bjh research paper

Identification of signal transduction pathways involved in the formation of platelet subpopulations upon activation

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Received 2 October 2011; accepted for publication 12 December 2011

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Summary

Platelets are formed elements of blood. Upon activation, they externalize phosphatidylserine, thus accelerating membrane-dependent reactions of blood coagulation. Activated platelets form two subpopulations, only one of which expresses phosphatidylserine. This study aimed to identify signalling pathways responsible for this segregation. Gel-filtered platelets, intact or loaded with calcium-sensitive dyes, were activated and labelled with annexin V and antibodies, followed by flow cytometric analysis. Calcium Green and Fura Red dyes were compared, and only the latter was able to detect calcium level differences in the platelet subpopulations. Phosphatidylserine-positive platelets produced by thrombin had stably high intracellular calcium level; addition of convulxin increased and stabilized calcium level in the phosphatidylserine-negative subpopulation. PAR1 agonist SFLLRN also induced calcium rise and subpopulation formation, but the resulting platelets were not coated with alpha-granule proteins. Adenylatecyclase activator forskolin inhibited phosphatidylserine-positive platelets formation several-fold, while its inhibitor SQ22536 had no effect. This suggests that adenylatecyclase inactivation is necessary, but not rate-limiting, for subpopulation segregation. Inhibition of mitogen-activated protein kinase kinase (U0126) and glycoprotein IIb-IIIa (Monafram®) was without effect, whereas inhibitors of phosphatidylinositol 3-kinase (wortmannin) and Src tyrosine kinase (PP2) decreased the procoagulant subpopulation threefold. These data identify the principal signalling pathways controlling platelet heterogeneity.

Keywords: platelet subpopulations, phosphatidylserine, thrombin, signal transduction, calcium signalling.

Blood platelets are small (2–4 μm) anucleate formed elements of blood that normally circulate at a concentration of 2–4×10^8/ml and become activated upon vessel wall injury. Activated platelets become able to form aggregates that prevent blood loss; they also acquire other properties, the most important of which is the ability to dramatically accelerate membrane-dependent reactions of blood coagulation (Panteleev *et al*, 2004; London *et al*, 2004) by means of phosphatidylserine (PS) externalization via some necrosis-like or, probably, apoptosis-like mechanisms (Jackson & Schoenwaelder, 2010; Rand *et al*, 2010; Suzuki *et al*, 2010).

Very intriguingly, only a subpopulation of platelets expresses PS upon activation (Dachary-Prigent *et al*, 1993; for recent reviews, see Munnix *et al*, 2009; Jackson & Schoenwaelder, 2010). In particular, the PS-positive subpopulation observed upon platelet stimulation with thrombin plus collagen (or some other agonist of the collagen receptor glycoprotein VI [GPVI]) is distinguished by retention of secreted alpha-granule proteins on the platelet membrane and was therefore called 'coated platelets' (Dale, 2005). Although dramatic differences in the properties of platelet subpopulations (Dale *et al*, 2002; Dachary-Prigent *et al*, 1993; London *et al*, 2004; Panteleev *et al*, 2005), as well as correlation observed between coated platelets formation in patients and the clinical manifestations (Prodan *et al*, 2010b,a, 2011), suggest the importance of platelet heterogeneity for thrombosis

First published online 16 January 2012
doi:10.1111/j.1365-2141.2011.09021.x

and haemostasis, the specific roles played by different subpopulations and mechanisms regulating their formation remain poorly understood.

Specifically, identification of signal transduction pathways that determine platelet segregation into subpopulations is of particular interest. Several studies have investigated the roles of thrombin (Keuren *et al*, 2005; Ramstrom *et al*, 2010) and adenosine 5′diphosphate (Kotova *et al*, 2008) receptors subtypes in this phenomenon. There have also been studies on calcium signalling in subpopulations formed after stimulation with the PAR1 agonist SFLLRN (London *et al*, 2006) or thrombin with collagen (Keuren *et al*, 2005). Involvement of activated factor XIII (FXIIIa) (Dale *et al*, 2002) and platelet mitochondrial permeability transition pore formation in the production of subpopulations were reported (Remenyi *et al*, 2005; Jobe *et al*, 2008). In addition to this, some indirect information about the regulation of subpopulations can be inferred from numerous studies on PS expression and functional procoagulant activity of the 'pre-subpopulation era', but this needs further testing and re-evaluation because of differences in activation conditions and detection methods.

Therefore, we attempted here to systematically investigate signal transduction mechanisms that could lead to platelet subpopulations' formation. The obtained data, together with prior observations, enabled us to map the platelet-heterogeneity-related pathways onto the currently accepted platelet signalling scheme.

Methods

Materials

The following materials were obtained from the sources shown: thrombin (Hematologic Technologies, Essex Junction, VT, USA); collagen receptor GPVI agonist convulxin (Pentapharm, Basel, Switzerland); prostaglandin E1 (MP Biochemicals, Irvine, CA, USA); phycoerythrin(PE)-conjugated annexin V, calcium-sensitive cell-permeable fluorescent dyes Calcium Green 1 and Fura Red (Molecular Probes, Eugene, OR, USA); fluorescein isothiocyanate (FITC)-conjugated annexin V (Biovision, CA, USA); U0126, SCH-23390, forskolin, AYPGKF, SCH79797, tcY-NH2 (Tocris Bioscience, Ellisville, MO, USA); GRGESP and GRGDSP (Bachem, Bubendorf, Switzerland); PP2, wortmannin, PPACK (EMD Chemicals, Gibbstown, NJ, USA); S2238 (Chromogenix, Milano, Italy); SFLLRN (either AnaSpec, San Jose, CA, USA, or Sigma-Aldrich, St Louis, MO, USA); HEPES, bovine serum albumin, Sepharose CL-2B, calcium ionophore A23187, SQ22536, apyrase (Sigma-Aldrich). The glycoprotein IIb-IIIa (GPIIb-IIIa) antagonist Monafram®, a F(ab')2 fragment of monoclonal antibody that blocks GPIIb-IIIa receptor activity (Byzova *et al*, 1994; Mazurov *et al*, 2001, 2002, 2004), was a generous gift from Professor A.V. Mazurov (Russian Cardiology Research and Production Centre, Moscow, Russia).

Thrombin active site titration

In order to determine an accurate molar concentration of thrombin, active site titration was performed using PPACK and S2238 essentially as described (Ramjee, 2000). Briefly, 10 μl aliquots of thrombin were mixed with an equal volume of PPACK at increasing concentrations, and the samples were incubated at room temperature for 20 min. The residual activity was estimated from the rate of S2238 hydrolysis measured by absorption at 405 nmol/l with the Thermomax microplate reader (Molecular Devices, Sunnyvale, CA, USA), thermoregulated at 37°C. Thrombin concentration was calculated from the intercept of a linear regression analysis of the residual activity *versus* PPACK concentration plot with the abscissa.

