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Heterogeneity in microparticle formation and exposure of anionic phospholipids at the plasma membrane of single adherent platelets

Jacob J. Briedé, Johan W.M. Heemskerk, H. Coenraad Hemker, Theo Lindhout *

Departments of Biochemistry and Human Biology, Cardiovascular Research Institute Maastricht, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands

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Abstract

Adherent platelets were examined for their ability to form microvesicles and procoagulant sites for thrombin formation. Epifluorescence and phase-contrast microscopy were employed to visualize shape changes, changes in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$), vesiculation of the plasma membrane and appearance of anionic phospholipids in the outer leaflet of the plasma membrane, as probed by annexin V binding. In the absence of extracellular Ca^{2+} two stable populations of adherent platelets were observed. The majority of the adherent platelets were fully spread and about 10% remained in a non-spread dendritic state. In the presence of extracellular Ca^{2+} vesiculation at the surface of spread platelets occurred at a rather slow rate (10% of the platelets after 20 min) concomitantly with an increase in $[Ca^{2+}]_i$ and binding of annexin V. However, a small fraction of the adherent platelets ($\sim$1%) responded much faster. Ionomycin-enhanced influx of Ca^{2+} in dendritic platelets resulted in a rapid transformation of these platelets into inflated, balloon-shaped, platelets having a diameter of 2.0 ± 0.7 μm without notable microvesicle formation. In contrast, fully spread platelets retained their shape but obtained frayed edges as a result of microvesicle formation. Confocal scanning fluorescence microscopy indicated that annexin V bound to very distinct sites at the outer plasma membrane of spread as well as balloon-shaped platelets. Inhibition of platelet calpain activity suppressed ionomycin-enhanced microvesicle formation and ballooning of platelets, but not annexin V binding. These findings indicate that vesiculation and ballooning, but not the exposure of phosphatidylserine at the outer leaflet of the adherent platelet membrane, are associated with cytoskeleton destruction. Altogether, the data suggest a similar relationship between $[Ca^{2+}]_i$ and the formation of platelet procoagulant sites as reported for platelets in suspension. However, the present investigations on single adherent platelets reveal for the first time that adhesion and spreading of platelets is not necessarily associated with the appearance of procoagulant sites. Secondly, an unexpected diversity was observed among adherent platelets with respect to sensitivity to Ca^{2+}-induced generation of procoagulant sites and Ca^{2+}-induced vesiculation of plasma membrane. It is tempting to speculate that this diversity is of importance for the procoagulant response of platelets to a hemostatic challenge elicited by an injured vessel wall.

Keywords: Platelet adhesion; Platelet morphology; Calcium response; Membrane vesiculation; Anionic phospholipid exposure

1. Introduction

The interaction of platelets with fibrinogen and extracellular matrix proteins, like collagen and fibronectin, is followed by a sequence of responses: (1)

* Corresponding author. Fax: +31-43-388-4159; E-mail: t.lindhout@bioch.unimaas.nl

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platelet shape change, (2) secretion of components from intracellular granula, (3) loss of phospholipid asymmetry in the plasma membrane and (4) membrane vesiculation (reviewed in [1–6]). These responses serve different biological functions: hemostatic plug formation and recruiting other blood cells to sites of injury. Loss of phospholipid asymmetry and vesiculation of the plasma membrane has been studied extensively because of the crucial importance of these processes in maintaining normal hemostasis [7–10].

There is good evidence that agonist-induced elevation of platelet $[Ca^{2+}]_i$ inhibits an ATP-dependent phospholipid pump (translocase) that specifically shuttles aminophospholipids from the outer to the inner leaflet of the cellular plasma membrane. In addition, high $[Ca^{2+}]_i$ activates a non-ATP-dependent phospholipid transporter (scramblase) that rapidly shuttles phospholipids from the inner to the outer leaflet. The net effect of increased $[Ca^{2+}]_i$ is surface exposure of anionic phospholipids like phosphatidylserine (see [11] for a review). Increased $[Ca^{2+}]_i$ also stimulates cytoplasmic calpain activity, which degrades the cytoskeleton [12]. Cytoskeleton degradation is thought to be responsible for microvesicle formation at the plasma membrane and shedding of microparticles that have anionic phospholipids at their surface [13,14].

Most of our understanding of the agonist-mediated generation of procoagulant platelets arises from studies with platelet suspensions. However, it remains to be seen whether platelets form a homogeneous population with respect to sensitivity to agonist-mediated activation. This question can only be answered when single platelets are studied. Studies on adherent single platelets are of special interest because they allow gathering of information about the contribution of outside-in signaling on platelet shape change, release reaction and even the development of procoagulant activity (reviewed in [15]). In this signaling process, the cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$ plays an essential role as a second messenger [16,17].

