CMLS, Cell. Mol. Life Sci. 62 (2005) 1514–1525
1420-682X/05/131514-12
DOI 10.1007/s00018-005-5099-y
© Birkhäuser Verlag, Basel, 2005

CMLS Cellular and Molecular Life Sciences

Research Article

Activated scramblase and inhibited aminophospholipid translocase cause phosphatidylserine exposure in a distinct platelet fraction

J. L. N. Wolfs[a], P. Comfurius[a], J. T. Rasmussen[b], J. F. W. Keuren[c], T. Lindhout[a], R. F. A. Zwaal[a] and E. M. Bevers[a,*]

[a] Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University, P.O. Box 616, 6200 MD Maastricht (The Netherlands), Fax: +31 43 3884159, e-mail: em.bevers@bioch.unimaas.nl
[b] Protein Chemistry Laboratory, Department of Molecular Biology, University of Aarhus (Denmark)
[c] Sanquin, Blood Bank Region South-East, Maastricht (The Netherlands)

Received 8 March 2005; received after revision 19 April 2005; accepted 13 May 2005
Online First 21 June 2005

Abstract. Platelet procoagulant activity is mainly determined by the extent of surface-exposed phosphatidylserine (PS), controlled by the activity of aminophospholipid translocase and phospholipid scramblase. Here, we studied both transport activities in single platelets upon stimulation with various agonists. Besides the formation of procoagulant microparticles, the results show that a distinct fraction of the platelets exposes PS when stimulated. The extent of PS exposure in these platelet fractions was similar to that in platelets challenged with Ca^{2+}-ionophore, where all cells exhibit maximal attainable PS exposure. The size of the PS-exposing fraction depends on the agonist and is proportional to the platelet procoagulant activity. Scramblase activity was observed only in the PS-exposing platelet fraction, whereas translocase activity was exclusively detectable in the fraction that did not expose PS. We conclude that, irrespective of the agonist, procoagulant platelets exhibit maximal surface exposure of PS by switching on scramblase and inhibiting translocase activity.

Key words. Procoagulant activity; scramblase; aminophospholipid translocase; thrombin; collagen; platelet; phosphatidylserine; microparticle.

Studies over the past two decades have revealed that different lipid transporters function in the maintenance and rearrangement of the transversal distribution of membrane phospholipids [1, 2]. In quiescent cells, aminophospholipid translocase is responsible for a rapid inward directed transport, which is specific for the aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE). The activity of this transporter causes an asymmetric distribution of the lipids in the plasma membrane in which the aminophospholipids, in particular PS, are primarily located in the inner leaflet, whereas the outer leaflet is dominated by the choline-containing lipids, phosphatidylcholine and sphingomyelin. Membrane lipid asymmetry dissipates upon activation of a protein termed 'scramblase', which facilitates bidirectional movement of phospholipids, irrespective of the chemical nature of the polar head group. A major consequence of activating the scramblase in cells is the appearance of PS in the membrane outer leaflet. When this occurs in blood cells, particularly in activated platelets, surface-exposed PS accelerates thrombus formation by offering a catalytic surface for several enzyme complexes of the coagulation process [1–3]. Lipid scrambling is accompanied by membrane blebbing and production of PS-exposing, procoagulant microparticles, shed from the plasma membrane [4–6]. Another major function of surface-exposed PS is to signal the scavenging of apoptotic cells by qualified macrophages [7, 8].

* Corresponding author.

The potency of different physiological platelet agonists to evoke a procoagulant response varies from collagen plus thrombin > collagen > thrombin; maximal activity is obtained with the non-physiological agonist Ca^{2+}-ionophore [9, 10]. The extent of procoagulant activity has been related to the amount of PS exposed at the cell surface. Since high cytoplasmic Ca^{2+} concentrations activate lipid scramblase and block aminophospholipid translocase, intermediate Ca^{2+} levels have been proposed to lead to a circumstance in which both transporters are active but oppose each other [2, 11]. Conceivably, these situations can accommodate a wide variety of PS mole fractions exposed at the cell surface.

Although some authors have proposed the involvement of specific protein receptors [12, 13], surface-exposed PS is widely appreciated to form an essential part of the procoagulant platelet surface. This is clearly illustrated by the fact that annexin A5 (previously known as annexin V), a protein with high affinity for PS, strongly inhibits the procoagulant activity of platelets or platelet-derived microparticles [14–16]. Lactadherin (MFG-E8), a glycoprotein of the milk fat globule membrane that binds to PS-containing lipid surfaces, has also been demonstrated to inhibit platelet prothrombinase and factor-X-activating activity [17, 18]. Platelet procoagulant activity increases two- to ten-fold after thrombin stimulation [9, 19, 20], suggesting an increased exposure of PS caused by this agonist. However, an increased outward movement of fluorescent-labelled PS could not be found in thrombin-stimulated platelets using a continuous assay to monitor transbilayer migration of lipids [21]. On the contrary, stimulation of platelets with thrombin caused a four-fold increased rate of inward transport of PS [22]. This increased aminophospholipid translocase activity has been used as an argument that components other than PS in the outer surface of thrombin-activated platelets are responsible for the increased procoagulant activity [13].

A possible explanation for these apparently paradoxical findings could be that platelet stimulation causes activation of scramblase in a minor fraction of the platelets, whereas in the majority of the platelets, aminophospholipid translocase is still active. We therefore analysed, by flow cytometry, both activities in single platelets using fluorescent lipid probes to monitor scramblase and translocase activity and fluorescent-labelled PS-binding proteins to detect surface-exposed endogenous PS.

Materials and methods

Reagents

The calcium ionophore ionomycin, N-ethylmaleimide (NEM) and bovine serum albumin, essentially fatty acid free (BSA) were obtained from Sigma (St Louis, Mo.). 1-Oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] hexanoyl]-*sn*-glycero-3-phospho-L-serine NBD-PS) was from Avanti Polar Lipids (Alabaster, Ala.). Thrombin was purified from bovine blood as previously described [23] and horse tendon collagen was from Horm, Nycomed (Munich, Germany). The coagulation proteins prothrombin, factor Xa and factor Va were purified from bovine blood as described before [23]. Thrombin-specific chromogenic substrate S2238 was obtained from Chromogenix (Mölndal, Sweden). FITC-conjugated anti-CD61 and control IgG were from BD Biosciences PharMingen (San Diego, Calif.). Alexa Fluor 647-conjugated annexin A5 and fluorescein isothiocyanate (FITC) were from Molecular Probes (Leiden, The Netherlands). Lactadherin (MGF-E8) was purified as described by Hvarregaard et al. [24] and labelled with FITC using the manufacturer's procedure.

Platelet isolation

Platelet isolation was performed as described elsewhere [25]. Briefly, 10 ml blood was collected in 2 ml anticoagulant ACD (80 mmol/l trisodium citrate, 52 mmol/l citric acid and 180 mmol/l glucose). Platelet-rich plasma was obtained by centrifuging whole blood for 15 min at 200 g. Platelets were spun down at 11,500 g for 1 min using a microfuge. The platelet pellet was resuspended and washed twice in HEPES buffer (10 mmol/l HEPES, 137 mmol/l NaCl, 2.7 mmol/l KCl, 2 mmol/l $MgCl_2$, 5 mmol/l glucose and 0.5 mg/ml BSA, adjusted to pH 6.6); before each centrifugation step, 100 µl ACD was added to each millilitre of resuspended platelets. Platelets were finally resuspended in the same HEPES buffer at pH 7.4, without BSA. The platelet count was adjusted to 2×10^8 platelets/ml.

Platelet activation

Platelet activation was performed at 37 °C at a concentration of 5×10^7 platelets/ml in a volume of 0.5 ml under continuous stirring (Teflon-coated stirring bar, 200 rpm) in the presence of 3 mmol/l $CaCl_2$. Platelet agonists used were thrombin (5 nmol/l), collagen (10 µg/ml), the combination of thrombin and collagen (5 nmol/l and 10 µg/ml, respectively) or ionomycin (1 µmol/l).

