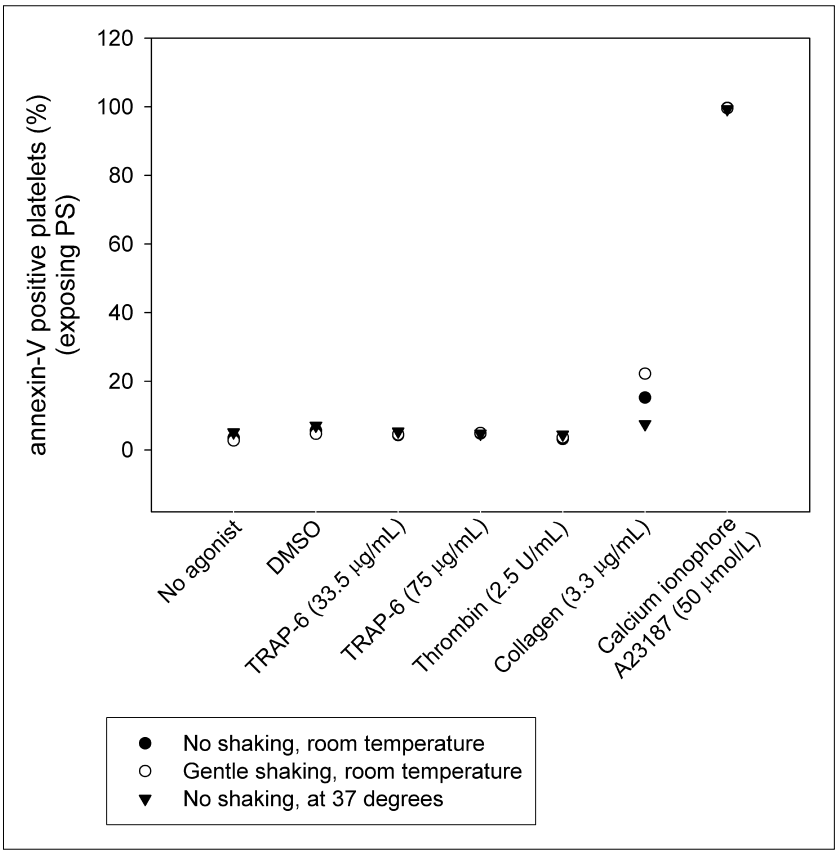

Figure 2: The influence of temperature and shaking on the exposure of PS by platelets from native blood incubated for 25 min with different platelet agonists. Fibrin polymerisation was inhibited by the presence of GPRP. Each point represents the mean value from measurements on platelets from three individuals.