In this paper we report on the interaction between single platelets and immobilized fibrinogen, one of the most potent platelet adhesive substrates, and the consequences for platelet morphology, $[Ca^{2+}]_i$ and exposure of anionic phospholipids at the outer leaflet of the plasma membrane, utilizing combined video-enhanced epifluorescence and phase-contrast microscopy. Digital image-processing techniques were employed to analyze and quantify the temporal relationship between the different platelet responses upon the addition of agents that control $[Ca^{2+}]_i$ and calpain activity of fibrinogen-adherent platelets.

2. Materials and methods

2.1. Materials

Fura-2 acetoxymethyl ester was purchased from Molecular Probes (Leiden, The Netherlands). Bovine serum albumin (BSA), bovine fibrinogen (fraction I, type IV), econazole nitrate (1-[2-([4-chlorophenyl]methoxy)-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole nitrate), 1,4-diazobicyclo[2.2.2]octane (DABCO), apyrase and ionomycin were from Sigma (St. Louis, USA). Calpain inhibitor (MDL-28170) carbamic acid, [1-(((1-formyl-2-phenylmethyl)amino)carboxyl)-2-methylpropyl]-phenylmethyl ester was from Meryll Dow (Cincinnati, OH, USA). Oregon Green 488-labeled annexin V (OG-annexin V) was a product of NeXins Research (Hoeven, The Netherlands).

2.2. Loading of platelets with fura-2 and MDL-28170

Suspensions of washed platelets were prepared as previously described [18]. Briefly, blood was drawn from healthy donors, who had not taken any antiplatelet medication in the preceding 2 weeks. Platelet-rich plasma, prepared by centrifugation, was incubated with 3 μmol/l fura-2 acetoxymethyl ester or 200 μmol/l MDL-28170 at 37°C for 1 h in the presence of apyrase (0.1 U/ml). The platelets were then sedimented by centrifugation, washed twice with Hepes buffer composed of 10 mmol/l Hepes, 136 mmol/l NaCl, 5 mmol/l glucose, 2.7 mmol/l KCl, 2 mmol/l $MgCl_2$, BSA (0.5 mg/ml) and apyrase (0.1 U/ml), pH 6.6. Finally, the platelets were resuspended in Hepes buffer of the same composition, but adjusted to pH 7.45. Platelets were counted on a Coulter counter (Coulter, Miami, USA) and the suspensions were adjusted to 5×10^7 platelets/ml.

2.3. Preparation of fibrinogen-coated coverslips

Fibrinogen-coated glass coverslips were prepared as previously described [19]. Briefly, cleaned glass coverslips (diameter 22 mm) were exposed for 1 h at room temperature to a solution of 10 mg/ml fibrinogen in 40 mmol/l phosphate buffer, 150 mmol/l NaCl, pH 7.4. The coverslips were rinsed with Hepes buffer (pH 7.45) and mounted in an incubation chamber made from Teflon (height 7.5 mm, inner diameter 19.5 mm).

2.4. Platelet adhesion to immobilized fibrinogen

The incubation chamber was filled with a suspension of washed platelets (300 μl, 1.5×10^7 platelets) and incubated at room temperature for 40 min. After incubation, non-attached platelets were removed by rinsing the chamber 5 times with 250 μl Hepes buffer, pH 7.45.

2.5. Phase-contrast imaging

Positive phase-contrast images were obtained with an inverted microscope (Nikon Diaphot 200; Nikon, Tokyo, Japan) using a Nikon 100×quartz oil-immersion objective containing a phase plate. The phase-contrast transmission images were recorded with an infrared camera (VPM 6132 monochrome high-resolution charge-coupled device camera, Vista, Norbain, UK). Quanticell 500 software (Applied Imaging, Newcastle, UK) was used for analyzing the phase-contrast images.

2.6. Fluorescence imaging

For fluorescence imaging, light from a xenon lamp passed a computer-controlled excitation and neutral density filter wheel, and reached the objective of the inverted microscope through an UV-transparent liquid light guide and a dichroic long-pass filter. The emission light passed a computer-controlled emission filter wheel, and finally reached a low-light level intensified, charge-coupled device camera working at standard video rate (Photonic Sciences, Robertsbridge, UK). Fura-2 signals were detected with alternating 340-nm and 380-nm excitation filters (bandpasses of 15 nm), a 400 nm dichroic mirror, and 510 nm emission filter (bandpass of 40 nm). Oregon Green 488 fluorescence was observed with a 485-nm excitation filter (bandpass of 40 nm), a 505-nm dichroic long-pass filter, and a 530-nm emission filter (bandpass of 30 nm). Oregon Green and fura-2 fluorescence images were digitized and averaged; background images were subtracted and stored. There was no interference between the fura-2 and the Oregon Green 488 fluorescence signals. The fluorescence images were off-line analyzed using Quanticell 700 software. The calibration of fluorescence ratio to molar concentrations of intracellular Ca^{2+} was according to Grynkiewicz et al. [20].