Platelet prothrombinase activity

Platelet procoagulant activity was assayed as described in more detail elsewhere [23]. Specifically, the following conditions were used: after activation at 5×10^7 platelets/ml, samples were diluted to 3×10^6 platelets/ml in HEPES buffer containing 3 mmol/l $CaCl_2$ and 0.5 mg/ml BSA and were incubated with 0.1 nmol/l factor Xa and 2 nmol/l factor Va for 1 min at 37 °C. Thrombin formation was initiated by addition of 1 µmol/l prothrombin and arrested after 2 min by addition of 5 mmol/l EDTA. Thrombin was measured using the chromogenic substrate S2238.

Separation of microparticles from remnant platelets after activation

Platelet activation has been demonstrated to be accompanied by the production of microparticles shed from the plasma membrane [4, 6]. To evaluate the contribution of microparticles in the present study, we used a previously described method to separate microparticles from remnant cells [5]. Briefly, after activation, platelet suspensions were centrifuged at 11,500 g for various time periods. Both the prothrombinase activity remaining in the supernatant and the lipid content of the supernatant appeared to approach constant values after 3 min centrifugation. This prothrombinase activity is operationally defined as derived from microparticles. The absence of platelets or remnant cells in the supernatant was confirmed by flow cytometry (see Results).

Measurement of scramblase and aminophospholipid translocase activity

The distribution of fluorescent lipid probes over the two leaflets of the plasma membrane was measured using a BSA back exchange protocol which rapidly extracts lipid probe from the external leaflet as described by Connor et al. [26]. To measure scramblase activity, platelets (2×10^8 platelets/ml) were preloaded at 37°C for 30 min with 1 µmol/l NBD-PS, added from a 0.5 mmol/l stock solution of this probe in dimethylsulphoxide. This concentration of added NBD-PS amounts to approximately 1% of the endogenous phospholipid concentration. Subsequently, 5×10^7 platelets/ml were stimulated at 37°C with the various agonists as described above. Five minutes after addition of the agonist, a sample of 50 µl was transferred to 50 µl HEPES buffer containing 2% (w/v) BSA, and another sample of 50 µl was added to 50 µl HEPES without BSA to monitor the amount of NBD-PS present in the inner leaflet and the total amount present in both leaflets, respectively. After an additional 5 min, 10 µl from both mixtures was diluted in 240 µl HEPES buffer containing 3 mmol/l $CaCl_2$; the platelet concentration in the diluted samples was 10^6 platelets/ml. Detection of surface-exposed endogenous PS was achieved by addition of 1 µl Alexa Fluor 647-conjugated annexin A5 and incubation for 5 min. Samples were analysed by flow cytometry.

To measure aminophospholipid translocase activity, the following protocol was used. Prior to addition of NBD-PS, platelets (5×10^7 platelets/mL) were activated for 5 min at 37°C as described above. Subsequently, 0.5 µmol/l NBD-PS was added and 50 µl samples were taken at different times, mixed with 50 µl HEPES with or without BSA and further processed as described for the scramblase assay.

For flow cytometry, all buffers were passed through a 0.2-µm Millipore filter before use.

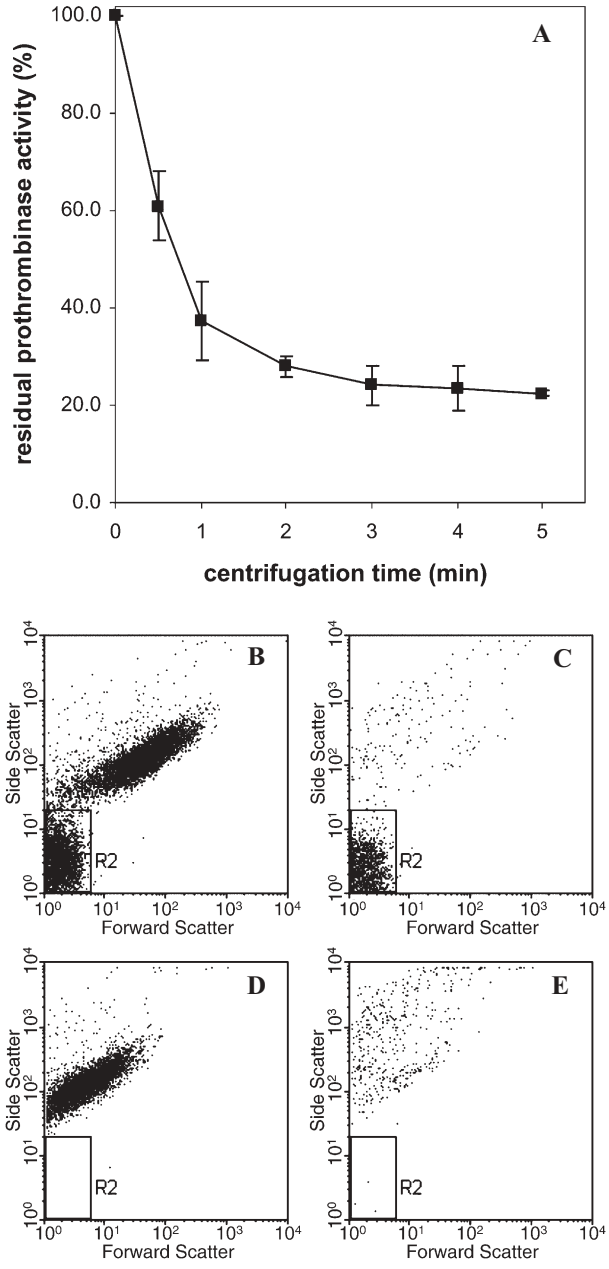

Figure 1. Platelet microparticle detection by flow cytometry. Platelets (5×10^7 platelets/ml) were activated with ionomycin (1 µmol/l) in the presence of 3 mmol/l $CaCl_2$ for 15 min. Samples from this incubation were centrifuged at 11,500 g for various time periods and prothrombinase activity of the supernatant was measured as described in materials and methods and expressed as percentage of the prothrombinase activity of the total platelet suspension (*A*) (data are mean values ± SD from three separate experiments). Samples from the total incubation (*B*, *D*) and the supernatant obtained after 3 min centrifugation (*C*, *E*) were stained with FITC-conjugated anti-CD61 and analysed in a Becton Dickinson FACScan flow cytometer using either the setting E01 (*B*, *C*) or E00 (*D*, *E*) for FSC. Represented are dot plots of FSC versus SSC exclusively for CD61-positive events. The region R2 is set to mark the area in which the microparticles appear using E01 for FSC. Note that events are no longer observed in this region when FSC is set at E00.

Flow cytometry

Samples were analysed with a FACScan flow cytometer (Beckton Dickinson, San Jose, Calif.) with light scatter and fluorescence channels set at logarithmic gain. Forward-angle light scatter (FSC) was detected at E00 and side scatter (SSC) was detected at 520 with no thresholds. The NBD and the Alexa Fluor 647 probes were excited with the 488-nm argon laser and the 633-nm HeNe laser, respectively, and their emission signals were measured simultaneously in FL 1 (530±30 nm bandpass filter) and FL 4 (661±16 nm bandpass filter). Scatter parameters and fluorescence intensities were collected from 10,000 platelets and were analysed using WinMDI version 2.8 software (http://facs.scripps.edu/software.html). For detection of microparticles, the setting for FSC was changed to E01 and 100,000 events were collected. FITC-conjugated anti-CD61, specific for the integrin ß$_3$ subunit of $\alpha_{IIb}\beta_3$, and FITC-conjugated non-specific IgG were used to discriminate platelet material from background and electronic noise. Only CD61-positive events above the control IgG background were collected and analysed.