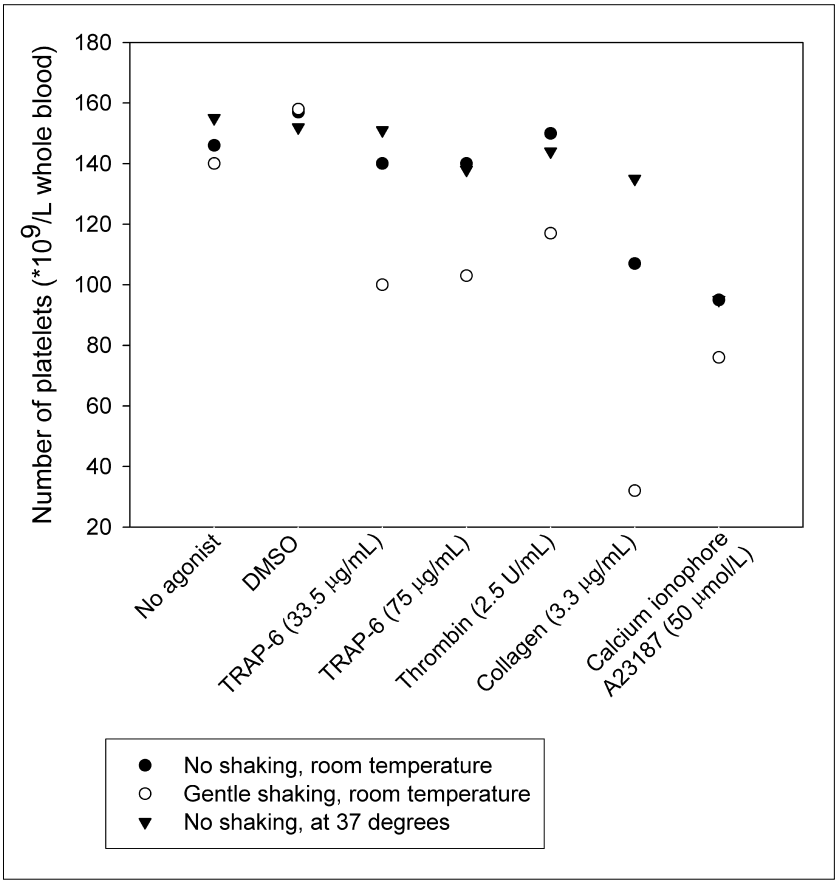

Figure 3: The influence of temperature and shaking during incubation on the number of platelets found in samples incubated for 25 min with different platelet agonists. Fibrin polymerisation was inhibited by the presence of GPRP. Each point represents the mean value from measurements on platelets from three individuals.

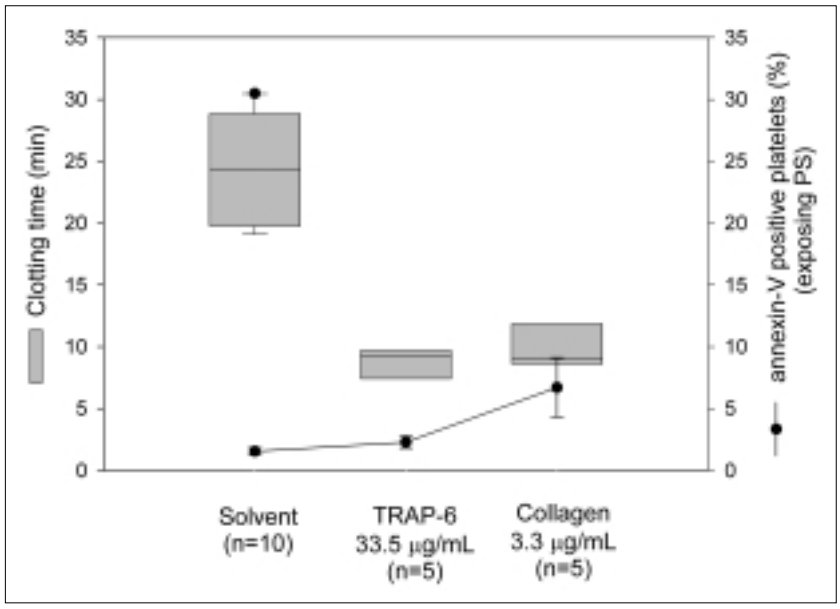

Figure 4: Comparison between clotting times and percentage platelets that exposed phosphatidylserine when TRAP-6 (33.5 μg/mL), collagen (3.3 μg/mL) or the corresponding solvents alone were added to native blood samples. The box plot shows the median, the 10th, 25th, 75th and 90th percentiles and all outliers. The error bars in the line and scatter plot show the standard deviation.

added, where 1.5 ± 0.2% of the cells were positive, $p < 0.05$. In collagen-stimulated samples, 6.7 ± 2.4% of the platelets were exposing PS. In samples with 33.5 μg/mL of TRAP-6, 2.3 ± 0.5% of the platelets exposed PS (mean ±SD, n = 5). The microparticle fraction was 2.3 ± 0.5% in the samples with TRAP-6, 2.8 ± 0.2% in samples with collagen and 22.3 ± 1.7% in samples with A23187. The microparticle fraction in samples with only buffer added was 2.0 ± 0.3% (n = 5).

To be able to perform clotting experiments, TRAP-6 had to be used to activate the platelet thrombin receptor, since the use of thrombin would have caused fibrin polymerisation regardless of platelet activation. Thrombin (2.5 U/mL) did, however, give similar amounts of PS-exposing platelets (4.9 ± 1.5%, (n = 6).