2.7. Confocal scanning laser microscopy of adherent platelets

Washed platelets were allowed to adhere at room temperature to fibrinogen, immobilized to a glass coverslip, during 40 min. Non-adhered platelets were removed by rinsing with Hepes buffer, pH 7.45. Then adherent platelets were incubated with 5 μmol/l ionomycin, directly followed by 3 mmol/l $CaCl_2$ and 1 μg/ml OG-annexin V. After 10 min, Hepes buffer pH 7.45 was replaced by Hepes buffer pH 8.0, containing 3 mmol/l $CaCl_2$ and 2% (w/v) DABCO. The fibrinogen-coated glass coverslip with adhered platelets was covered with a concave glass coverslip and sealed. Oregon Green 488 fluorescent platelets were visualized using a MRC 600 confocal scanning laser device (Bio-Rad, Hemel Hempstead, UK) mounted onto an Axiophot microscope (Zeiss, Oberkochen, Germany) with a Nikon 63×quartz oil-immersion objective. Z-Scans with steps of 0.5 μm were made. Every scan was 6-times averaged using Kalman filtering [21].

3. Results

3.1. Platelet responses following adhesion to immobilized fibrinogen in the absence of extracellular Ca^{2+}

Platelets that adhere to immobilized fibrinogen in the absence of extracellular Ca^{2+} first developed pseudopodia and within 40 min 90% of the platelets were fully spread (platelets 1, 2 and 3 in Fig. 1A).