Results

Scramblase and aminophospholipid translocase activity in platelet microparticles

Activation of platelets is usually accompanied by production of microparticles shed from the plasma membrane [4, 6]. We have previously defined microparticles based on procoagulant activity which remained in the supernatant of activated platelets after centrifugation at 17,000 g/min [5]. As shown in figure 1A, the residual prothrombinase activity of a suspension of ionomycin-stimulated platelets approached constant values after 90 s centrifugation at 11,500 g. We found that this fraction of non-sedimentable procoagulant activity varied between 20–30% of the total and was independent of the agonist used, in agreement with observations of Sims et al. [4]. When microparticles isolated from NBD-PS-loaded platelets were incubated with BSA, the NBD-PS appeared completely extractable by BSA, indicative of an activated scramblase. On the other hand, microparticles were unable to accumulate non-exchangeable NBD-PS when added exogenously, indicating that aminophospholipid translocase activity is inhibited or unable to oppose the scramblase activity (data not shown).

Because of their small size, the fluorescence signal of microparticles labelled with Alexa 647-conjugated annexin A5 or NBD-PS will generate events in a flow cytometer with a low fluorescence signal relative to that of activated platelets. To avoid interference of microparticles in the flow cytometric approach to detect scramblase and aminophospholipid translocase activity in activated platelet suspensions, flow cytometer settings were chosen to exclude these events. To establish the position of microparticles in the dot plots of forward versus side scatter, we used a fluorescence-gated detection based on anti-CD61 as a marker for platelets and platelet-derived microparticles. Figure 1B shows a dot plot of forward versus side scatter of a suspension of ionomycin-activated platelets in which the microparticles form a population of events

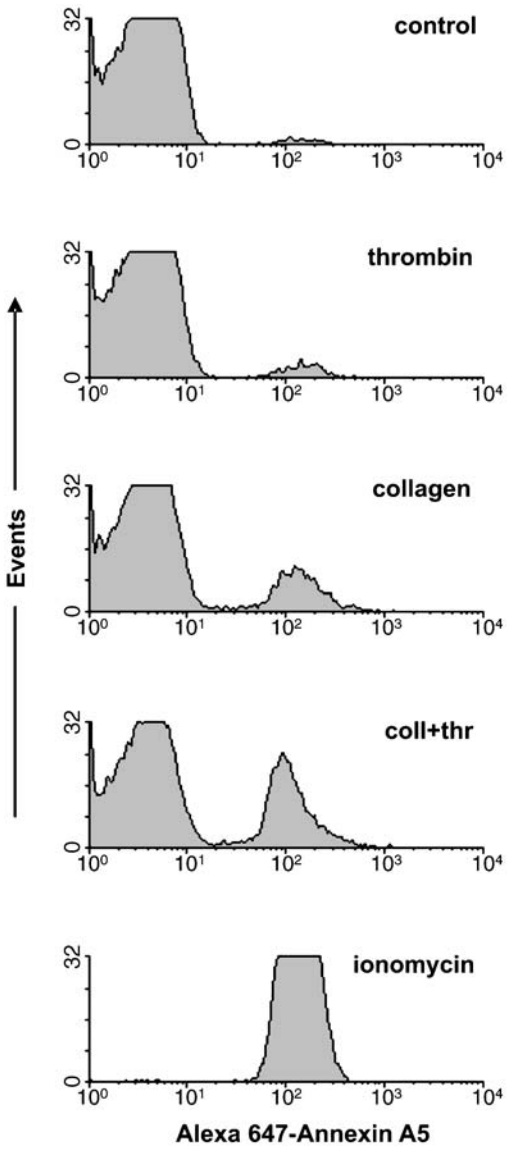

Figure 2. Histograms of annexin A5 binding to platelets stimulated with various agonists. Platelets (5×10^7 platelets/ml) in suspension were activated with thrombin (5 nmol/l), collagen (10 µg/ml), collagen plus thrombin (10 µg/ml and 5 nmol/l, respectively) or ionomycin (1 µmol/l) in the presence of 3 mmol/l $CaCl_2$ for 5 min under continuous stirring. Samples were 50-fold diluted and analysed with Alexa 647-conjugated annexin A5. A scale of maximal 32 events was chosen to better visualise the fraction annexin A5-positive events induced in the control and thrombin-stimulated platelets. Shown are the histograms of a representative experiment out of five performed.

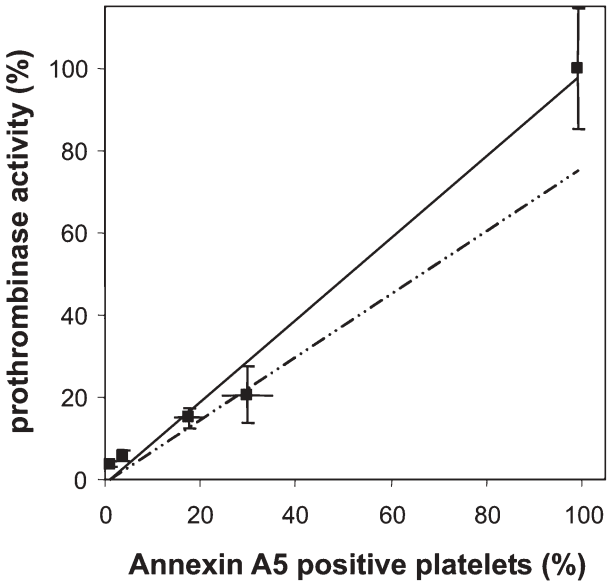

Figure 3. Correlation between platelet prothrombinase activity and binding of Alexa 647-conjugated annexin A5. Platelets (5×10^7 platelets/ml) were stimulated for 5 min with various agonists after which prothrombinase activity and annexin A5 binding were measured as described in Materials and methods. Prothrombinase activity is expressed as a percentage of the maximal activity observed for ionomycin-activated platelets, which was 224±33 nmol/l thrombin per minute. Note that prothrombinase activity includes that of the microparticles. When corrected for the contribution of the microparticles to the prothrombinase activity, the actual correlation between prothrombinase activity and number of PS-exposing remnant cells is represented by the dashed line. Data are mean values ± SD from five independent experiments.

distinct from that of the remnant platelets (region R2 in fig. 1B). The position of the microparticles in the dot plots of FSC versus SSC was further confirmed by analysis of a supernatant of ionomycin-treated platelets obtained after 3 min centrifugation at 11,500 g (region R2 in fig. 1C). Changing the setting for FSC to E00 caused the microparticles to become undetectable in the flow cytometer as illustrated in figure 1D, E. All subsequent experiments to measure scramblase and translocase activity were performed using these settings for flow cytometry.

PS exposure occurs in a distinct platelet fraction

PS exposure as a result of platelet activation was measured in single platelets using fluorescent marker proteins, which selectively recognize PS. Figure 2 shows histograms of Alexa 647-conjugated annexin A5 staining of platelets in suspension, activated with thrombin, collagen or collagen plus thrombin. Only a fraction of the platelets became annexin A5 positive, the amount depending on the agonist used. Remarkably, the mean fluorescence intensity of the annexin A5-positive platelet fraction is comparable for the different agonists and coincides with the mean fluorescence intensity of annexin A5 binding found upon stimulation with Ca^{2+}-ionophore, in which case virtually all cells become annexin A5 positive. Figure 3 shows that there is a good correlation ($r=0.99$) between the percentage of annexin A5-positive cells and the corresponding procoagulant activity, measured as prothrombinase activity. Thus, the differences in procoagulant activity observed for various platelet stimuli do not seem to be determined by a gradual increase in the extent of surface-exposed PS of each platelet, but rather by a fraction of the platelets that exhibits maximal PS exposure such as elicited by Ca^{2+}-ionophore. It should be noted in figure 3 that the prothrombinase activity is related to the whole platelet suspension, including the microparticles, whereas, due to the settings of the flow cytometer, the annexin A5-positive events represent exclusively platelets or remnant cells. The contribution of the microparticles to the prothrombinase activity (approximately 20–30%) appeared to be independent of the agonist used. The dashed line in figure 3 shows the relationship between prothrombinase activity and annexin A5 binding corrected for the microparticle contribution to the prothrombinase activity.