PS exposure vs. clotting time for different GPIIb/IIIa antagonists

We have earlier reported about differences in the action of the GPIIb/IIIa antagonists MK-852 and abciximab influencing the clotting time for collagen-stimulated samples (7). We therefore wanted to see how these inhibitors influenced the number of PS-exposing platelets. The percentage of PS-exposing platelets as compared to the clotting times earlier reported is shown in Figure 5. An inverse correlation was observed between the clotting time and PS exposure in these samples. Addition of

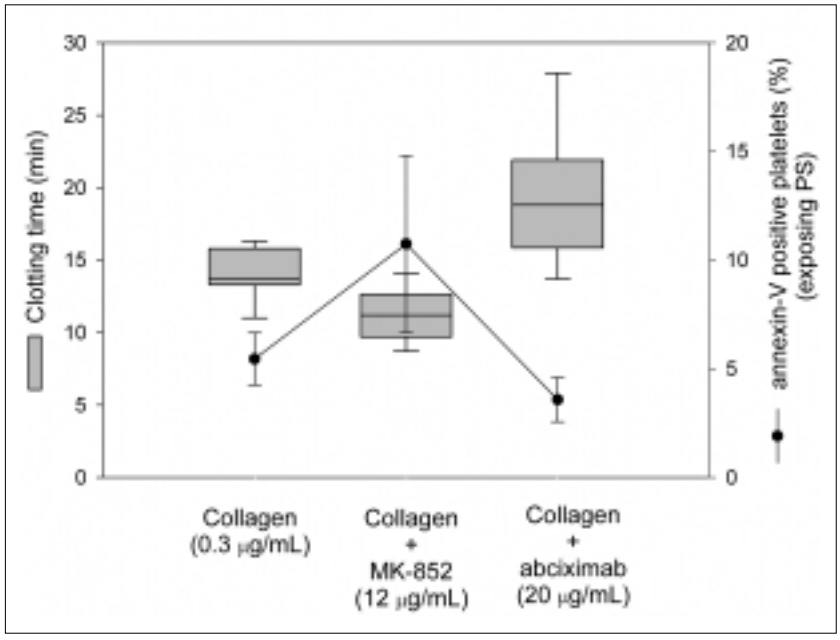

Figure 5: Comparison between clotting times and percentage platelets that exposed phosphatidylserine in native blood samples with collagen (0.3 μg/mL) alone and in combination with the GPIIb/IIIa inhibitors MK-852 (12 μg/mL) or abciximab (20 μg/mL).

abciximab to collagen-stimulated samples decreased the percentage of PS-exposing platelets ($p < 0.05$) to levels similar to the ones in control samples with only buffer added. However, when MK-852 was used instead of abciximab, the percentage of PS-exposing platelets was increased as compared to the samples with collagen alone ($p < 0.05$).

PS exposure vs. clotting time with different dilutions of calcium ionophore A23187

The clotting times were similar in samples with 5, 3.75 and 2.5 μmol/L A23187 (15.7 ± 1.8, 15.6 ± 1.9 and 16.2 ± 2.0 min, respectively) even though the amount of platelets exposing PS ranged from 5- to 60% (Fig. 6). The clotting times for these samples were only slightly decreased as compared to the solvent (DMSO, final concentration 0.5%) alone, where the clotting time was 20.4 ± 3.2 min and where 3.0 ± 1.1% of the platelets were exposing PS. Only the addition of 50 μmol/L A23187, with 99.9 ± 0.1% annexin V-positive platelets, gave clotting times (10.4 ± 0.9 min) similar to the ones in blood with TRAP-6 or collagen.

Experiments with PC/PS vesicles

The PC/PS vesicle preparation used in these experiments shortened the clotting time for plasma with 33.3 μg/mL of celite from 3.93 ± 0.81 to 1.84 ± 0.17 min (n = 6).