Platelet isolation

Platelets were isolated from freshly drawn human blood of healthy volunteers or patients in accordance with the Declaration of Helsinki essentially as described (Kotova *et al*, 2008; Panteleev *et al*, 2005). Blood was collected following written informed consent, under a protocol approved by the Centre for Theoretical Problems of Physicochemical Pharmacology and National Research Centre for Haematology Ethical Committees, into 3·8% sodium citrate (pH 5·5) at 9:1 blood/anticoagulant volume ratio and supplemented with prostaglandin E1 (1 μmol/l) and apyrase (0·1 unit/ml) to prevent platelet activation. To obtain platelet-rich plasma, the blood was centrifuged at 100 ***g*** for 10 min at room temperature. The obtained platelet-rich plasma was supplemented with 3·8% sodium citrate (pH 5·5) at citrate/plasma ratio of 1:3 to decrease pH and prevent aggregation. Platelets were sedimented by brief centrifugation, resuspended in buffer A (150 mmol/l NaCl, 2·7 mmol/l KCl, 1 mmol/l $MgCl_2$, 0·4 mmol/l NaH_2PO_4, 20 mmol/l HEPES, 5 mmol/l glucose, 0·5% bovine serum albumin) and subjected to gel filtration on a chromatography column packed with Sepharose CL-2B and equilibrated with buffer A.

Patients

The Glanzmann thrombastenia patient was a female of Caucasian (Russian) origin, aged 32 years at the time of the study. She had type 1 disease (<5% of activated GPIIb-IIIa level by flow cytometry), suffered from occasional life-long bleeds, and did not routinely take medication.

Flow cytometry

The indicated concentrations of platelets were activated by incubation with indicated agonists in buffer A with 2·5 mmol/l $CaCl_2$ for 15 min in the presence of labelling antibodies and/or annexin V, diluted 20-fold, and immediately analysed using a FACSCalibur (BD Biosciences, San Jose, CA, USA) flow

cytometer. The acquired data were processed using WinMDI 2.8 software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA).

Calcium signalling

Prior to gel filtration, resuspended platelets were incubated with 10 μmol/l Fura Red/AM or 10 μmol/l Calcium Green-1 for 45 min at room temperature in the presence of apyrase (0·1 unit/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 vehicle did not affect the results. Samples were collected at timed intervals, diluted 20-fold, and immediately analysed by flow cytometry.

Statistics

All experiments were reproduced at least in triplicate with platelets from different donors except for studies with patients. Comparisons were carried out with the paired Student's *t*-test. Statistical significance was set as $P < 0{\cdot}05$. Values are reported as mean ± standard error of the mean (SEM) unless specified otherwise.

Results

Comparison of Calcium Green 1 and Fura Red as detectors of cytoplasmic calcium levels in platelet subpopulations

The first step was to validate the intracellular calcium detection system used in the study. Although Fura Red has been previously used for investigation of calcium signalling in platelet subpopulations (London *et al*, 2006), there were also reports that Calcium Green-1 has essential advantages over other fluorescent indicators for measurement of intracellular calcium levels in platelets (Lee *et al*, 1999). Therefore, platelets were loaded alternatively with Calcium Green-1 or Fura Red, and the fluorescence level was compared using flow cytometry for either intact platelets, calcium ionophore A23187-stimulated platelet, or platelets activated with thrombin plus convulxin (Fig 1).

Stimulation with thrombin plus convulxin led to the formation of two different subpopulations with regard to PS expression within several minutes (Fig 1A). We did not observe any significant difference between these subpopulations using Calcium Green-1 (Fig 1B), and their fluorescence was also similar to A23187-activated platelets, which were expected to have an intracellular calium level similar to that of

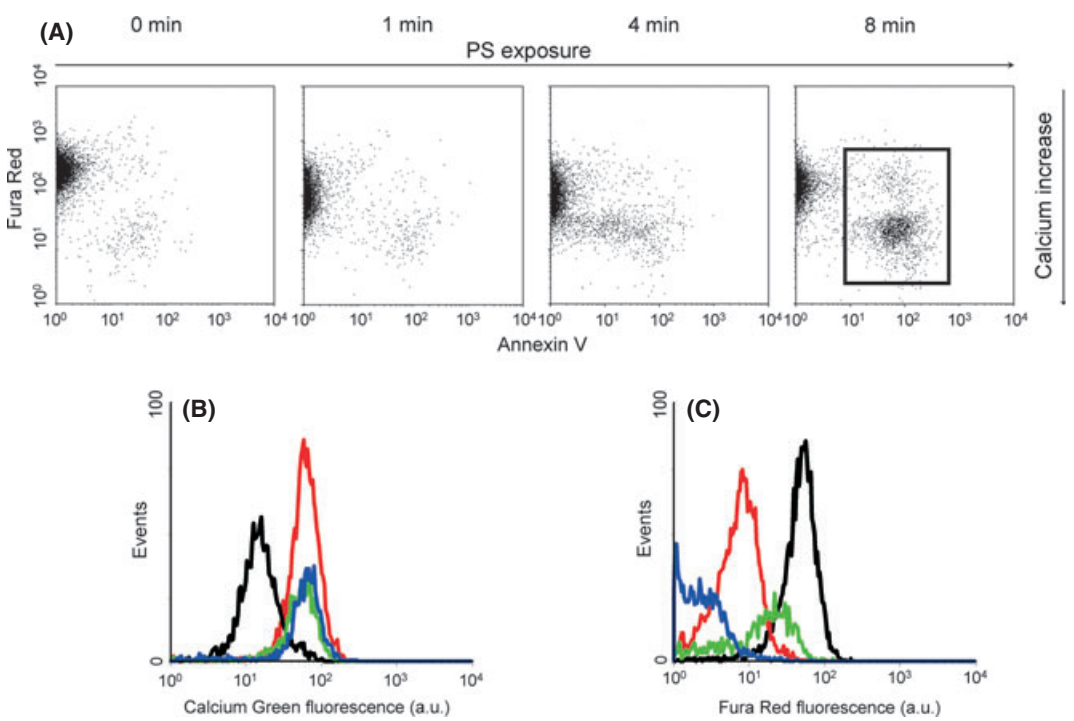

Fig 1. Calcium signalling in platelet subpopulations: comparison of Calcium Green 1 and Fura Red. (A) Design of a typical experiment. Dot plots show binding, at indicated timepoints, of annexin V *versus* Fura Red dye fluorescence for Fura Red-loaded platelets activated at 4×10^4/μl with thrombin (100 nmol/l). The square in the plot for $t = 8$ min indicates the PS-positive subpopulation. (B, C) Distribution of calcium-sensitive dye fluorescence in platelet subpopulations for Calcium Green 1 (B) and Fura Red (C). Platelets were either left unstimulated as a negative control, or stimulated for 10 min with 10 nmol/l of thrombin and 100 ng/ml convulxin, or with 10 μmol/l calcium ionophore A23187 as a positive control. The curves in the histogram are for resting platelets (black), A23187-activated platelets (red), and for the PS-negative (green) and PS-positive (blue) subpopulations of the thrombin-plus-convulxin-activated platelets.

the extracellular milieu, i.e. 2·5 mmol/l. In contrast, fluorescence of the Fura Red-loaded platelets was significantly different for the two subpopulations: the PS-positive platelets had fivefold smaller fluorescence, which indicated higher calcium levels for Fura Red (Fig 1C). Based on this, Fura Red was chosen for all subsequent studies.

Of note, Fura Red fluorescence in the PS-positive platelets was even somewhat smaller than in the A23187-activated platelets. Most likely, this did not indicate a higher calcium level, but was due to the smaller size (Panteleev *et al*, 2005) and specific shape (Kulkarni & Jackson, 2004) of the PS-positive platelets. It seems reasonable to assume that the fluorescence was actually in saturation, and Fura Red could not be used to reliably distinguish these concentrations.