Under the conditions used in the present study, the threshold for binding of annexin A5 is about 4 mol% PS [17, 27], from which one may argue that the abrupt increase in the fluorescence signal to the level of ionomycin-activated platelets might be an artefact of the detection limit of PS by annexin A5. Therefore, we used FITC-conjugated lactadherin to analyse the number of positive platelets obtained with the different stimuli. This protein was recently described as an alternative PS-binding protein, which, in contrast to annexin A5, binds proportionally to membranes with a PS content varying from 0 to 2% [17]. The results, summarized in table 1 demonstrate that the percentage positive cells, detected with either annexin A5 or lactadherin, are very similar for all agonists used, suggesting that both probes label the same platelets. This was confirmed by a double-label experiment in which low, non-saturating concentrations of both Alexa-conjugated annexin A5 and FITC-conjugated lactadherin were added to avoid mutual interference of the binding (table 1, fig. 4).

Table 1. Binding of annexin A5 and lactadherin to stimulated platelets

Agonist	Percent positive platelets		
	annexin A5	lactadherin	anx+lact
None	1.4 ± 0.4	2.3 ± 0.1	2.1 ± 0.1
Thrombin	3.8 ± 0.9	4.4 ± 0.2	4.7 ± 0.2
Collagen	17.5 ± 2.9	24.7 ± 3.1	20.3 ± 4.2
Collagen+thrombin	29.7 ± 5.2	30.4 ± 2.2	27.3 ± 1.6
Ionomycin	99.2 ± 0.2	98.5 ± 2.9	94.4 ± 2.6

Platelets were stimulated with the indicated agonists for 5 min and subsequently incubated with Alexa 647-conjugated annexin A5, FITC-conjugated lactadherin or both labelled proteins and analysed by flow cytometry. For further details refer to the legend of figure 4. Data represent mean values ± SD (n = 3).

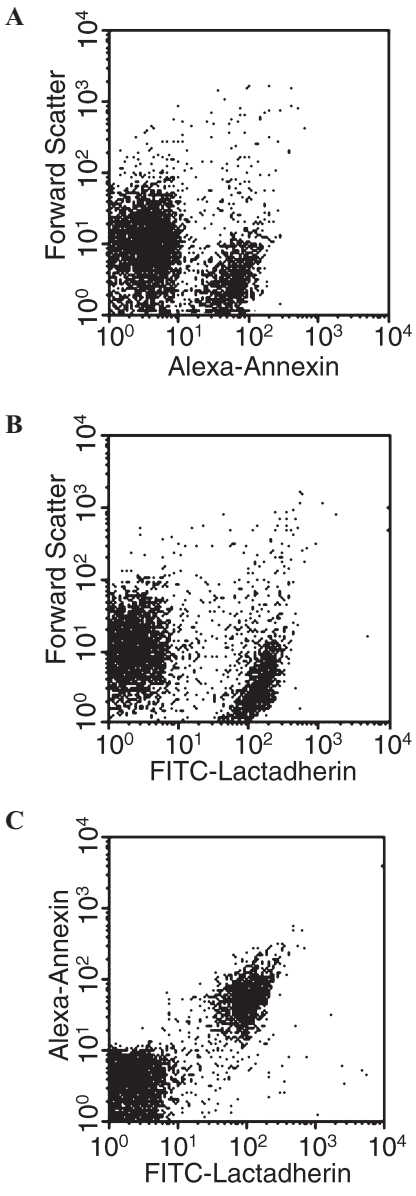

Figure 4. Dot plots of lactadherin and annexin A5 binding to collagen-stimulated platelets. Platelets (5×10^7 platelets/ml) were stimulated for 5 min with collagen (10 µg/ml) + thrombin (5 nmol/l) under continuous stirring. From the same incubation, three samples were 50-fold diluted and analysed in the flow cytometer for binding of Alexa 647-conjugated annexin A5, FITC-conjugated lactadherin or the combination of both labels. Concentrations of both annexin A5 and lactadherin were chosen to avoid appreciable mutual interference of the binding of each of these proteins: Alexa 647-conjugated annexin A5 was added at a dilution of 1 in 250 of the commercial preparation; FITC-conjugated lactadherin was added at a final concentration of 2 nmol/l. Shown are the dot plots of a typical experiment out of three performed. (*A*) Binding of annexin A5 versus Forward scatter. (*B*) Binding of lactadherin versus Forward scatter. (*C*) Binding of both annexin A5 and lactadherin.

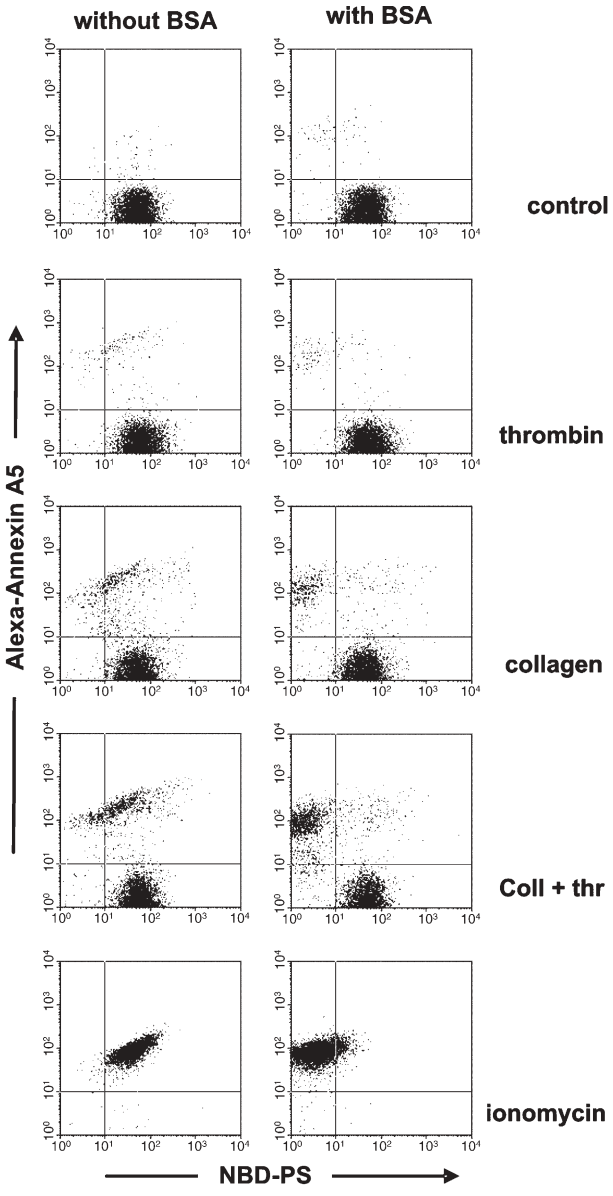

Figure 5. Scramblase activity in platelets stimulated with various agonists. Platelets, preloaded with NBD-PS, were stimulated at 5×10^7 platelets/mL with thrombin (5 nmol/l), collagen (10 µg/ml), collagen plus thrombin (10 µg/ml and 5 nmol/l, respectively) or ionomycin (1 µmol/l) under continuous stirring. After 5 min, samples were 50-fold diluted in buffer with and without BSA, and Alexa 647-conjugated annexin A5 was added. After an additional 5 min, samples were analysed by flow cytometry. Represented are dot plots of NBD-PS fluorescence versus Alexa 647-annexin A5; left panels represent samples in the absence of BSA, right panels show samples taken in the presence of BSA. Based on control platelets in the absence of BSA, quadrants are set to demarcate platelets that retain NBD-PS (upper- and lower-right quadrants) or do not become annexin A5 positive (lower-left and lower-right quadrants). Shown are the dot plots of a representative experiment out of three performed.