The clotting times induced by addition of PC/PS vesicles (0.003-100 μmol/L) to native whole blood (24.9 ± 3.0 min) were not significantly different from the times for samples with only buffer added (20.8 ± 3.7 min, n = 4). When 50 μg/mL of TRAP-6 was present, the clotting times decreased to 10.5 ± 2.0 min (n = 5), but the addition of PC/PS vesicles did not shorten these clotting times any further (9.7 ± 2.4 min). The addition of 0.003-100 μmol/L PC/PS could not induce clotting of platelet-free plasma within 2 h (n = 6), even though the calcium levels were between 1 and 2 mmol/L. This plasma was the same as used for the celite experiments, where it showed clotting times of 2 min with and 4 min without vesicles added.

Inhibition of clotting by addition of annexin V

The addition of annexin V to samples stimulated with TRAP-6 or collagen led to an almost linear increase in clotting times for doses up to 5 μg/mL. A less steep increase was then seen, resulting in clotting times over 20 min, which is in the lower range of the ones normally obtained without any agonist, with doses above 10 μg/mL (Fig. 7). The clotting time reduction with 50 μmol/l A23187 was also affected by annexin V addition, but here the effects were smaller, as could be expected. For samples without agonist, no clear effects were seen at doses below 10 μg/mL. Total anticoagulation was never achieved, but a clear prolongation was seen with higher doses. The collagen-stimulated samples were the ones most affected by annexin V addition.

Discussion

It is well known that platelets are important in several aspects of the coagulation process. Addition of TRAP-6 or collagen shortened the clotting time for native whole blood, indicating an important role for the platelet in the amplification of the coagulation process. But still only a fraction of the platelets exposed

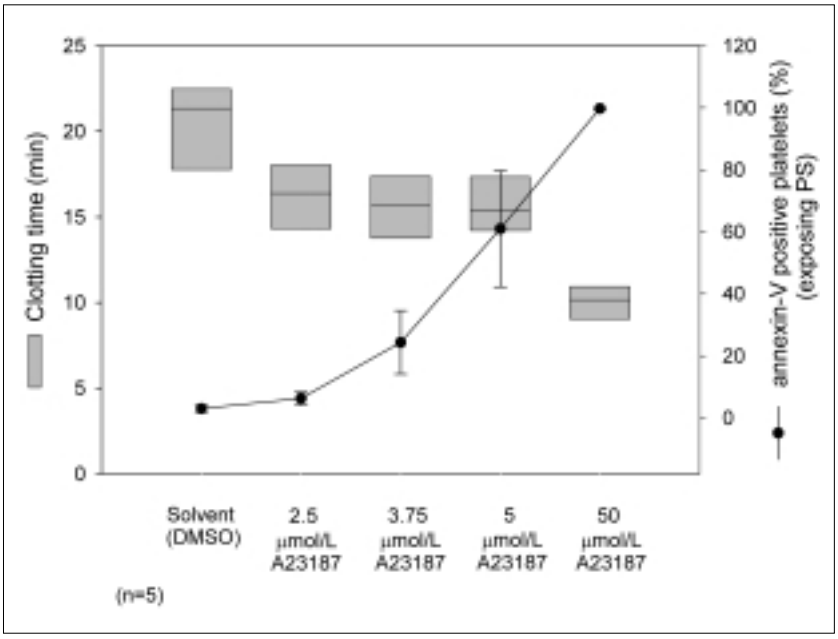

Figure 6: Comparison between clotting times and percentage platelets that exposed phosphatidylserine in native blood samples with different concentrations of the calcium ionophore A23187 (final whole blood concentration 50, 5, 3.75 or 2.5 μmol/L) or the corresponding solvent (DMSO) added.