Intracellular calcium kinetics and platelet segregation into subpopulations upon stimulation

As a next step, we investigated calcium concentration changes in platelets activated by a range of potent agonists: thrombin, convulxin, the PAR1 agonist peptide SFLLRN, and their combinations (Fig 2). PS-positive activated platelets had higher intracellular calcium levels than PS-negative ones, independent of activation type. The fluorescent signal in this subpopulation remained stable from the time when this subpopulation could be clearly identified (2 min after beginning the stimulation); however, this does not necessarily mean that the calcium concentration was sustained because the concentration values are likely to be outside the range that can be measured with Fura Red. On the contrary, calcium responses in the PS-negative platelet subpopulation formed upon thrombin- or SFLLRN-induced activation were reversible: after an initial increase, the calcium concentration returned to basal levels (Fig 2A,B). Addition of convulxin not only increased the number of the procoagulant PS-positive platelets by up to 10-fold, but also increased and stabilized the calcium concentration in the PS-negative platelet subpopulation (Fig 2C,D).

Role of protease-activated receptors in the formation of platelet subpopulations and their coating with alpha-granule proteins

An important characteristic of the subpopulations formed in the presence of thrombin is alpha-granule protein coating (Dale *et al*, 2002; Kotova *et al*, 2008). In order to detemine if this requires thrombin enzymatic activity, we stimulated platelets with thrombin, SFLLRN, PAR4 agonist AYPGKF, or SFLLRN plus AYPGKF, either in the absence or presence of convulxin, and double-labelled them with the PS marker, annexin V, and with the antibody against fibrinogen (Fig 3A). All types of activation induced the formation of a PS-positive subpopulation. However, high levels of fibrinogen were determined only in the PS-positive platelets produced in the presence of thrombin. Without thrombin, any combination of agonists resulted in the formation of PS-positive and PS-negative subpopulations that were indistinguishable by their level of surface-associated fibrinogen.

In order to better elucidate possible roles of the two PAR receptor subtypes in the subpopulation formation, we studied formation of PS-positive platelet formation as a function of thrombin concentration (Fig 3B) and then supplemented the activation mixture with either agonists or antagonists of PARs (Fig 3C). There was no complete saturation even at 1000 nmol/l thrombin, and the dependence appeared biphasic in the double-reciprocal plot (Fig 3B, inset), which agrees well with participation of both PAR1 and PAR4 in the phenomenon. For potent activation in the presence of thrombin, the addition of either agonists or antagonists of individual PARs had only a minor effect, if any, on the platelet divergence into subpopulations (Fig 3C) indicating that the receptors complement each other.

Roles of major platelet signal transduction pathways in the subpopulation formation

To evaluate possible contributions of other signal transduction pathways in the formation of subpopulations, pharmacological inhibition was used (Fig 4). Incubation with the adenylate cyclase activator forskolin decreased the number of PS-positive platelets formed upon thrombin stimulation by *c.* 80%; and its effect exceeded 90% for platelets stimulated with thrombin plus convulxin (Fig 4A). In contrast, adenylate cyclase inhibitor SQ22536 had no effect under any conditions (Fig 4A). This indicates that cAMP concentration decrease upon platelet activation is necessary, but is not rate-limiting for the platelet subpopulation formation. Furthermore, inhibitors of phosphatidylinositol 3-kinase (wortmannin) and of Src tyrosine kinase (PP2) both decreased the coated platelets subpopulation approximately threefold (Fig 4B). Finally, inhibition of either mitogen-activated protein kinase kinase (with U0126) or inwardly rectifying potassium channels (with SCH23390) had no effect on the formation of the PS-positive platelets (Fig 4C).

Role of GPIIb-IIIa in the formation of platelet subpopulations

There are conflicting reports on the role of GPIIb-IIIa in the PS expression in platelets (Hamilton *et al*, 2004; Jones *et al*, 2010; Razmara *et al*, 2007). Therefore, we tested its possible contribution to platelet subpopulation formation in three ways: by addition of RGD-containing peptides that are known to block binding of most GPIIb-IIIa ligands (Fig 5A); by addition of a macromolecular antagonist, the anti-GPIIb-IIIa antibody fragment Monafram® (Fig 5B); or by studying formation of subpopulations in platelets from a type 1 Glanzmann thrombasthenia patient (Fig 5C). None of the GPIIb-IIIa antagonists influenced formation of the PS-positive platelet subpopulation under conditions of our study. The results obtained with a Glanzmann thrombasthenia patient platelets were also within