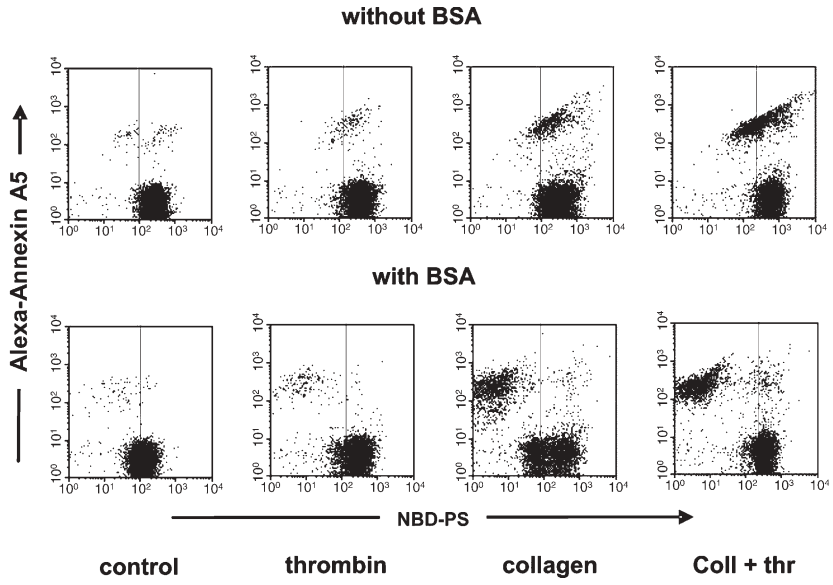

Figure 6. Translocase activity in platelets stimulated with various agonists. Platelets were stimulated at 5×10^7 ml with thrombin (5 nmol/l), collagen (10 μg/ml), collagen plus thrombin (10 μg/ml and 5 nmol/l, respectively) or ionomycin (1 μmol/l) under continuous stirring. After 5 min, NBD-PS was added and translocase was allowed to proceed for another 5 min. Subsequently, samples were taken and diluted 50-fold in buffer with and without BSA, followed by addition of Alexa 647-conjugated annexin A5. Represented are dot plots of NBD-PS fluorescence versus Alexa 647-annexin A5, upper panels represent samples in the absence of BSA, lower panels represent samples taken on BSA. The vertical line demarcates platelets with the total amount of NBD-PS that was added, irrespective of the localisation of this probe in the inner or outer leaflet of the plasma membrane. Events left of this demarcation line represent platelets that have lost NBD-PS upon extraction with BSA, indicating incomplete translocation to the inner leaflet. Note that, also in the upper panel, annexin A5-positive platelets have lost NBD-PS signal, caused by carry over of BSA from the annexin A5 preparation (see text). Shown are the dot plots of a representative experiment out of three.

Next to the population of platelets that binds neither annexin A5 nor lactadherin, there is only one population of cells that becomes positive for both labels. No platelets were found to be labelled exclusively with lactadherin (fig. 4C), indicating that the labelled platelet fraction exposes at least 4 mol% PS. As expected and in agreement with other studies [14, 15, 17, 18], both annexin A5 and lactadherin can fully inhibit platelet prothrombinase activity, irrespective of the agonist used (data not shown).

Scramblase is active in the platelet fraction which exposes PS and inactive in the fraction that does not expose PS

To relate PS exposure to activation of phospholipid scramblase, we measured this activity in annexin A5-positive and annexin A5-negative cells as depicted in figure 5. Scramblase was measured in platelets incubated with NBD-PS for a sufficient time to allow accumulation in the inner leaflet. After stimulation with the various agonists for 5 min, the NBD fluorescence signal decreases due to extraction by BSA of NBD-PS that appears in the outer leaflet by means of an activated scramblase. From the dot plots of NBD fluorescence versus Alexa 647 staining, the annexin A5-negative cells (lower quadrants of the right panels in fig. 5) clearly did not lose NBD-PS upon addition of BSA, whereas in the annexin A5-positive cells, virtually all NBD-PS could be extracted by BSA (upper-left quadrant of the right panels in fig. 5). This appears true for all agonists tested. Note that also in the absence of BSA (left panels in fig. 5), the NBD fluorescence signal in the annexin A5-positive cells was slightly shifted to the left when compared to that of the annexin A5-negative cells. We could demonstrate that this is caused by the presence of BSA in the Alexa 647-annexin A5 conjugate, which is added by the manufacturer (Molecular Probes) to stabilise the protein preparation (data not shown).

Aminophospholipid translocase can only be observed in the platelet fraction that does not expose PS

Next, we focused on the aminophospholipid translocase activity. Platelets were activated with various agonists for 5 min before adding NBD-PS to monitor inward transport by the translocase. The residual NBD fluorescence after extraction with BSA can be taken as a measure for NBD-PS that has been transported to the inner leaflet of the plasma membrane by the aminophospholipid translocase. An example of such an experiment is presented in fig. 6**,** showing typical dot plots of the NBD-PS fluorescence versus Alexa 647-annexin A5 binding after 5 min inward transport of NBD-PS in the various stimulated platelet suspensions. The upper panels show the fluorescence signal of NBD-PS in the absence of BSA, i.e. the total

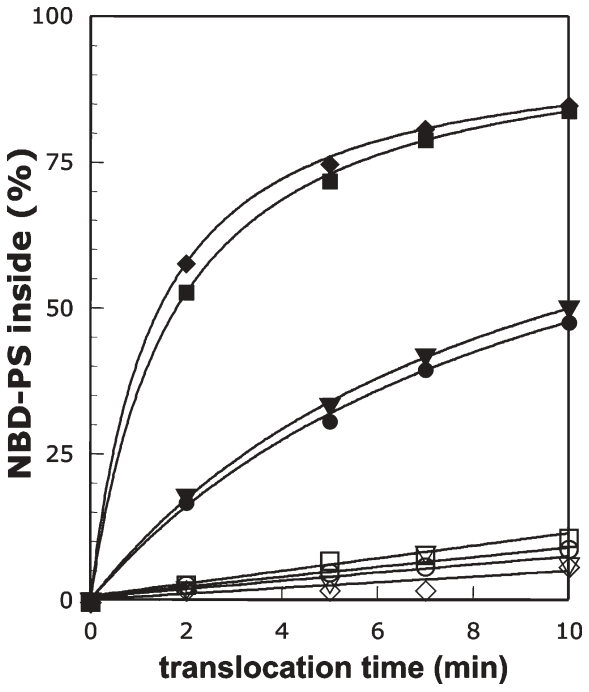

Figure 7. Aminophospholipid translocase activity in annexin A5-positive and annexin A5-negative platelets. NBD-PS was added to platelet suspensions that were activated with thrombin (5 nmol/l), collagen (10 μg/ml) or collagen plus thrombin (10 μg/ml and 5 nmol/l, respectively) or for 5 min. Timed samples were 50-fold diluted in buffer with and without BSA, and Alexa 647-annexin A5 was added. Translocase activities of the annexin A5-positive and annexin A5-negative platelet fraction were analysed by flow cytometry as described in the legend to figure 5 and in Materials and methods. Translocase activity is expressed as the percentage of NBD-PS that is not extractable with BSA (NBD-PS inside). Symbols: ● ○, control; ■ □, thrombin; ▼ ▽, collagen; ◆ ◇, collagen plus thrombin. Open symbols, annexin A5-positive platelets; closed symbols, annexin A5-negative platelets.

amount of probe that was added. Note that the probe distributes equally over the annexin A5-positive and -negative platelets. The lower panels depict the residual NBD-PS fluorescence after extraction with BSA, i.e. the non-exchangeable NBD-PS present in the inner leaflet. Clearly, aminophospholipid translocase appeared active exclusively in the annexin A5-negative platelet population. In the annexin A5-positive fraction, NBD-PS remained completely extractable by BSA. Time courses of the inward transport of NBD-PS in the annexin A5-negative and annexin A5-positive platelet fractions obtained with the various agonists are depicted in figure 7. The results show that for all agonists used, aminophospholipid translocase was virtually absent in the annexin A5-positive fraction of the platelets and active in the annexin A5-negative platelets. Note that the rate of NBD-PS transport was significantly increased in platelets stimulated with thrombin or collagen plus thrombin in comparison to unstimulated platelets or platelets stimulated with collagen alone. A similar increase in rate of aminophospholipid transport was found when platelets were stimulated via the protease-activated receptors PAR 1 and 4 by the peptide agonists SFLLRN and AYPGKF, respectively (data not shown).