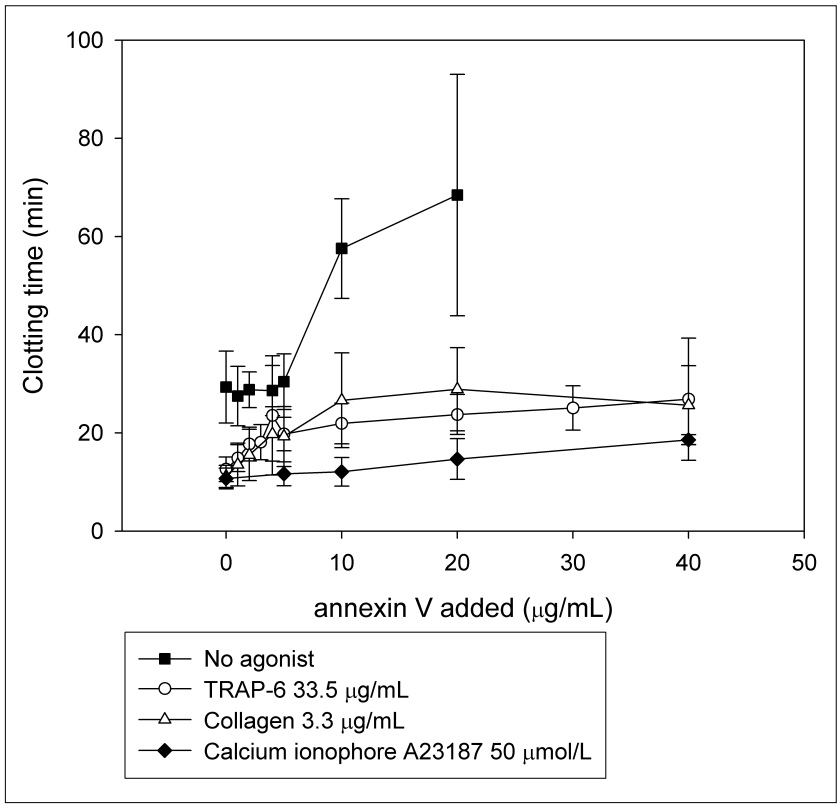

Figure 7: Clotting times for native whole blood samples activated with different platelet agonists in the presence of recombinant annexin V (0 to 40 μg/mL). Each point represents the mean and standard deviation of 3 to 6 measurements from different donors.

any measurable PS in samples with these agonists. It is, however, worth noting that the agonist concentrations examined had large effects on whole blood clotting times. This observation is in accordance with previously published flow cytometry studies, where only a small population of PS-exposing cells were found after stimulation with thrombin (16) or a combination of thrombin and collagen (17, 18). In contrast other groups have reported that 60-80% of the platelets became annexin V-positive when stimulated with 0.2-0.4 U/mL of thrombin at room temperature with gentle shaking (19). This was, however, done on gel-filtered platelets from citrate-anticoagulated blood, which perhaps made the platelets more easily activated. This could also be the case when annexin V binding was measured in PRP during aggregometry with TRAP as activator (20). It has been postulated that the combination of thrombin and fibrin fibers is needed to stimulate platelet PS exposure (21), but this can not be the case, since no fibrin fibers should be present in gel-filtered platelet suspensions or in PRP anticoagulated with hirudin. In our setting, with GPRP as anticoagulant, we may get thrombin formation and fibrin monomers, but no fibrin fibers, and we did not see any large PS exposure following stimulation with TRAP or thrombin. The influence of fibrin fibers is impossible to investigate with flow cytometry, as is the investigation of aggregated or adhered platelets, since larger aggregates will be excluded from the single cell population investigated in the analysis.

Other studies reporting about 35-40% positive platelets have used thrombin in combination with convulxin (22) (which activates the platelet collagen receptor GPVI), or collagen (6) for activation. Another hypothesis is that platelet adhesion or aggregation is preceding PS exposure (21, 23). Gentle shaking both increased the aggregation and percentage of platelets exposing PS with collagen, but for the other agonists aggregation increased but not PS exposure (Figs. 2 and 3).

The differences in incubation conditions, platelet preparation procedures and methods of defining the positive population make it very hard to compare the absolute numbers of PS-exposing cells obtained in the studies mentioned above. We believe results obtained with native or minimally altered blood are more relevant for the physiology of platelets than results obtained after centrifugation and/or gel filtration at extremely low calcium ion concentrations.