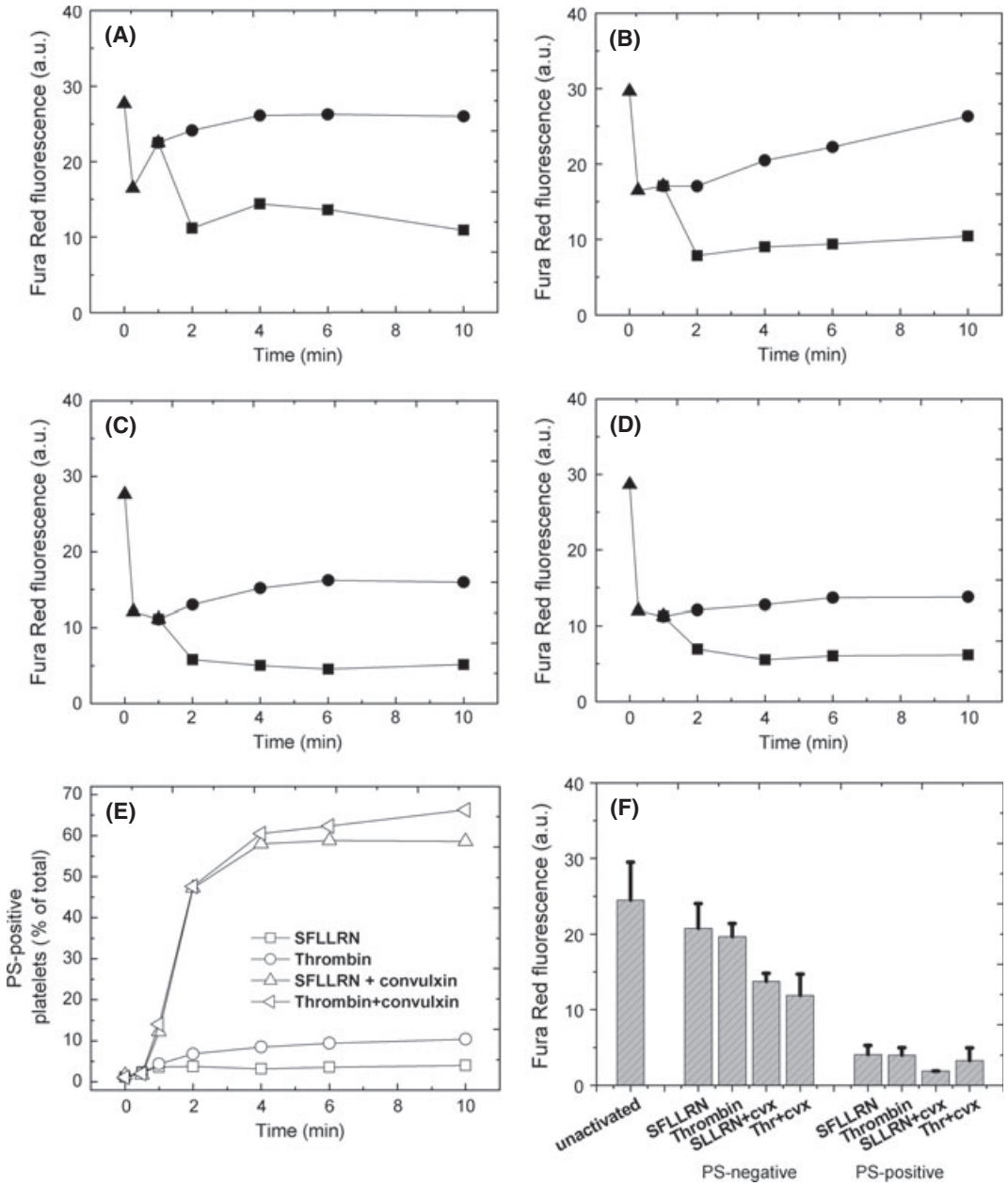

Fig 2. Calcium signalling in platelet subpopulations: kinetics. Calcium kinetics is shown for platelets activated with (A) 150 micromol/l SFLLRN, (B) 100 nmol/l thrombin, (C) 150 micromol/l SFLLRN plus 10 ng/ml convulxin, or (D) 10 nmol/l thrombin plus 10 ng/ml convulxin (cvx). Fura Red-loaded, gel-filtered platelets were stimulated at 2×10^4/μl with 2·5 mmol/l $CaCl_2$ and 0·5% (vol/vol) fluorescein isothiocyanate -labelled annexin V. Time samples were diluted 10-fold and immediately analysed by flow cytometry. Mean Fura Red fluorescence was measured in the total platelet population before segregation (▲), and then separately for phosphatidylserine (PS)-negative (●) and PS-positive (■)subpopulations. Data for a typical representative experiment are shown for each panel. (E) Kinetics of PS-positive platelets formation for the same four cases. (F) Mean Fura Red fluorescence in the subpopulations of platelets stimulated for 10 min with different agonists as described in (A–D). Means ± SD values are shown for n = 2–6 experiments with platelets from different donors.

the normal range (determined as mean ± 2standard deviations), although it is of interest that the outcome induced by thrombin (at 100 nmol/l) in these platelets was similar to that obtained with a lower concentration of thrombin (10 nmol/l) plus convulxin (10 ng/ml). Taken together, these data indicate that GPIIb-IIIa does not participate in the platelet segregation into the PS-positive and PS-negative subpopulations following stimulation with either thrombin or thrombin with convulxin under conditions of our study.

Discussion

The main findings of this study are schematically represented in Fig 6. It depicts major signal transduction pathways in

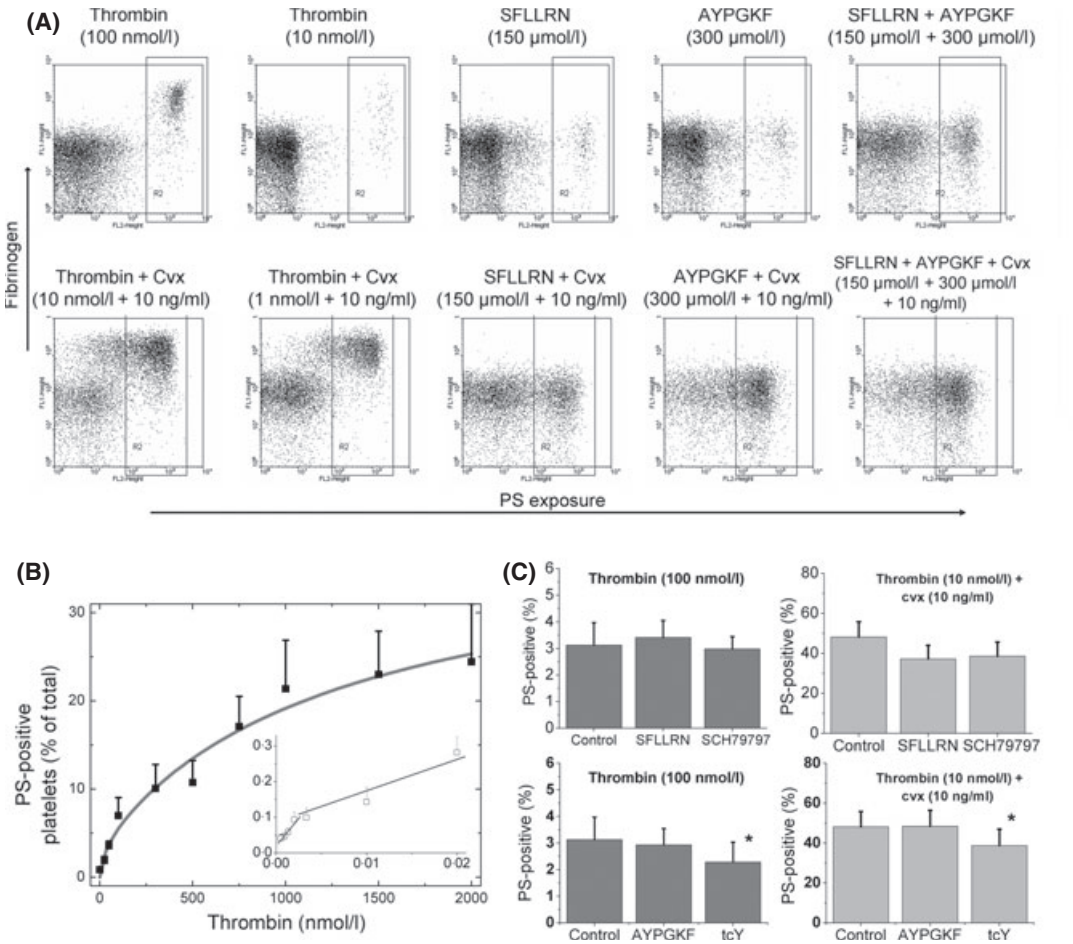

Fig 3. Role of protease-activated receptors in the formation of platelet subpopulations and in their coating with alpha-granule proteins. (A) Dot plots of phycoerythrin-labelled annexin V fluorescence *versus* fluorescein isothiocyanate-labelled anti-fibrinogen antibody fluorescence for platelets activated at 5×10^4/μl as indicated in the figure. (B) Fraction of phosphatidylserine (PS)-positive platelets activated at 1×10^5/μl as a function of thrombin concentration. Mean values ± SEM are shown. The inset shows a double reciprocal plot of the same data. (C) Effect of agonists and antagonists of the protease-activated receptors on the course of subpopulation formation in platelets stimulated at 2×10^4/μl. Concentrations used were: PAR1 agonist SFLLRN, 150 μmol/l; PAR4 agonist AYPGKF, 300 μmol/l; PAR1 antagonist SCH79797, 3 μmol/l; PAR4 antagonist tcY-NH2, 400 μmol/l. Mean values ± SEM for $n = 4$ experiments with platelets from four donors are shown. Asterisks indicate significant difference from controls, $P < 0{\cdot}05$. cvx, convulxin.

platelets, with those found to be important for the subpopulations' formation shown clearly and those judged to be non-participating in this process shaded with grey.

In agreement with the previous reports (Dale *et al*, 2002; Keuren *et al*, 2005; Ramstrom *et al*, 2010), there are three possible ways to initiate platelet activation so that two subpopulations with different PS expression will be ultimately produced: via either GPVI, PAR1, or PAR4. The sizes of the subpopulations can be additionally modulated by adenosine 5′disposphate acting via the P2Y12 receptor, while thromboxane A2 signalling and the P2Y1 receptor do not appear to contribute significantly (Kotova *et al*, 2008). Of note, stimulation of GPVI, PAR1, and PAR4 in any combinations was sufficient to induce PS expression in a platelet subpopulation, but did not lead to the coating of this subpopulation with alpha-granule proteins, which could be achieved only with thrombin (Figure 3A). Some other activity of thrombin, in addition to the ability to activate PAR1 and PAR4, is involved in this process: possible candidacies discussed previously include activation of transglutaminases (Dale, 2005) and cleavage of platelet-derived fibrinogen into fibrin (Munnix *et al*, 2009).