Effect of inactivation of aminophospholipid translocase on PS exposure in platelets

The virtual absence of aminophospholipid translocase activity in the annexin A5-positive platelets raises the question as to whether mere inhibition of this transporter suffices to dissipate lipid asymmetry and cause surface exposure of PS as a result of a spontaneous transbilayer movement of this lipid. Being dependent on reduced sulfhydryl groups [5, 28, 29], aminophospholipid translocase activity can be inhibited by sulfhydryl reactive agents such as NEM. Since NEM treatment of platelets affects the permeability barrier of the plasma membrane for Ca^{2+} (unpublished observations), annexin A5 could not be used to monitor the appearance of PS in the external membrane leaflet. We therefore used lactadherin, since this protein does not require Ca^{2+} for binding to PS-containing lipid surfaces [17]. Treatment of platelets for 5 min with 1 mmol/l NEM in the presence of 1 mmol/l EGTA completely abrogated the inward transport of NBD-PS (data not shown). It should be emphasized that under these conditions, scramblase can still be activated with Ca^{2+}-ionomycin. Analysis of platelets with FITC-lactadherin showed no appreciable PS exposure for at least 5 min after addition of NEM, when compared with untreated platelets (fig. 8A). Thus, the exposure of PS evoked within 5 min by thrombin, collagen, collagen plus thrombin or ionomycin, is not merely caused by inhibition of aminophospholipid translocase, but indeed requires the activity of a phospholipid scramblase. Prolongation of the time of NEM treatment to 60 min caused a gradual increase in lactadherin binding of the entire platelet population, in contrast to the observations with platelets stimulated with thrombin, collagen or ionomycin, where the increased binding of annexin A5 relates to an increased fraction of 'high-binding' platelets (compare fig. 2). Figure 8B shows a time course of ionomycin treatment of platelets as analysed by lactadherin. Scrambling of lipids was arrested by addition of EDTA [21] and PS-exposing platelets were monitored by lactadherin. In contrast to the gradual surface accumulation of PS induced by NEM, which occurs in the entire platelet population, Ca^{2+}-ionophore induced maximal PS exposure in only a fraction of the platelets, which increased in time to become maximal (100%) in less than 3 min. No platelets with an intermediate extent of PS exposure could be observed during these 3 min.

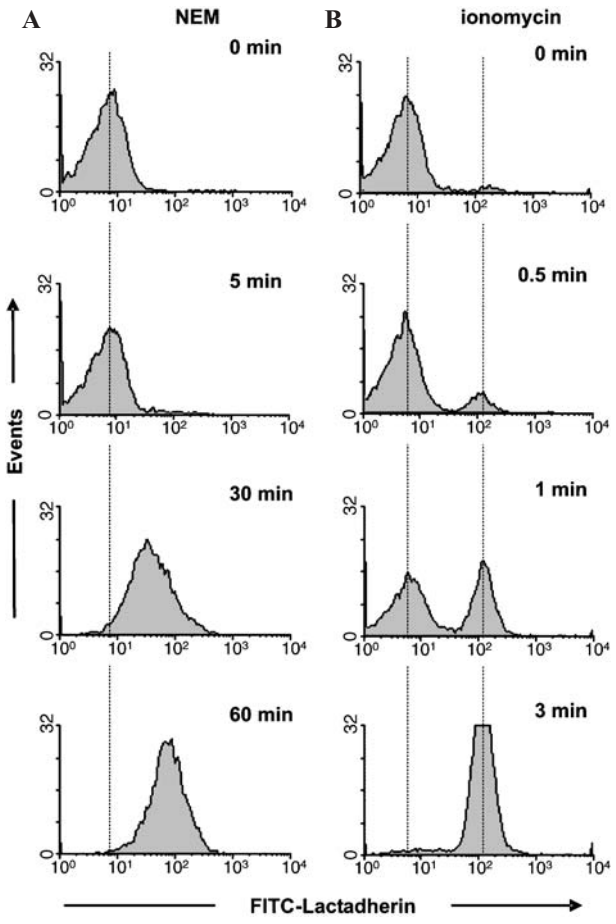

Figure 8. Binding of lactadherin to platelets treated with NEM and ionomycin. (*A*) Platelets at 5×10^7 ml were treated with 1 mmol/l NEM in the presence of 1 mmol/l EGTA. At the times indicated, samples were 50-fold diluted and after addition of FITC-lactadherin analysed by flow cytometry. (*B*) Platelets at 5×10^7 ml were treated with 1 μmol/l ionomycin in the presence of 3 mmol/l $CaCl_2$. At the times indicated, samples were 50-fold diluted in buffer with 1 mmol/l EGTA to arrest the process of lipid scrambling. After addition of FITC-lactadherin, samples were analysed by flow cytometry. Shown are the histograms of a representative experiment out of two.

Discussion

Procoagulant activity is caused by a fraction of maximal PS-exposing platelets

The platelet procoagulant response involves the exposure of PS, required for the acceleration of the lipid-dependent coagulation reactions that lead to the formation of factor Xa and thrombin. Both the lipid concentration, i.e. the number of platelets, and the phospholipid composition of the outer leaflet of the plasma membrane, in particular the mole fraction PS, determine the extent of procoagulant activity of a platelet suspension. It is well appreciated that the platelet procoagulant response is dependent on the agonist used; maximal procoagulant activity is evoked when the membrane is selectively permeabilized for Ca^{2+} by means of a Ca^{2+}-ionophore, e.g. ionomycin. This corresponds to maximal surface exposure of PS since, as was shown previously, PS in ionomycin-treated platelets is completely randomized over both membrane leaflets [5, 30]. The most powerful physiological trigger to induce PS exposure in platelets is the combined action of collagen and thrombin, while each of these agonists alone appears less potent. Because a linear relationship was found between the percentage PS-exposing platelets and the prothrombinase activity (fig. 3), we conclude that the increased procoagulant activity of a platelet suspension as induced by the different agonists is related to an increase in the fraction of platelets exhibiting *maximal* PS exposure (i.e. as induced by Ca^{2+}-ionophore), rather than to the entire platelet suspension in which each platelet gradually accumulates PS in the outer leaflet of the plasma membrane (fig. 2, 3). The pivotal role of surface exposed PS to the procoagulant activity is supported by the observation that, in concordance with the literature [14–16], annexin A5 completely blocked this activity (data not shown).

A heterogeneous response of platelets has also been observed previously by Dachary-Prigent et al. [10] and recently by London et al. [31]. The formation of two platelet populations, i.e. one with and one without surface-exposed PS, has been ignored in most equilibrium binding studies in the past, which has led to erroneous estimations of the number of binding sites per platelet for PS-binding proteins. Recently, the number of binding sites for factor IXa on thrombin- or SFLLRN-stimulated platelets has been refined in view of these findings [31]. A heterogeneous response of platelets to various agonists, *e.g.* thrombin, may also compromise interpretations on the comparison of procoagulant activity between platelets and synthetic phospholipid vesicles [12, 13].