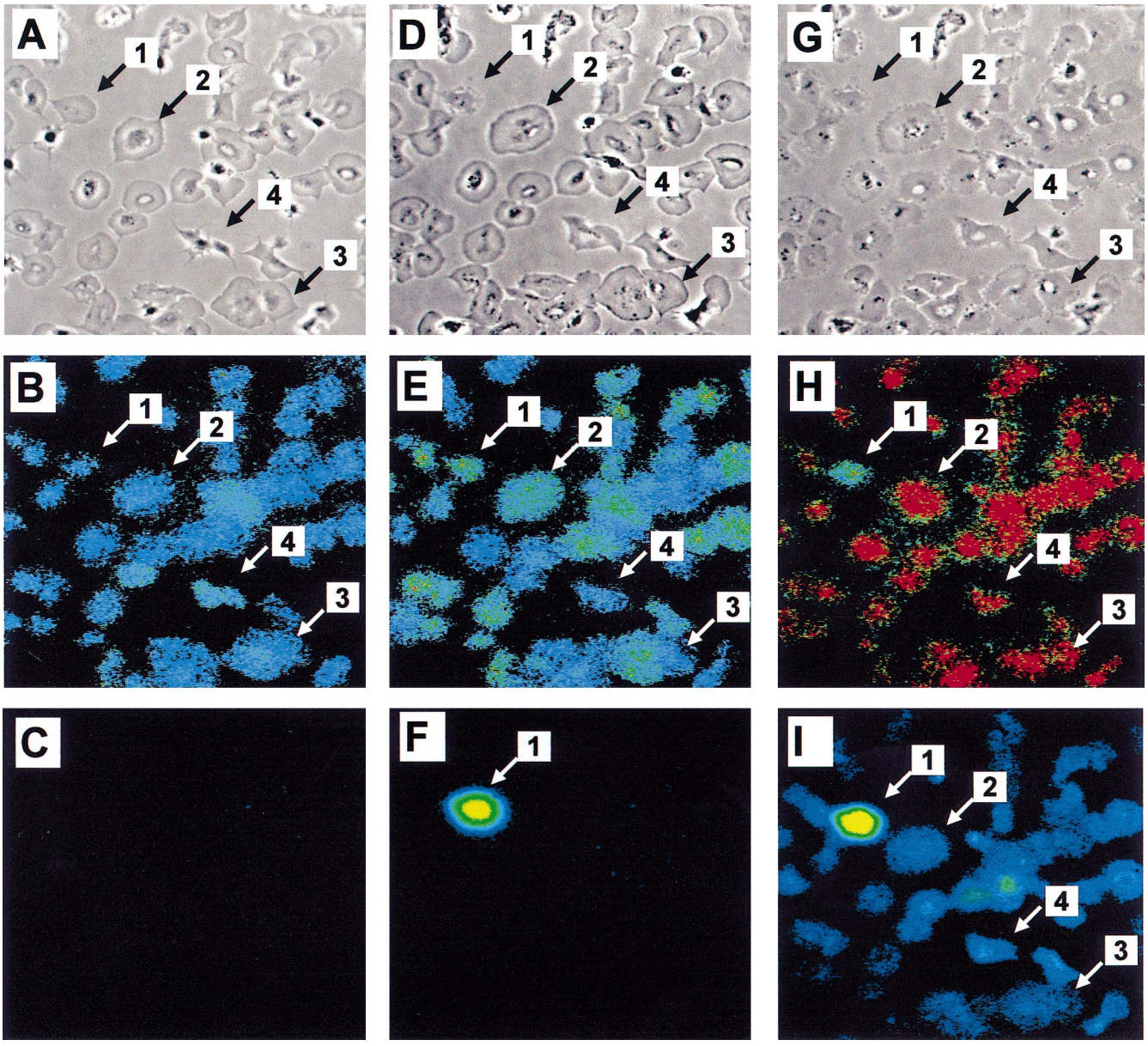

Fig. 1. Effect of extracellular Ca^{2+} on morphology, $[Ca^{2+}]_i$ and annexin V binding of adherent platelets. Platelets loaded with fura-2 were exposed to immobilized fibrinogen for 40 min. The first row of images show phase-contrast images of the adherent platelets in absence of extracellular Ca^{2+} (A), followed by a 10-min incubation with 3 mmol/l $CaCl_2$ and 1 μg/ml Oregon Green 488-labeled annexin V (D) and a 5-min incubation with 5 μmol/l ionomycin (G). The second row are the fura-2 fluorescence ratio images and the third row represents the OG-annexin V fluorescence images of the corresponding microscopic fields shown in A, D and G, respectively. Further experimental details are given in Section 2.

The other 10% of the platelets remained in a non-spread dendritic state (platelet 4 in Fig. 1A). Prolongation of the incubation time did not alter the ratio of fully spread over dendritic platelets. The findings as shown in Fig. 1A are representative for all experiments performed under these conditions and thus donor-independent. In spite of the dramatic shape change of the platelets, from spherical non-adhering to a fully spread state, the intracellular free calcium concentration, $[Ca^{2+}]_i$, remained low as indicated by the blue/green pseudocolor of the fura-2 fluorescence ratio image (Fig. 1B). Continuous monitoring of the

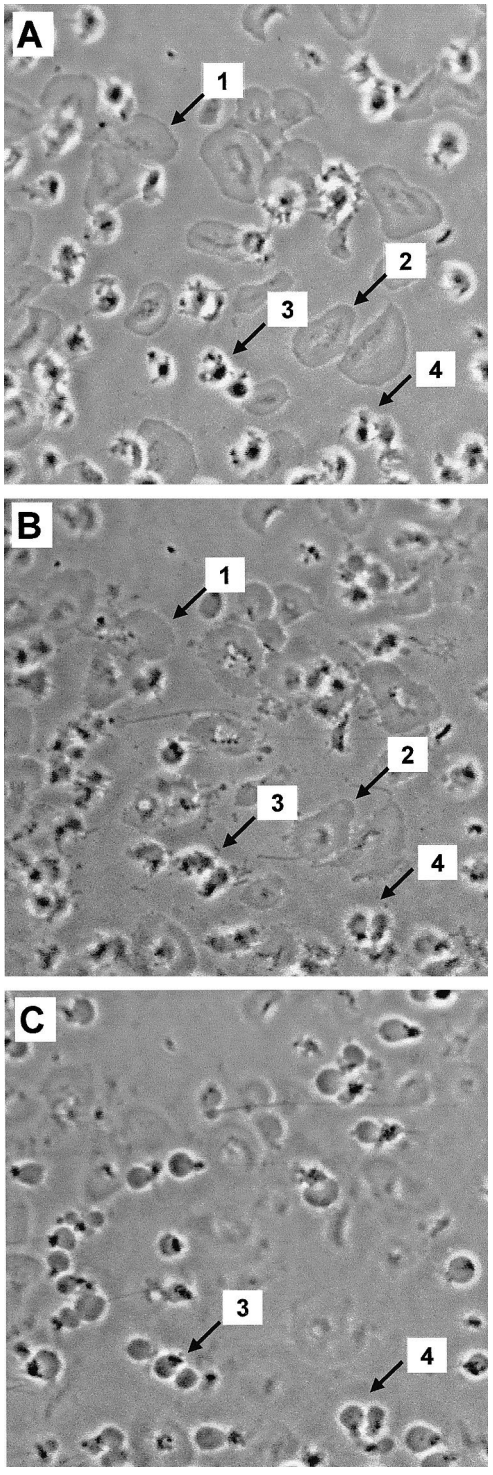

Fig. 2. Ionomycin-induced morphological changes in dendritic and fully spread platelets. Platelets adhering to immobilized fibrinogen were incubated with Hepes buffer (pH 7.45) containing 3 mmol/l $CaCl_2$ and 5 μmol/l ionomycin. Phase-contrast images show: one particular microscopic field prior to the addition of $CaCl_2$ and ionomycin (A), 5 min after the addition of $CaCl_2$ and ionomycin (B) and focused 1–2 μm above surface of the coverslip (C).
←

fura-2 signal showed that a vast majority of the platelets had an invariable $[Ca^{2+}]_i$ of about 50 nmol/l. Some platelets (less than 10% and irrespective of their morphology) showed small, irregular oscillations in $[Ca^{2+}]_i$ between 30 and 100 nmol/l. Together, these data indicate that when no extracellular Ca^{2+} is present two adherent platelet populations can be identified: dendritic non-spread platelets and fully spread platelets. In spite of a different morphology, both stable populations have similar low $[Ca^{2+}]_i$. Thus, if platelet shape change is triggered by the interaction of platelet receptors (integrin $\alpha_{IIb}\beta_3$) with immobilized fibrinogen, it seems not to be related to calcium signaling.