Comparative contribution of PAR1 and PAR4 to thrombin-induced procoagulant activity has been a subject of some controversy. Different reports suggested either that PAR1 is predominant and determines the effects of thrombin (Andersen *et al*, 1999; Keuren *et al*, 2005; London *et al*, 2004), or that PAR1 has a minor role, if any (Dicker *et al*, 2001), or that PAR1 and PAR4 are comparable (Ramstrom *et al*, 2010), or that even stimulation of both receptors cannot explain the effects of thrombin (Dorsam *et al*, 2004). These studies differ by the platelet isolation method (unless performed directly in whole blood), the type and degree of platelet activation (with or without stimulation of collagen receptors), the methods of

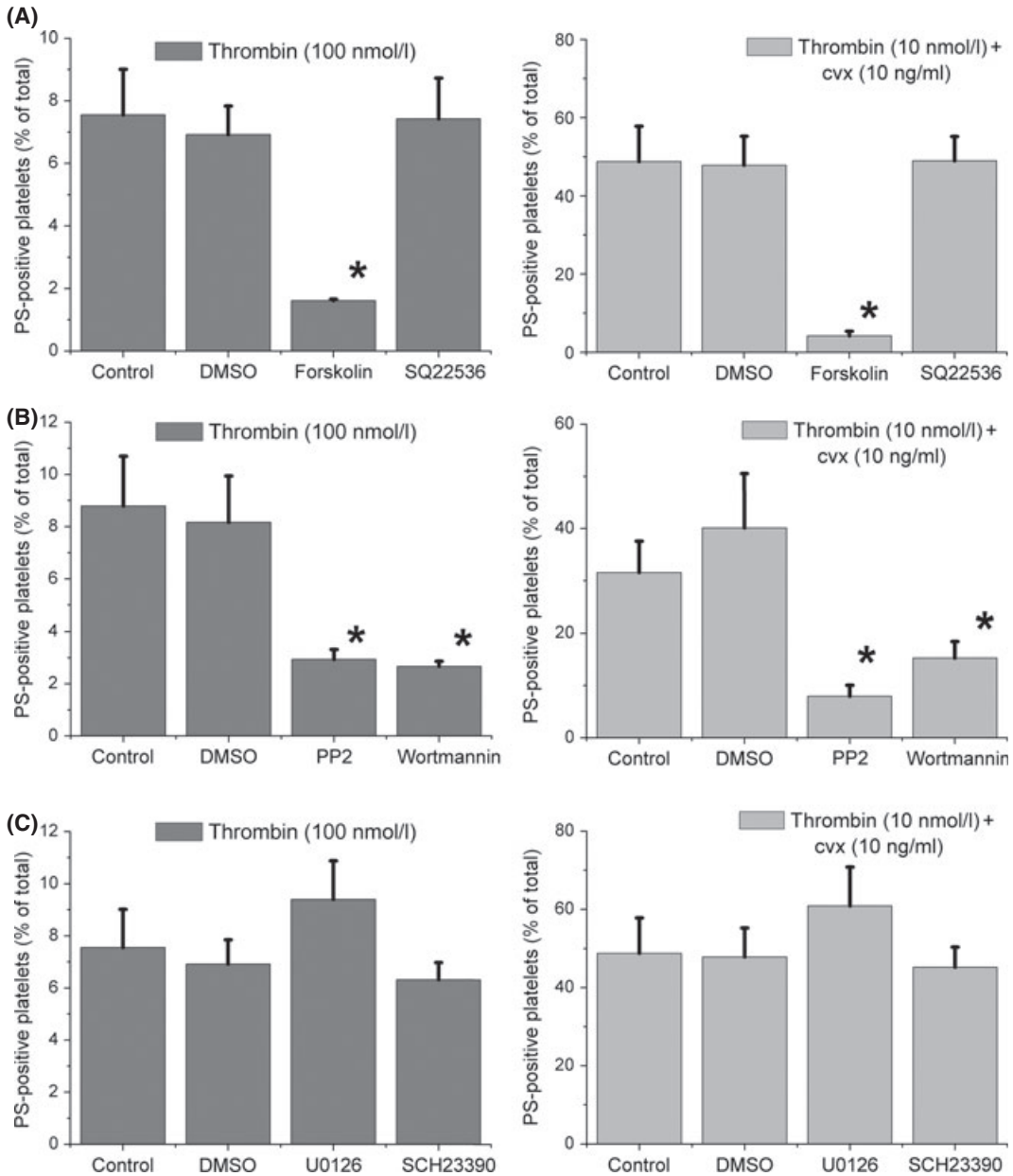

Fig 4. Roles of the major platelet signal transduction pathways in the formation of subpopulations. (A) Effect of platelet pre-incubation with either adenylate cyclase activator forskolin (20 μmol/l) or inhibitor SQ22536 (30 μmol/l) on platelet segregation into subpopulations. Mean values ± SEM are shown. (B) Effect of specific antagonists of PI3K (wortmannin, 500 μmol/l) and Src tyrosine kinase (PP2, 10 μmol/l) for 30 min. Mean values ± SEM are shown. (C) Effect of specific antagonists of MAP kinase kinase U0126 (20 μmol/l) and of G protein-gated potassium channels SCH23390 (200 μmol/l). Platelets were activated at 2×10^4/μl. Mean values ± SEM for $n = 4$ experiments with platelets from different donors are shown. In all experiments, platelets were pre-incubated with inhibitors for 30 min prior to addition of agonists. Asterisks indicate significant difference from controls, $P < 0{\cdot}05$. DMSO, dimethylsulphoxide; cvx, convulxin.

receptor stimulation/inhibition, and the methods of procoagulant activity characterization (PS detection or coagulation factor activation). Our results obtained for gel-filtered platelets stimulated with thrombin both in the absence or in the presence of convulxin are consistent with the latter point of view: i.e. that PAR1 and PAR4 are indeed comparable in that the responses caused by their saturating concentrations are similar. They also provide a possible explanation for the great difference between prothrombin activation velocity on platelets stimulated with thrombin and platelets stimulated with a combination of PAR agonist peptides reported earlier (Dorsam *et al*, 2004): it is likely that the difference is caused by the platelet coating with alpha-granule proteins (Figure 3A). The dependence of the PS-positive subpopulation formation on the concentration of thrombin is biphasic in a double-reciprocal plot (Figure 3B). This could indicate that both receptors are