In our experiments with the glycoprotein IIb/IIIa antagonists MK-852 and abciximab, an inverse correlation was found between clotting time and percentage of platelets exposing PS. GPIIb/IIIa has been shown to bind prothrombin and to accelerate its conversion to thrombin, a mechanism that could explain how the blocking of this receptor could prolong the clotting time (24). Other GPIIb/IIIa antagonists have been shown to decrease both prothrombinase and tenase activity and PS exposure by stimulated platelets (16), but the efficiency was reported to vary between different antagonists (6). MK-852 has

earlier been reported to induce a slight increase in procoagulant activity in platelets stimulated with collagen and thrombin, but not when thrombin was used as single stimulus (25), a finding that is supportsed by our observation of shortened clotting times and increased PS exposure in samples with MK-852 and collagen.

For TRAP-6, collagen and A23187 (50 μmol/L) almost the same coagulation times were obtained, in spite of huge differences in PS expression. An explanation for this might be that the amount of PS-exposing platelets just had to exceed a certain threshold level to ensure proper coagulation amplification. However, the results with different concentrations of A23187 did not support this view, since the clotting times were much longer for samples with A23187 exposing the same amounts of PS as the samples stimulated with TRAP-6 or collagen. Additionally the clotting times did not change when the PS exposure was varied between 5 and 60% using different concentrations of A23187. It has been shown that even though A23187 induces high expression of PS, it is unable to promote significant expression of factor V on the platelet surface (22), an expression possibly important for the procoagulant properties.

The addition of a phospholipid vesicle preparation containing vesicles with 20% PS and 80% PC to native whole blood or to a platelet-free plasma did not affect the clotting times. The vesicles were also unable to shorten the clotting times of native blood samples with TRAP-6 added. This indicates that the exposure of PS on phospholipid surfaces is not enough in itself to affect the clotting of native whole blood, or to induce clotting in a recalcified plasma depleted of platelets. In a plasma sample without platelets or any added coagulation activators, coagulation has to proceed through the intrinsic pathway. The plasma preparation, recalcification process and experimental conditions by itself does not necessarily produce fXIIa, as shown by the fact that our plasma did not clot spontaneously if no coagulation activator was added. Then it is not strange why coagulation in this plasma is not accelerated by the phospholipid vesicles. In the whole blood experiments, we believe that the effects caused by the blood cells probably override the eventual effects by the phospholipid vesicles, even in samples with only buffer added. This confirms previous knowledge: it is reported that the addition of PC/PS vesicles alone did not affect the coagulation of whole blood, unless the vesicles were supplemented with tissue factor (26).

The experiments with addition of annexin V to native whole blood samples did, however, indicate that PS exposure is involved in the platelet response leading to the shortening of the clotting time observed in native blood. Addition of annexin V prolonged the clotting times for all agonists, but could not prevent coagulation. The largest inhibiting effect was seen with collagen, which might indicate that PS exposure is an important response to this agonist, as is also indicated by flow cytometry studies performed (27).

Platelets from different healthy donors have been shown to differ in their ability to support the formation of factor Xa and thrombin, a difference not correlated to their exposure of phosphatidylserine, indicating that the platelets exhibit additional features important for the assembly of these complexes (28). The platelets contain several proteins involved in the haemostasis, such as fibrinogen, von Willebrand factor and factor V (29), which can be released upon activation. A disputed finding is factor Xa activity on washed and gel-filtered platelets after activation (30). Another possible explanation is the suggested presence of receptors for factors Va and VIIIa on platelets, contributing to the procoagulant activity observed (31). Low level expression of factor V on the platelet surface has been reported to be induced by all platelet agonists without exposure of PS (22). The existence of a receptor for factor Xa, EPR-1, on activated platelets (32) together with the finding that factor IXa is differently accommodated on platelets and artificial vesicles containing PS (33), also confirms the theory of additional binding possibilities on platelet membranes. Another finding supporting additional mechanisms is the description of patients with a platelet defect with spontaneous PS exposure, the "Stormorken syndrome". These patients have a bleeding tendency tendency and no increase in thrombin formation (34-35).