3.2. *Responses of adherent platelets to increased extracellular Ca^{2+} concentration*

Adherent platelets from the previous experiment (Fig. 1A) were incubated for 10 min with 3 mmol/l $CaCl_2$. The averaged intracellular calcium levels of the adherent platelets increased from 50 to 70 nmol/l over a time period of 10 min. It is clearly seen that following the addition of extracellular Ca^{2+} the dendritic non-spread platelets did spread to some extent (Fig. 1D, platelet 4). However, $[Ca^{2+}]_i$ of these platelets did not change, indicating that spreading of these platelets is not signaled by an increased $[Ca^{2+}]_i$.

When besides extracellular calcium OG-annexin V (1 μg/ml) was also added, one platelet in the microscopic field under examination bound annexin V (Fig. 1F, platelet 1). Close inspection of the corresponding phase-contrast image of this platelet (Fig. 1D, platelet 1) revealed that this particular platelet showed frayed edges, indicating vesiculation of the plasma membrane. Inspection of other microscopic fields for OG-annexin V fluorescence revealed that about 1% of the adherent platelets bound annexin V, showed frayed edges and had somewhat elevated $[Ca^{2+}]_i$ of about 150 nmol/l. On prolonged incubation with 3 mmol/l $CaCl_2$ an increasing number of OG-annexin V-binding platelets were seen. After 30