The cause of this differential response of platelets to various stimuli is unknown. Studies from Dale and coworkers have demonstrated the existence of a population of platelets with high levels of surface-bound alpha granule factor V on stimulation with dual agonists such as collagen plus thrombin, which they termed ‘coat platelets’ [32, 33]. Although coinciding with the expression of ‘coat-factor V’, surface exposure of PS is not sufficient to generate coat platelets, as illustrated by the observation that stimulation with Ca-ionophore did not cause high levels of factor V binding. We emphasize that under our experimental conditions, the procoagulant activity was measured under saturating conditions of (exogenously added) factor Va, showing a linear correlation with the fraction of PS-exposing platelets (fig. 3) independent of the agonist. Since PS exposure appears to relate to levels of intracellular Ca^{2+} [21, 34, 35], the heterogeneity may reflect differences in Ca^{2+} homeostasis of the platelets. Alternatively, differences in agonist receptor density, possibly related to age, may explain the heterogeneous procoagulant response.

Scramblase switched on and translocase switched off in PS-exposing platelets

In principle, a concomitant partial activation of the scramblase and partial inhibition of aminophospholipid translocase, oppositely regulated by intracellular Ca^{2+}, would suffice to accommodate a wide variety of PS mole fractions to become exposed in the exofacial leaflet of the platelet plasma membrane [2, 11]. In the present study, we clearly demonstrate that upon stimulation, scramblase is fully activated in the PS-exposing platelet fraction. Whether or not aminophospholipid translocase is completely inhibited in this fraction cannot be decided from these experiments. Some residual translocase activity may go unnoticed in platelets in which the scramblase is fully switched on, because all NBD-PS that appears in the outer leaflet will be extracted by BSA. Platelets that become scramblase positive abruptly reach a level of maximal PS exposure, as obtained when platelets are challenged with ionomycin. Experiments with ionomycin-activated platelets from a patient with Scott syndrome, deficient in phospholipid scramblase, have demonstrated that aminophospholipid translocase activity is completely abrogated upon a rise in intracellular Ca^{2+} [36]. Therefore, in the fraction of PS-exposing platelets, aminophospholipid translocase is very likely fully blocked. In contrast, in the annexin A5-negative platelets, scramblase activity is absent, whereas translocase activity appears to be even higher than observed for unstimulated control platelets. This fully 'on-off' phenomenon of the scramblase and the oppositely regulated apparent 'on-off' mechanism of the aminophospholipid translocase are compatible with the jump in PS exposure shown in the histograms of figure 2 and figure 8B. There is apparently no partial activation of scramblase combined with a partial inactivation of translocase that can give rise to platelets with intermediate degrees of PS exposure. This is supported by the binding of lactadherin and annexin A5 in the stimulated platelet suspensions. Although binding of annexin A5 has a threshold of approx 4 mol% PS and lactadherin binding was demonstrated to be proportional in the range from 0–2%, both lactadherin and annexin A5 label the same platelet fraction. This means that the platelet procoagulant response involves an abrupt exposure of at least 4 mol% PS. It should be emphasized that the sudden exposure of PS in a fraction of the platelets is not caused by cell lysis (data not shown).

Inhibition of aminophospholipid translocase is insufficient to cause PS exposure during platelet activation

Mere inhibition of aminophospholipid translocase does not readily result in loss of lipid asymmetry. Platelets, treated with NEM to completely block aminophospholipid translocase activity, do not expose PS within 5 min (fig. 8), the time in which thrombin and/or collagen induce a procoagulant response. Thus, the mechanism of PS exposure upon stimulation with these agonists is not related to a spontaneous outward migration and concomitant inhibition of the translocase to prevent PS from being pumped back to the inner leaflet. Also, prolonged incubation with NEM shows a gradual increase in PS exposure in the entire platelet population, contrary to the abrupt increase in a fraction of the platelets seen with the various agonists. The different time scale of PS exposure in NEM-treated platelets is reminiscent of PS exposure observed in cells undergoing apoptosis. A recent paper by Augereau et al. [37] showed that local anaesthetics induce apoptotic-like mitochondrial events associated with PS exposure in blood platelets. However, using tetracaine to induce these apoptotic-like effects, we observed an increasing fraction of high PS-exposing cells rather than a situation where all platelets gradually accumulate PS in their outer leaflets (results not shown). This may suggest that PS exposure in this apoptotic-like process is also an 'all or none' event in activating scramblase and inactivating translocase, similar to that observed with the various platelet agonists. Nevertheless, the mechanism of PS exposure during cell activation is distinct from that during apoptosis, as demonstrated in Scott syndrome, where PS exposure induced by Ca^{2+}-ionophore is deficient and that induced by apoptosis is not [38]. The mechanism through which PS accumulates at the outer surface of the platelet after treatment with NEM may reflect spontaneous dissipation of membrane lipid asymmetry.

Translocase activity is enhanced in the platelet fraction that does not expose PS

The separate analysis of aminophospholipid translocase activity and scramblase activity in the annexin A5-positive and annexin A5-negative fraction in a suspension of platelets offers an explanation for the previous observation that stimulation with thrombin leads both to an increased prothrombinase activity and an increased aminophospholipid translocase activity [22]. Only a small, scramblase-positive, PS-exposing fraction of the platelets contributes to the procoagulant response, whereas the majority of the platelets do not expose PS due to an active aminophospholipid translocase activity. This may also put a previous study by Larson et al. [13] into a different perspective. These authors claimed that the increased aminophospholipid translocase activity indicated that thrombin activated platelets do not expose PS, and therefore components other than PS must be responsible for the procoagulant activity of thrombin-activated platelets. Our results clearly show that surface-exposed PS is required for the procoagulant activity of thrombin activated platelets.

Remarkably, the rate of inward transport of PS in the annexin A5-negative fraction of a platelet suspension stimulated with thrombin or collagen plus thrombin is at least four-fold increased compared to the rate in unstim-

ulated platelets. This increased rate is not observed when platelets are stimulated with collagen alone. One may speculate that the enhanced aminophospholipid translocase activity could be caused by additional copies of translocase originating from intracellular granula membranes that become part of the plasma membrane as a result of the release reaction. This is supported by the different extent of serotonin release observed between collagen- and thrombin-stimulated platelets [39], although this parameter relates to dense granule membranes only. Considering, however, that less than half of the total amount of membrane in the platelet is intracellular [40], even a complete release of all intracellular granules would not account for a four-fold increased rate of translocase activity. Apparently, other changes in the platelet plasma membrane induced by the action of thrombin must cause alterations in the properties of the aminophospholipid transporter. It cannot be excluded either that the increased rate of translocase results from a looser packing of phospholipids in the membrane of thrombin-activated platelets.

In summary, we have demonstrated that PS exposure upon platelet stimulation does not involve the entire platelet population but rather a fraction of the platelets, the size of the fraction depending on the agonist used. The fraction of PS-exposing platelets always shows the same extent of PS exposure, irrespective of the agonist. Apparently, platelets with an intermediate PS exposure are not detectable. From this we infer that in the PS-exposing platelet fraction, aminophospholipid translocase might be switched off before scramblase becomes fully activated. Thus, once platelets reach the threshold for a procoagulant response, they suddenly change into maximal PS-exposing cells.