In conclusion, platelet PS exposure seems to be necessary, but not sufficient for the coagulation amplification seen when platelets are stimulated via physiological receptors in a whole blood environment. The mechanisms behind this additional procoagulant activity will be the focus for upcoming studies.

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References

1. Bevers EM, Comfurius P, Zwaal RF. Regulatory mechanisms in maintenance and modulation of transmembrane lipid asymmetry: pathophysiological implications. Lupus 1996; 5: 480-7.
2. Stenflo J. Structure-function relationships of epidermal growth factor modules in vitamin K-dependent clotting factors. Blood 1991; 78: 1637-51.
3. Sunnerhagen M, Drakenberg T, Forsen S, Stenflo J. Effect of Ca2+ on the structure of vitamin K-dependent coagulation factors. Haemostasis 1996; 26: 45-53.
4. Blockmans D, Deckmyn H, Vermylen J. Platelet activation. Blood Rev 1995; 9: 143-56.
5. Carr ME, Jr., Carr SL, Hantgan RR, Braaten J. Glycoprotein IIb/IIIa blockade inhibits platelet-mediated force development and reduces gel elastic modulus. Thromb Haemost 1995; 73: 499-505.
6. Furman MI, Krueger LA, Frelinger AL, Barnard MR, Mascelli MA, Nakada MT, Michelson AD. GPIIb-IIIa antagonist-induced reduction in platelet surface factor V/Va binding and phosphatidylserine expression in whole blood. Thromb Haemost 2000; 84: 492-8.
7. Ramström S, Rånby M, Lindahl T. The role of platelets in blood coagulation –- effects of platelet agonists and of GPIIb/IIIa inhibitors studied by free oscillation rheometry. Thromb Res 2002; 105: 165-72.
8. Bohlin L. Method of measuring rheological properties and rheometer for carrying out the method. In: PCT (Patent Cooperation Treaty) publication number WO94/08222; 1994.
9. Bohlin L, Bearing device. In: PCT (Patent Cooperation Treaty) publication number WO98/54475; 1998.
10. Laudano AP, Cottrell BA, Doolittle RF. Synthetic peptides modeled on fibrin polymerization sites. Ann NY Acad Sci 1983; 408: 315-29.
11. Kawasaki K, Hirase K, Miyano M, Tsuji T, Iwamoto M. Amino acids and peptides. XVI. Synthesis of N-terminal tetrapeptide analogs of fibrin α-chain and their inhibitory effects on fibrinogen/thrombin clotting. Chem Pharm Bull 1992; 40: 3253-60.
12. Coughlin SR. How the protease thrombin talks to cells. Proc Natl Acad Sci USA 1999; 96: 11023-7.
13. Kehrel B, Wierwille S, Clemetson KJ, Anders O, Steiner M, Knight CG, Farndale RW, Okuma M, Barnes MJ. Glycoprotein VI is a major collagen receptor for platelet activation: it recognizes the platelet-activating quaternary structure of collagen, whereas CD36, glycoprotein IIb/IIIa, and von Willebrand factor do not. Blood 1998; 91: 491-9.
14. Lindahl TL, Festin R, Larsson A. Studies of fibrinogen binding to platelets by flow cytometry: an improved method for studies of platelet activation. Thromb Haemost 1992; 68: 221-5.
15. Morrissey J. Morrissey Lab Protocol for Preparing Phospholipid Vesicles by Extrusion. At: http://tf7.org/Extrude.html; Accessed 2001 09/06.
16. Pedicord DL, Thomas BE, Mousa SA, Dicker IB. Glycoprotein IIb/IIIa receptor antagonists inhibit the development of platelet procoagulant activity. Thromb Res 1998; 90: 247-58.
17. Pasquet JM, Dachary-Prigent J, Nurden AT. Microvesicle release is associated with extensive protein tyrosine dephosphorylation in platelets stimulated by A23187 or a mixture of thrombin and collagen. Biochem J 1998; 333: 591-9.