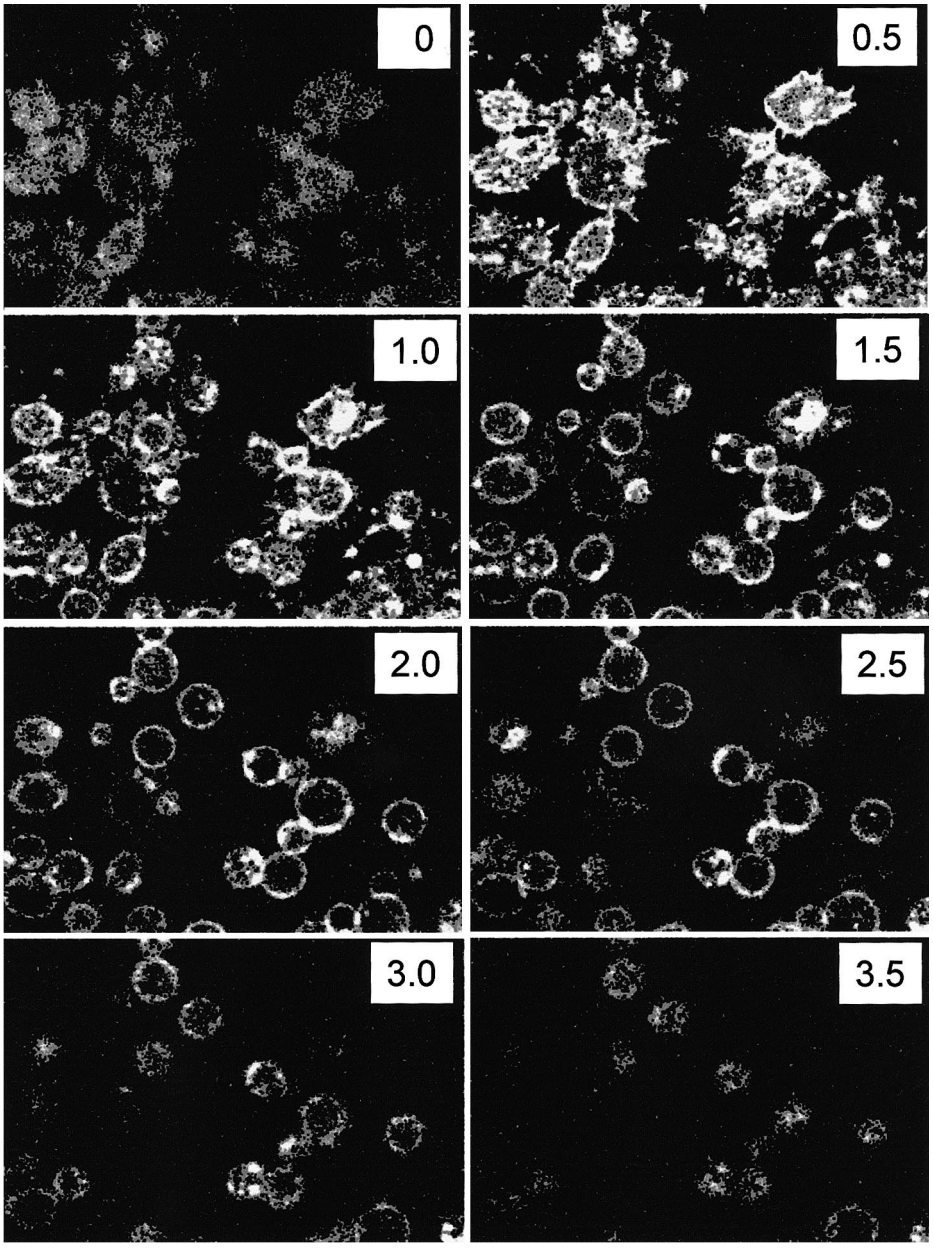

Fig. 3. Confocal laser microscopy of ionomycin-activated platelets. Platelets were exposed to immobilized fibrinogen for 40 min and incubated for 10 min with Hepes buffer (pH 7.45) containing 3 mmol/l $CaCl_2$, 5 μmol/l ionomycin and 1 μg/ml Oregon Green 488-labeled annexin V. Confocal scanning laser microscopy was performed with steps of 0.5 μm. The images show OG-annexin V fluorescence at the indicated distance from the surface (in μm) of the coverslip.

min about 10% of all adherent platelets were stained with OG-annexin V. Annexin V binding was preceded by a rapid increase of $[Ca^{2+}]_i$ from 70 to 150 nmol/l. Non-annexin V binding platelets had $[Ca^{2+}]_i$ of about 70 nmol/l. Thus, whereas spreading of platelets appeared not to be signaled by cytosolic calcium, vesiculation and loss of phospholipid asymmetry can be associated with elevated $[Ca^{2+}]_i$ of adherent platelets. To see whether vesiculation of the membrane and/or exposure of PS could be induced in all adherent platelets, the Ca^{2+} ionophore ionomycin (5 μmol/l) was used to increase the rate of inflow of extracellular Ca^{2+}. Within 5 min after the addition of ionomycin, all spread platelets showed frayed edges

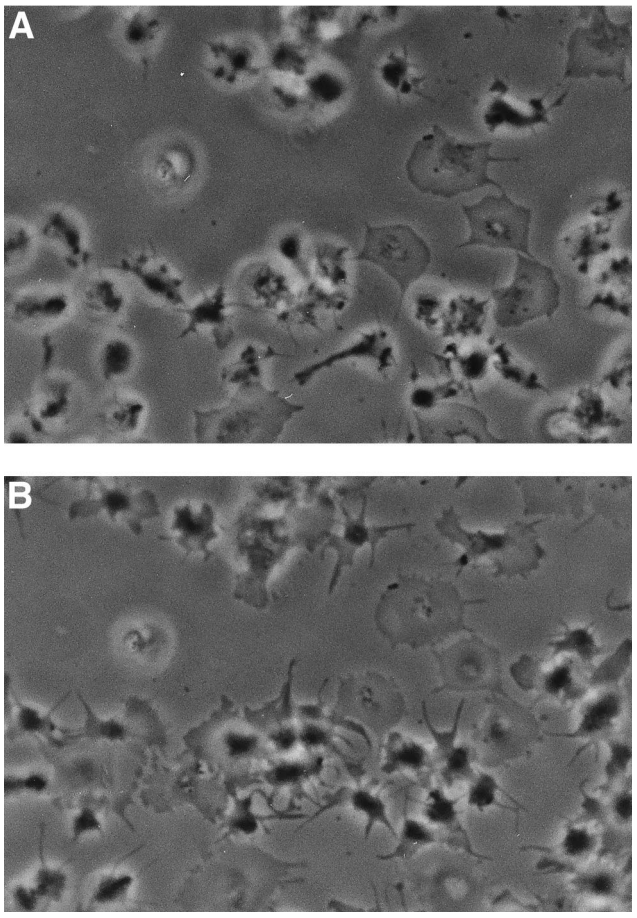

Fig. 4. Effect of calpain inhibitor on microvesicle formation of adherent platelets. Adherent platelets loaded with the calpain inhibitor MDL-28170 were exposed to 3 mmol/l $CaCl_2$ and 5 μmol/l ionomycin. The phase-contrast images show: (A) adherent platelets prior the addition of $CaCl_2$ and ionomycin; (B) the same microscopic field 5 min after the addition of $CaCl_2$ and ionomycin.

(Fig. 1G) and bound annexin V (Fig. 1I). The $[Ca^{2+}]_i$ of all cells was highly increased as indicated by the red pseudo color in the fura-2 fluorescence ratio image (Fig. 1H) and was found to vary around 700 nmol/l.