1 Sims P. J. and Wiedmer T. (2001) Unraveling the mysteries of phospholipid scrambling. Thromb. Haemost. **86:** 266–275
2 Bevers E. M., Comfurius P., Dekkers D. W. and Zwaal R. F. (1999) Lipid translocation across the plasma membrane of mammalian cells. Biochim. Biophys. Acta **1439:** 317–330
3 Heemskerk J. W., Bevers E. M. and Lindhout T. (2002) Platelet activation and blood coagulation. Thromb. Haemost. **88:** 186–193
4 Sims P. J., Wiedmer T., Esmon C. T., Weiss H. J. andShattil S. J. (1989) Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity. J Biol Chem **264:** 17049–17057
5 Comfurius P., Senden J. M., Tilly R. H., Schroit A. J. and Bevers E. M., Zwaal, R. F. (1990) Loss of membrane phospholipid asymmetry in platelets and red cells may be associated with calcium-induced shedding of plasma membrane and inhibition of aminophospholipid translocase. Biochim. Biophys. Acta **1026:** 153–160
6 Morel O., Toti F., Hugel B. and Freyssinet J. M. (2004) Cellular microparticles: a disseminated storage pool of bioactive vascular effectors. Curr. Opin. Hematol. **11:** 156–164
7 Fadok V. A., Xue D. and Henson P. (2001) If phosphatidylserine is the death knell, a new phosphatidylserine-specific receptor is the bellringer. Cell Death Differ. **8:** 582–587
8 Schlegel R. A. and Williamson P. (2001) Phosphatidylserine, a death knell. Cell Death Differ. **8:** 551–563
9 Bevers E. M., Comfurius P. and Zwaal R. F. (1983) Changes in membrane phospholipid distribution during platelet activation. Biochim. Biophys. Acta **736:** 57–66
10 Dachary-Prigent J., Freyssinet J. M., Pasquet J. M., Carron, J. C. and 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
11 Diaz C. and Schroit A. J. (1996) Role of translocases in the generation of phosphatidylserine asymmetry. J. Membr. Biol. **151:** 1–9
12 Monroe D. M., Hoffman M. and Roberts H. R. (2002) Platelets and thrombin generation. Arterioscler. Thromb. Vasc. Biol. **22:** 1381–1389
13 Larson P. J., Camire R. M., Wong D., Fasano N. C., Monroe D. M. Tracy P. B. et al. (1998) Structure/function analyses of recombinant variants of human factor Xa: factor Xa incorporation into prothrombinase on the thrombin-activated platelet surface is not mimicked by syntheti phospholipid vesicles. Biochemistry **37:** 5029–5038.
14 Thiagarajan, P. and Tait J. F. (1991) Collagen-induced exposure of anionic phospholipid in platelets and platelet-derived microparticles. J Biol Chem **266:** 24302–24307
15 Thiagarajan P. and Tait J. F. (1990) Binding of annexin V/placental anticoagulant protein I to platelets: evidence for phosphatidylserine exposure in the procoagulant response of activated platelets. J. Biol. Chem. **265:** 17420–17423
16 Pasquet J. M., Toti F., Nurden A. T. and Dachary-Prigent J. (1996) Procoagulant activity and active calpain in platelet-derived microparticles. Thromb Res **82:** 509–522
17 Shi J., Heegaard C. W., Rasmussen J. T. and Gilbert G. E. (2004) Lactadherin binds selectively to membranes containing phosphatidyl-l-serine and increased curvature. Biochim. Biophys. Acta **1667:** 82–90
18 Gilbert G. E., Shi J., Rasmussen J. T., Heegaard C. W., Zhao J. and Price P. (2003) Limited exposure of phosphatidylserine on stimulated platelets is necessary for procoagulant activity and provides binding sites for lactadherin. J. Thromb. Haemost. **1(S1):** Abstr. P1274
19 Dörmann D., Clemetson K. J. and Kehrel B. E. (2000) The GPIb thrombin-binding site is essential for thrombin-induced platelet procoagulant activity. Blood **96:** 2469–2478
20 Dorsam R. T., Tuluc M. and Kunapuli S. P. (2004) Role of protease-activated and ADP receptor subtypes in thrombin generation on human platelets. J. Thromb. Haemost. **2:** 804–812
21 Williamson P., Bevers E. M., Smeets E. F., Comfurius P., Schlegel R. A. and Zwaal R. F. (1995) Continuous analysis of the mechanism of activated transbilayer lipid movement in platelets. Biochemistry **34:** 10448–10455
22 Tilly R. H., Senden J. M., Comfurius P., Bevers E. M. and Zwaal R. F. (1990) Increased aminophospholipid translocase activity in human platelets during secretion. Biochim. Biophys. Acta **1029:** 188–190
23 Rosing J., Rijn J. L. van, Bevers E. M., Dieijen G. van, Comfurius P. and Zwaal R. F. (1985) The role of activated human platelets in prothrombin and factor X activation. Blood **65:** 319–332
24 Hvarregaard J., Andersen M. H., Berglund L., Rasmussen J. T., Petersen T. E. (1996) Characterization of glycoprotein PAS-6/7 from membranes of bovine milk fat globules. Eur. J. Biochem. **240:** 628–636
25 Heemskerk J. W., Comfurius P., Feijge M. A. and Bevers E. M. (2004) Measurement of the platelet procoagulant response. Methods Mol. Biol. **272:** 135–144

26 Connor J., Pak C. H., Zwaal R. F. and Schroit A. J. (1992) Bidirectional transbilayer movement of phospholipid analogs in human red blood cells: evidence for an ATP-dependent and protein-mediated process. J. Biol. Chem. **267:** 19412–19417

27 Stuart M. C., Bevers E. M., Comfurius P., Zwaal R. F., Reutelingsperger C. P. and Frederik P. M. (1995) Ultrastructural detection of surface exposed phosphatidylserine on activated blood platelets. Thromb. Haemost. **74:** 1145–1151

28 Jong K. de, Geldwerth D. and Kuypers F. A. (1997) Oxidative damage does not alter membrane phospholipid asymmetry in human erythrocytes. Biochemistry **36:** 6768–6776

29 Connor J. and Schroit A. J. (1991) Transbilayer movement of phosphatidylserine in erythrocytes. Inhibitors of aminophospholipid transport block the association of photolabeled lipid to its transporter. Biochim. Biophys. Acta **1066:** 37–42

30 Rijn J. van, Rosing J. and Dieijen G. van (1983) Activity of human blood platelets in prothrombin and in factor X activation induced by ionophore A23187. Eur. J. Biochem. **133:** 1–10

31 London F. S., Marcinkiewicz M. and Walsh P. N. (2004) A subpopulation of platelets responds to thrombin- or SFLLRN-stimulation with binding sites for factor IXa. J. Biol. Chem. **279:** 19854–19859

32 Alberio L., Safa O., Clemetson K. J., Esmon C. T. and Dale G. L. (2000) Surface expression and functional characterization of alpha-granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. Blood **95:** 1694–1702

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

34 Smeets E. F., Heemskerk J. W., Comfurius P., Bevers E. M. and Zwaal R. F. (1993) Thapsigargin amplifies the platelet procoagulant response caused by thrombin. Thromb. Haemost. **70:** 1024–1029

35 Dachary-Prigent J., Pasquet J. M., Freyssinet J. M. and Nurden A. T. (1995) Calcium involvement in aminophospholipid exposure and microparticle formation during platelet activation: a study using Ca^{2+}-ATPase inhibitors. Biochemistry **34:** 11625–11634

36 Bevers E. M., Wiedmer T., Comfurius P., Zhao J., Smeets E. F., Schlegel R. A. et al. (1995) The complex of phosphatidylinositol 4,5-bisphosphate and calcium ions is not responsible for Ca(2+)-induced loss of phospholipid asymmetry in the human erythrocyte: a study in Scott syndrome, a disorder of calcium-induced phospholipid scrambling. Blood **86:** 1983–1991

37 Augereau O., Rossignol R., DeGiorgi F., Mazat J. P., Letellier T. and Dachary-Prigent J. (2004) Apoptotic-like mitochondrial events associated to phosphatidylserine exposure in blood platelets induced by local anaesthetics. Thromb. Haemost. **92:** 104–113

38 Williamson P., Christie A., Kohlin T., Schlegel R. A., Comfurius P., Harmsma M. et al. (2001) Phospholipid scramblase activation pathways in lymphocytes. Biochemistry **40:** 8065–8072

39 Hsu-Lin S., Berman C. L., Furie B. C., August D. and Furie B. (1984) A platelet membrane protein expressed during platelet activation and secretion: studies using a monoclonal antibody specific for thrombin-activated platelets. J. Biol. Chem. **259:** 9121–9126

40 Chap H. J., Zwaal R. F. and Deenen L. L. van (1977) Action of highly purified phospholipases on blood platelets: evidence for an asymmetric distribution of phospholipids in the surface membrane. Biochim. Biophys. Acta **467:** 146–164

To access this journal online:
http://www.birkhauser.ch