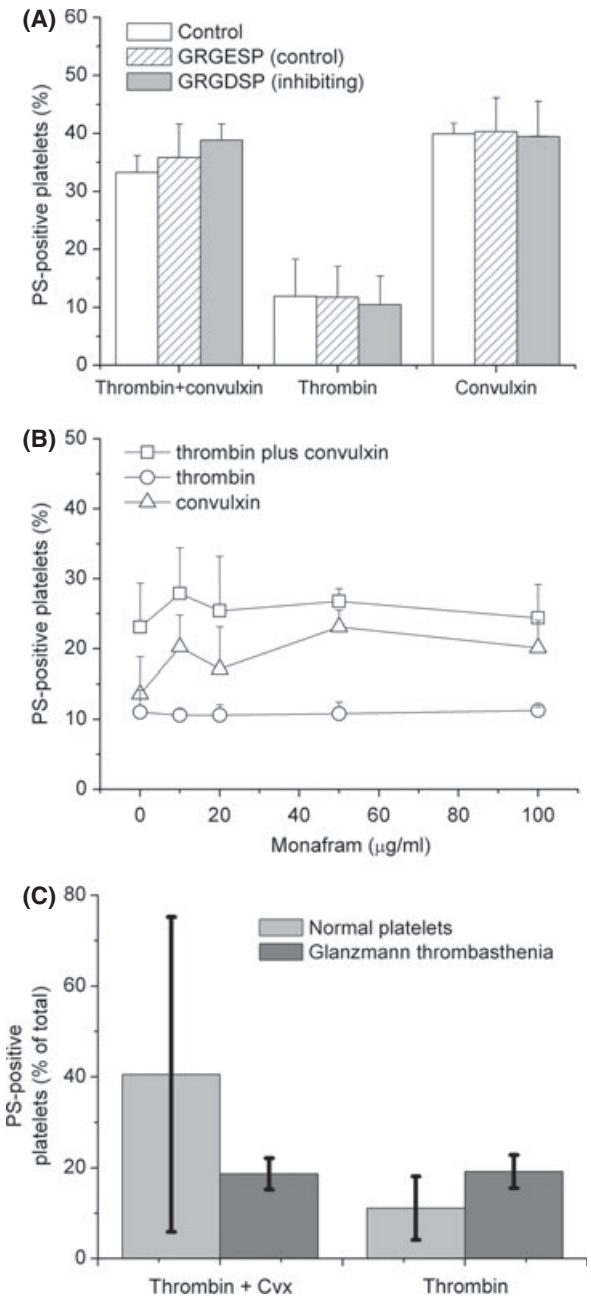

Fig 5. Role of GPIIb-IIIa in the formation of platelet subpopulations. (A) GPIIb-IIIa antagonist peptide GRGDSP at 0·5 mg/ml has no effect on PS-positive platelets compared with the control peptide GRGESP. Platelets were activated at 1×10^5/μl with either 10 nmol/l thrombin plus 10 ng/ml convulxin, 100 nmol/l thrombin, or 100 ng/ml convulxin. Mean values ± SEM are shown. (B) GPIIb-IIIa antagonist Monafram® has no effect on PS-positive platelets. Platelets are activated at 1×10^5/μl with either 10 nmol/l thrombin plus 10 ng/ml convulxin, 100 nmol/l thrombin, or 100 ng/ml convulxin. Mean values ± SEM are shown. (C) PS-positive platelets formation in a patient with severe Glanzmann's thrombasthenia proceeds similarly to that in normal donors. Platelets were activated at 5×10^4/μl with either 10 nmol/l thrombin plus 10 ng/ml convulxin (Cvx) or 100 nmol/l thrombin. The control data are means ± 2SD for $n = 4$ experiments with platelets from different donors; the sample data are means ± SEM for $n = 2$ experiments with platelets from a single patient.

involved in the process, with PAR1 working at low thrombin concentrations and PAR4 taking over as thrombin concentration increases, thus allowing to combine potent response in the lower concentration range with retention of dose-dependence at higher concentrations. These concentration ranges, which can be estimated from the inset in Figure 3B, are consistent with the dissociation constants for the thrombin receptors (Harmon & Jamieson, 1986) and half-maximal efficiency concentration reported (Nieman, 2008). Under the typical conditions of the present study, effects of both receptors are close to saturation, with PAR1 completely saturated (Figure 3C).

Our data on the regulation of platelet subpopulation by the kinase-dependent signalling pathways are in agreement with previous studies that identified the importance of adenylate cyclase (Yan *et al*, 2009) and phosphoinositide 3-kinases (Bucki *et al*, 2001; van der Meijden *et al*, 2008) in platelet PS expression. Lack of effect of the adenylatecyclase inhibitor SQ22536 on subpopulations suggests, however, that adenylate cyclase, cAMP, and cAMP-dependent protein kinase A are necessary, but not controlling elements (Panteleev *et al*, 2010) for this platelet response. The importance of the Src-kinase family for PS expression was previously reported in studies of platelet adhesion to collagen in flow chambers (Munnix *et al*, 2005). Our experimental model also shows their critical involvement in the formation of the procoagulant platelet subpopulation for all types of activation. Identification of the specific isoforms of phosphoinositide 3-kinases and Src kinases requires special investigation. Finally, the fact that mitogen-activated protein kinase kinase or inwardly rectifying potassium channels do not contribute to the subpopulation formation has not been to our knowledge reported previously, but lack of contribution of these relatively minor pathways does not seem very surprising.

The possible role of outside-in signalling via GPIIb-IIIa (integrin $\alpha_{IIb}\beta_3$) in platelet procoagulant activity development has been a subject of several reports over the last 10 years. Initially, it was implicated as a major component of platelet procoagulant response, which was diminished by GPIIb-IIIa antagonists (Dicker *et al*, 2001); this was supported by an independent recent report (Razmara *et al*, 2007). In contrast, another group has shown that some antagonists paradoxically stimulate formation of the procoagulant platelet subpopulation (Hamilton *et al*, 2004), which was recently confirmed and investigated in more detail by another group (Jones *et al*, 2010). While these studies generally used different methods to affect this pathway, three of them employed abciximab or c7E3 Fab that was reported to either inhibit (Dicker *et al*, 2001; Razmara *et al*, 2007) or not to affect (Hamilton *et al*, 2004) procoagulant activity. In the present study, we employed two GPIIb-IIIa antagonists and did not find any significant effects; subpopulation formation in a severe Glanzmann thrombosthenia patient did not differ significantly from the normal one either. The data indicate that outside-in signalling does not normally contribute to the PS externalization under our study

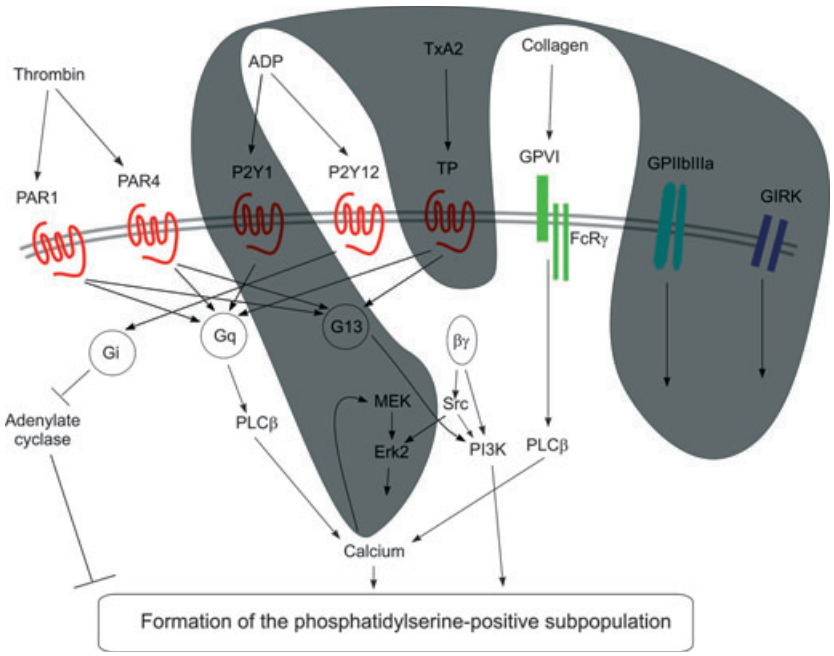

Fig 6. Signal transduction pathways that control formation of the PS-positive and PS-negative platelet subpopulations. The main signalling events in platelet activation are depicted. Those that are not important for the formation of the subpopulations based on this study and other reports are shaded in grey.

conditions (i.e. gel-filtered platelets, potent activation and no stirring conditions), although this in no ways interferes with the suggestion (Hamilton *et al*, 2004; Jones *et al*, 2010) that some specific ligands can stimulate this pathway and modulate procoagulant activity.