The question can be raised whether the OG-annexin V-stained platelets, as platelet 1 in Fig. 1F, had already exposed anionic phospholipid before the addition of extracellular Ca^{2+}. Because binding of annexin V requires Ca^{2+}, we addressed this question by utilizing econazole, a drug that has been shown to block influx of extracellular Ca^{2+} [22]. After an incubation of 20 min with 10 μmol/l econazole, we found that all adherent platelets showed constant low $[Ca^{2+}]_i$ of about 75 nmol/l and none of the adherent platelets showed binding of annexin V, indicating that influx of extracellular Ca^{2+} is essential to make that some platelets (less than 1% of all adherent cells) rapidly loose their phospholipid asymmetry. That the econazole-treated platelets could react on an influx of extracellular Ca^{2+} was confirmed by the addition of the Ca^{2+} ionophore ionomycin (5 μmol/l) which resulted in high and constant elevated $[Ca^{2+}]_i$ of 700 nmol/l, fraying of the plasma membrane and binding of OG-annexin V to all platelets within 5 min.

3.3. Vesiculation of the plasma membrane of adherent platelets

So far the observations on vesiculation of the plasma membrane of adherent platelets were made with fully spread and spread-dendritic platelets. Next, we investigated the ability of non-spread dendritic platelets to form microparticles. To this end $CaCl_2$ (3 mmol/l) and ionomycin (5 μmol/l) were added simultaneously to platelets that were adhered to immobilized fibrinogen in the absence of extracellular Ca^{2+}. Phase-contrast microscopy showed that fully spread platelets (like platelets 1 and 2 in Fig. 2A) retained this shape but with frayed edges (Fig. 2B, platelets 1 and 2). The non-spread dendritic platelets (like platelets 3 and 4 in Fig. 2A) immediately changed into balloon-shaped structures (Fig. 2B, platelets 3 and 4). The balloon-like shape of these platelets became evident when focused 1–2 μm above the glass coverslip with immobilized fibrinogen (Fig. 2C).

To confirm the balloon-like shape and to find out how the anionic phospholipids are distributed in the outer leaflet of the plasma membrane, confocal scanning laser microscopy of adherent platelets, exposed to 3 mmol/l $CaCl_2$ and 5 μmol/l ionomycin, was performed in the presence of OG-annexin V (Fig. 3). Scans were started at the surface of the coverslip with steps of 0.5 μm. The first 1.5 μm above the glass coverslip surface showed OG-annexin V fluorescence associated with spread platelets forming microvesicles at their plasma membranes. The next 2 μm showed circular patterns of fluorescence with some heavily stained spots. Fig. 3 indicates two interesting features. Firstly, dendritic, non-spread platelets were indeed transformed to inflated balloons with an esti-

mated diameter of 2.0 ± 0.7 μm. Secondly, the spot-like distribution of annexin V binding sites indicates a heterogeneous distribution of the anionic phospholipids in the plasma membrane.

Investigations with fura-2-loaded platelets showed that when balloon-shaped platelets were treated with ionomycin the $[Ca^{2+}]_i$ rapidly increased to values around 700 nmol/l. Because intracellular calpain is activated by high $[Ca^{2+}]_i$ it is feasible that the transition of dendritic-shaped platelets into inflated balloon-like, without notable spreading, might be the result of a rapid destruction of the cytoskeleton. Strong support for this notion was obtained from experiments with platelets loaded with a calpain inhibitor, MDL-28170. In the presence of MDL-28170 a rapid influx of extracellular Ca^{2+} did not result in the appearance of balloon-shaped platelets, but the hyaloplasma did spread between pseudopodia of the dendritic platelets (Fig. 4). In addition, MDL-28170 also blocked vesiculation of the plasma membrane of the spread platelets. The same experiments with fura-2- and MDL-28170-loaded platelets showed that MDL-28170 did not affect the increase in $[Ca^{2+}]_i$ when these platelets were incubated with ionomycin. Also, the annexin V binding of MDL-28170-loaded platelets after stimulation with ionomycin was not different from those not loaded with the calpain inhibitor (data not shown). Thus, as has been reported for platelets in suspension [8], loss in phospholipid asymmetry is not necessarily associated with vesiculation of the plasma membrane of adherent platelets.

4. Discussion

In the absence of extracellular Ca^{2+} the majority of washed platelets readily spread on a surface coated with immobilized fibrinogen once they adhered to the surface and had formed pseudopodia. Some platelets (about 10% of those that adhered), however, did not spread but kept a dendritic shape. All adhesion experiments under these conditions showed this phenomenon, suggesting that the existence of two classes of adhering platelets is not donor dependent. According to the fura-2 measurements no striking differences were found in the intracellular calcium concentration ($[Ca^{2+}]_i$) between the two platelet populations. As a matter of fact, a low $[Ca^{2+}]_i$ of about 50 nmol/l was found in all adherent platelets. Surprisingly, addition of extracellular Ca^{2+} initiated the spreading of the dendritic platelets, but was not accompanied with a significant increase in $[Ca^{2+}]_i$. The finding that spreading of platelets does not require an elevated $[Ca^{2+}]_i$ is in agreement with earlier reports [18,23,24], but are in contrast with work reported by Ikeda et al. [25]. These authors found that $[Ca^{2+}]_i$ increased when platelets adhered to immobilized fibrinogen and concluded that the increased $[Ca^{2+}]_i$ plays a key role in achieving morphological changes of platelets that adhere to immobilized fibrinogen.