18. Dachary-Prigent J, Pasquet J-M, Nurden A. Simultaneous detection of changes in cytoplasmic Ca2+, aminophospholipid exposure and microvesiculation in activated platelets. Platelets 1997; 8: 405-12.
19. Dormann D, Clemetson KJ, Kehrel BE. The GPIb thrombin-binding site is essential for thrombin-induced platelet procoagulant activity. Blood 2000; 96: 2469-78.
20. Storey RF, Sanderson HM, White AE, May JA, Cameron KE, Heptinstall S. The central role of the P(2T) receptor in amplification of human platelet activation, aggregation, secretion and procoagulant activity. Br J Haematol 2000; 110: 925-34.
21. Beguin S, Kumar R, Keularts I, Seligsohn U, Coller BS, Hemker HC. Fibrin-dependent platelet procoagulant activity requires GPIb receptors and von Willebrand factor. Blood 1999; 93: 564-70.
22. Alberio L, Safa O, Clemetson KJ, Esmon CT, Dale GL. Surface expression and functional characterization of alpha-granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. Blood 2000; 95: 1694-702.
23. Siljander P, Farndale RW, Feijge MA, Comfurius P, Kos S, Bevers EM, Heemskerk JW. Platelet adhesion enhances the glycoprotein VI-dependent procoagulant response: Involvement of p38 MAP kinase and calpain. Arterioscler Thromb Vasc Biol 2001; 21: 618-27.
24. Byzova TV, Plow EF. Networking in the hemostatic system. Integrin alphaiibbeta3 binds prothrombin and influences its activation. J Biol Chem 1997; 272: 27183-8.
25. Lages B, Weiss HJ. Greater inhibition of platelet procoagulant activity by antibody-derived glycoprotein IIb-IIIa inhibitors than by peptide and peptidomimetic inhibitors. Br J Haematol 2001; 113: 65-71.
26. Rand M, Lock J, van't Veer C, Gaffney D, Mann K. Blood clotting in minimally altered whole blood. Blood 1996; 88: 3432-45.
27. Dachary-Prigent J, Freyssinet JM, Pasquet JM, Carron JC, Nurden AT. Annexin V as a probe of aminophospholipid exposure and platelet membrane vesiculation: a flow cytometry study showing a role for free sulfhydryl groups. Blood 1993; 81: 2554-65.
28. Sumner WT, Monroe DM, Hoffman M. Variability in platelet procoagulant activity in healthy volunteers. Thromb Res 1996; 81: 533-43.
29. Niewiarowski S. Secreted platelet proteins. In: Haemostasis and Thrombosis. Bloom A, Forbes C, Thomas D, Tuddenham E, eds: Churchill Livingstone 1994; 167-81.
30. Holme PA, Brosstad F, Solum NO. Platelet-derived microvesicles and activated platelets express factor Xa activity. Blood Coagul Fibrinolysis 1995; 6: 302-10.
31. Nesheim ME, Furmaniak-Kazmierczak E, Henin C, Cote G. On the existence of platelet receptors for factor V(a) and factor VIII(a). Thromb Haemost 1993; 70: 80-6.
32. Bouchard BA, Catcher CS, Thrash BR, Adida C, Tracy PB. Effector cell protease receptor-1, a platelet activation-dependent membrane protein, regulates prothrombinase-catalyzed thrombin generation. J Biol Chem 1997; 272: 9244-51.
33. London F, Ahmad SS, Walsh PN. Annexin V inhibition of factor IXa-catalyzed factor X activation on human platelets and on negatively-charged phospholipid vesicles. Biochemistry 1996; 35: 16886-97.
34. Solum NO. Procoagulant expression in platelets and defects leading to clinical disorders. Arterioscler Thromb Vasc Biol 1999; 19: 2841-6.
35. Stormorken H, Holmsen H, Sund R, Sakariassen KS, Hovig T, Jellum E, Solum O. Studies on the haemostatic defect in a complicated syndrome. An inverse Scott syndrome platelet membrane abnormality? Thromb Haemost 1995; 74: 1244-51.