Calcium signalling is recognized to be tightly related to PS exposure in platelet subpopulations (London *et al*, 2006; Keuren *et al*, 2005). It is not clear why Calcium Green 1, previously suggested to be optimal for intracellular calcium measuring in platelets (Lee *et al*, 1999), did not distinguish between the subpopulations despite sharing similar calcium binding parameters with Fura Red. This could probably explained by different cell permeability that might lead to different intracellular concentrations. Our data suggest that Fura Red has some disadvantages as well: the signal from the PS-positive subpopulation appears to be in saturation and thus calcium dynamics in this subpopulation cannot be reconstructed reliably.

Except for this minor reservation, we could not distinguish between calcium kinetics in platelet subpopulations induced by thrombin or by the PAR1 agonist SFLLRN. In all cases, platelets of the PS-positive subpopulation were characterized by sustained intracellular calcium increase, in agreement with previous reports (London *et al*, 2006; Keuren *et al*, 2005). It is of interest that stimulation of GPVI with convulxin had a pronounced effect on the PS-negative subpopulation by means of increasing and stabilizing the intracellular calcium level, that otherwise returned to normal. This increase and stabilization were not sufficient to turn these platelets into procoagulant ones: the subpopulations differed between 1 and 2 min after activation, and their numbers remained generally stable after 5 min. It cannot be excluded that intracellular calcium in the PS-positive subpopulation was also affected by convulxin, but the above-described limitation of Fura Red prevented observation of this effect.

Although a preliminary picture of the role of platelet signal transduction networks that has influence on PS expression and platelet segregation into subpopulations can be proposed (Figure 6), several key elements are still missing. The foremost of these include: (i) pathways from calcium to PS expression that have been just recently illuminated by identification of a protein responsible for Scott syndrome (Suzuki *et al*, 2010); (ii) links between calcium and mitochondria-related events that are critical for the subpopulation formation (Jobe *et al*, 2008; Remenyi *et al*, 2005); (iii) position of the actual trigger(s) that determine(s) platelet fate upon activation by assigning it to one or other subpopulation. In addition, the contributions of various protein kinase isoforms, cAMP-dependent pathways, molecular mechanisms and kinetics of signalling are also far from clear. The data of the present study, which attempted to characterize these signal transduction mechanisms, emphasize the importance of understanding complicated signalling networks that are responsible for platelet activation and are critically important for haemostasis and thrombosis.

Author Contributions

NNT and YNK performed research and analysed the data, SAV recruited and characterized the patients, MAP planned research and wrote the manuscript with contributions from all authors.

Conflict of Interest

The authors have no conflicts of interest.

Acknowledgements

The authors are indebted to the Glanzmann thrombasthenia patient for her kind agreement to participate in the study. We thank Prof. A.V. Mazurov for his generous gift of Monafram®.

We are grateful to Natalya V. Zakharova for reading the manuscript and valuable comments. The study was supported by the Russian Foundation for Basic Research grants 10-01-91055, 11-04-00303, 11-04-12080, by the Russian Academy of Sciences Presidium Basic Research Program 'Molecular and Cellular Biology', and by the Russian Federation President Grant for Young Scientists MK-155·2010·4.

References

Andersen, H., Greenberg, D.L., Fujikawa, K., Xu, W., Chung, D.W. & Davie, E.W. (1999) Protease-activated receptor 1 is the primary mediator of thrombin-stimulated platelet procoagulant activity. *Proceedings of the National Academy of Sciences of the USA*, **96,** 11189–11193.

Bucki, R., Janmey, P.A., Vegners, R., Giraud, F. & Sulpice, J.C. (2001) Involvement of phosphatidylinositol 4,5-bisphosphate in phosphatidylserine exposure in platelets: use of a permeant phosphoinositide-binding peptide. *Biochemistry*, **40,** 15752–15761.

Byzova, T.V., Vlasik, T.N. & Mazurov, A.V. (1994) Inhibition of platelet aggregation with monoclonal antibodies to the glycoprotein IIb-IIIa complex (in Russian). *Biulleten' eksperimental'noĭ biologii i meditsiny*, **118,** 402–405.

Dachary-Prigent, J., Freyssinet, J.M., Pasquet, J.M., Carron, J.C. & Nurden, A.T. (1993) Annexin V as a probe of aminophospholipid exposure and platelet membrane vesiculation: a flow cytometry study showing a role for free sulfhydryl groups. *Blood*, **81,** 2554–2565.

Dale, G.L. (2005) Coated-platelets: an emerging component of the procoagulant response. *Journal of Thrombosis and Haemostasis*, **3,** 2185–2192.

Dale, G.L., Friese, P., Batar, P., Hamilton, S.F., Reed, G.L., Jackson, K.W., Clemetson, K.J. & Alberio, L. (2002) Stimulated platelets use serotonin to enhance their retention of procoagulant proteins on the cell surface. *Nature*, **415,** 175–179.

Dicker, I.B., Pedicord, D.L., Seiffert, D.A., Jamieson, G.A. & Greco, N.J. (2001) Both the high affinity thrombin receptor (GPIb-IX-V) and GPIIb/IIIa are implicated in expression of thrombin-induced platelet procoagulant activity. *Thrombosis and Haemostasis*, **86,** 1065–1069.

Dorsam, R.T., Tuluc, M. & Kunapuli, S.P. (2004) Role of protease-activated and ADP receptor subtypes in thrombin generation on human platelets. *Journal of Thrombosis and Haemostasis*, **2,** 804–812.

Hamilton, S.F., Miller, M.W., Thompson, C.A. & Dale, G.L. (2004) Glycoprotein IIb/IIIa inhibitors increase COAT-platelet production in vitro. *The Journal of Laboratory and Clinical Medicine*, **143,** 320–326.

Harmon, J.T. & Jamieson, G.A. (1986) Platelet activation by alpha-thrombin is a receptor-mediated event. *Annals of the New York Academy of Sciences*, **485,** 387–395.

Jackson, S.P. & Schoenwaelder, S.M. (2010) Procoagulant platelets: are they necrotic? *Blood*, **116,** 2011–2018.

Jobe, S.M., Wilson, K.M., Leo, L., Raimondi, A., Molkentin, J.D., Lentz, S.R. & Di, P.J. (2008) Critical role for the mitochondrial permeability transition pore and cyclophilin D in platelet activation and thrombosis. *Blood*, **111,** 1257–1265.

Jones, M.L., Harper, M.T., Aitken, E.W., Williams, C.M. & Poole, A.W. (2010) RGD-ligand mimetic antagonists of integrin alphaIIbbeta3 paradoxically enhance GPVI-induced human platelet activation. *Journal of Thrombosis and Haemostasis*, **8,** 567–576.