The dendritic and spread platelets were further analyzed for a (partially) loss of their asymmetric distribution of plasma membrane phospholipids by examining their annexin V binding properties after the addition of 3 mmol/l $CaCl_2$. This concentration was chosen because the binding of annexin V requires 2–3 mmol/l Ca^{2+} [26]. None of the adherent platelets that were incubated with extracellular calcium and econazole, a calcium channel blocker [22], bound annexin V. This clearly indicates that the outer leaflet of the plasma membrane of adherent platelets, either non-spread dendritic or fully spread, does not contain the anionic phospholipid, phosphatidylserine. On prolonged incubation with extracellular Ca^{2+}, in the absence of econazole, an increasing number of adherent platelets showed increased $[Ca^{2+}]_i$ (about 150 nmol/l), vesiculation of the plasma membrane and could be stained with annexin V. It is apparent, as reported for platelets in suspension, that elevated $[Ca^{2+}]_i$ evokes dramatic shape changes of the plasma membrane and the loss of phospholipid asymmetry [14]. It is of interest to note that we repeatedly observed that 1% of the adherent platelets responded within a few minutes to addition of extracellular calcium with vesiculation of the membrane and annexin V binding. These few platelets may be of importance for a rapid onset of thrombin generation because membranes with exposed anionic phospholipids may serve as sites for the assembly of the prothrombin and factor X converting enzyme complexes (see [27] for a review). Traces of thrombin thus formed are assumed to stimulate the generation of additional procoagulant sites, which in turn enables an explosive thrombin generation.

Because of the different susceptibilities platelets

may have in responding to agonists, like extracellular Ca^{2+} and thrombin, we addressed the question whether the two platelet populations with different spreading abilities respond differently in surface exposure of procoagulant phospholipid. This work confirms that the formation of procoagulant sites at the platelet surface is signaled by $[Ca^{2+}]_i$ (reviewed in [11]). Changes in $[Ca^{2+}]_i$ in its turn is induced by a variety of physiological important agonists, e.g., thrombin. Thrombin induces a repetitive spiking of $[Ca^{2+}]_i$ in individual fibrinogen-adherent platelets [18], so in this study we used the calcium ionophore, ionomycin, for a constant influx of extracellular Ca^{2+}. As expected, the constant high $[Ca^{2+}]_i$ was associated with the appearance of binding sites for annexin V. We further confirmed that vesiculation of the membrane of spread platelets, but not binding of annexin V, could be prevented when platelets were loaded with the membrane permeable calpain inhibitor MDL-28170. These observations are compatible with the notion that Ca^{2+}-dependent activation of calpain results in destruction of cytoskeleton and hence microvesicle formation [14,28]. However, what remained unnoticed in other studies was the finding that a rapid increase of $[Ca^{2+}]_i$ of dendritic platelets did not result in spreading of these platelets as was observed under conditions of a slowly increasing $[Ca^{2+}]_i$, nor in vesiculation of the membrane, but was associated with a rapid transformation into inflated balloon-like platelets with diameter of 2.0 ± 0.7 μm. It is tempting to speculate that the lesser extent of integrin $\alpha_{IIb}\beta_3$-mediated interaction of the dendritic platelets with the fibrinogen-coated surface allows after destruction of the cytoskeleton the formation of a balloon-shaped platelet rather than the appearance of small microvesicles at the plasma membrane. Indeed, when adherent dendritic platelets loaded with the calpain inhibitor MDL-28170 were incubated with ionomycin and extracellular Ca^{2+} these platelets spread and were not transformed in a balloon-like shape.

Confocal microscopy demonstrated that the annexin V binding sites, and thus the procoagulant sites, were not uniformly distributed over the plasma membrane of adherent platelets. The confocal scans revealed a few intensively stained spots at the surface of the balloon-shaped platelets, whereas for spread platelets the fluorescence seemed to be associated with the many sites where microvesicles were formed.

In summary, this study utilizing washed platelets and immobilized fibrinogen demonstrated an unexpected diversity in the rate of appearance of procoagulant sites on adherent platelets, a difference in the ability of these platelets to form microparticles and a very heterogeneous distribution of procoagulant sites at the plasma membrane of single surface-bound platelets. The consequences of this diversity for the initiation and propagation of thrombin formation are the subject of further investigations.

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