Keuren, J.F., Wielders, S.J., Ulrichts, H., Hackeng, T., Heemskerk, J.W., Deckmyn, H., Bevers, E.M. & Lindhout, T. (2005) Synergistic effect of thrombin on collagen-induced platelet procoagulant activity is mediated through protease-activated receptor-1. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **25,** 1499–1505.

Kotova, Y.N., Ataullakhanov, F.I. & Panteleev, M.A. (2008) Formation of coated platelets is regulated by the dense granule secretion of adenosine 5′diphosphate acting via the P2Y12 receptor. *Journal of Thrombosis and Haemostasis*, **6,** 1603–1605.

Kulkarni, S. & Jackson, S.P. (2004) Platelet factor XIII and calpain negatively regulate integrin alphaIIbbeta3 adhesive function and thrombus growth. *The Journal of Biological Chemistry*, **279,** 30697–30706.

Lee, S.K., Lee, J.Y., Lee, M.Y., Chung, S.M. & Chung, J.H. (1999) Advantages of calcium green-1 over other fluorescent dyes in measuring cytosolic calcium in platelets. *Analytical Biochemistry*, **273,** 186–191.

London, F.S., Marcinkiewicz, M. & Walsh, P.N. (2004) A subpopulation of platelets responds to thrombin- or SFLLRN-stimulation with binding sites for factor IXa. *The Journal of Biological Chemistry*, **279,** 19854–19859.

London, F.S., Marcinkiewicz, M. & Walsh, P.N. (2006) PAR-1-stimulated factor IXa binding to a small platelet subpopulation requires a pronounced and sustained increase of cytoplasmic calcium. *Biochemistry*, **45,** 7289–7298.

Mazurov, A.V., Pevzner, D.V., Khaspekova, S.G., Dudnik, O.A., Byzova, T.V., Vlasik, T.N., Bakharev, V.N., Pistsov, M.I., Staroverov, I.I. & Ruda, M.I. (2001) Inhibition of thrombocyte aggregation by F(ab')2-fragments of monoclonal antibodies FraMon (CRC64) to glycoproteins IIb-IIIa(In Russian). *Terapevticheskiĭ Arkhiv*.,**73,** 66–73.

Mazurov, A.V., Pevzner, D.V., Antonova, O.A., Byzova, T.V., Khaspekova, S.G., Semenov, A.V., Vlasik, T.N., Samko, A.N., Staroverov, I.I. & Ruda, M.Y. (2002) Safety, inhibition of platelet aggregation and pharmacokinetics of Fab'2 fragments of the anti-glycoprotein IIb-IIIa monoclonal antibody FRaMon in high-risk coronary angioplasty. *Platelets*, **13,** 465–477.

Mazurov, A.V., Pevzner, D.V., Vlasik, T.N. & Ruda, M.I. (2004) Antiplatelet effects of glycoproteins IIb-IIIa antagonist monafram (in Russian). *Rossiiskii Fiziologicheskii Zhurnal imeni I.M. Sechenova (Russian Journal of Physiology)*, **90,** 586–599.

van der Meijden, P.E., Schoenwaelder, S.M., Feijge, M.A., Cosemans, J.M., Munnix, I.C., Wetzker, R., Heller, R., Jackson, S.P. & Heemskerk, J.W. (2008) Dual P2Y 12 receptor signaling in thrombin-stimulated platelets – involvement of phosphoinositide 3-kinase beta but not gamma isoform in Ca2+ mobilization and procoagulant activity. *The FEBS Journal*, **275,** 371–385.

Munnix, I.C., Strehl, A., Kuijpers, M.J., Auger, J.M., van der Meijden, P.E., van Zandvoort, M.A., oude Egbrink, M.G., Nieswandt, B. & Heemskerk, J.W. (2005) The glycoprotein VI-phospholipase Cgamma2 signaling pathway controls thrombus formation induced by collagen and tissue factor in vitro and in vivo. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **25,** 2673–2678.

Munnix, I.C., Cosemans, J.M., Auger, J.M. & Heemskerk, J.W. (2009) Platelet response heterogeneity in thrombus formation. *Thrombosis and Haemostasis*, **102,** 1149–1156.

Nieman, M.T. (2008) Protease-activated receptor 4 uses anionic residues to interact with alpha-thrombin in the absence or presence of protease-activated receptor 1. *Biochemistry*, **47,** 13279–13286.

Panteleev, M.A., Saenko, E.L., Ananyeva, N.M. & Ataullakhanov, F.I. (2004) Kinetics of Factor X activation by the membrane-bound complex of Factor IXa and Factor VIIIa. *The Biochemical Journal*, **381,** 779–794.

Panteleev, M.A., Ananyeva, N.M., Greco, N.J., Ataullakhanov, F.I. & Saenko, E.L. (2005) Two subpopulations of thrombin-activated platelets differ in their binding of the components of the

intrinsic factor X-activating complex. *Journal of Thrombosis and Haemostasis*, **3,** 2545–2553.

Panteleev, M.A., Balandina, A.N., Lipets, E.N., Ovanesov, M.V. & Ataullakhanov, F.I. (2010) Task-oriented modular decomposition of biological networks: trigger mechanism in blood coagulation. *Biophysical Journal*, **98,** 1751–1761.

Prodan, C.I., Stoner, J.A., Cowan, L.D. & Dale, G.L. (2010a) Lower coated-platelet levels are associated with early hemorrhagic transformation in patients with non-lacunar brain infarction. *Journal of Thrombosis and Haemostasis*, **8,** 1185–1190.

Prodan, C.I., Vincent, A.S. & Dale, G.L. (2010b) Coated platelet levels correlate with bleed volume in patients with spontaneous intracerebral hemorrhage. *Stroke*, **41,** 1301–1303.

Prodan, C.I., Ross, E.D., Stoner, J.A., Cowan, L.D., Vincent, A.S. & Dale, G.L. (2011) Coated-platelet levels and progression from mild cognitive impairment to Alzheimer disease. *Neurology*, **76,** 247–252.

Ramjee, M.K. (2000) The use of fluorogenic substrates to monitor thrombin generation for the analysis of plasma and whole blood coagulation. *Analytical Biochemistry*, **277,** 11–18.

Ramstrom, S., O'Neill, S., Dunne, E. & Kenny, D. (2010) Annexin V binding to platelets is agonist, time and temperature dependent. *Platelets*, **21,** 289–296.

Rand, M.L., Wang, H., Bang, K.W., Teitel, J.M., Blanchette, V.S., Freedman, J. & Nurden, A.T. (2010) Phosphatidylserine exposure and other apoptotic-like events in Bernard-Soulier syndrome platelets. *American Journal of Hematology*, **85,** 584–592.

Razmara, M., Hu, H., Masquelier, M. & Li, N. (2007) Glycoprotein IIb/IIIa blockade inhibits platelet aminophospholipid exposure by potentiating translocase and attenuating scramblase activity. *Cellular and Molecular Life Sciences*, **64,** 999–1008.

Remenyi, G., Szasz, R., Friese, P. & Dale, G.L. (2005) Role of mitochondrial permeability transition pore in coated-platelet formation. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **25,** 467–471.

Suzuki, J., Umeda, M., Sims, P.J. & Nagata, S. (2010) Calcium-dependent phospholipid scrambling by TMEM16F. *Nature*, **468,** 834–838.

Yan, R., Wang, Z., Yuan, Y., Cheng, H. & Dai, K. (2009) Role of cAMP-dependent protein kinase in the regulation of platelet procoagulant activity. *Archives of Biochemistry and Biophysics*, **485,** 41–48.