Changes in Cytosolic Calcium Concentrations and Cell Morphology in Single Platelets Adhered to Fibrinogen-Coated Surface Under Flow

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Changes in intracellular calcium concentration \([\text{Ca}^{2+}]_i\) of fura-2-loaded human platelet during its adhesion to a fibrinogen-coated surface were studied, using a flow chamber mounted on an epifluorescence microscope equipped with digital-ratio imaging. Adherent platelets were individually mapped under a scanning electron microscope to establish the possible correlation between adhesion-associated shape alterations and \([\text{Ca}^{2+}]_i\) changes. We found that 1) there was no immediate \([\text{Ca}^{2+}]_i\) elevation on platelet adhesion; 2) \([\text{Ca}^{2+}]_i\) increased in most adherent platelets with a lag time ranging 10 to 200 s, averaging about 1 minute; 3) the pattern of \([\text{Ca}^{2+}]_i\) changes varied drastically among individual adherent platelets; 4) the degree of \([\text{Ca}^{2+}]_i\) elevation appeared to correlate with the extent of morphological change, with the vast majority (>90%) of spread platelets showed detectable \([\text{Ca}^{2+}]_i\) changes; 5) neither morphological nor \([\text{Ca}^{2+}]_i\) changes correlated with the lag time; 6) platelets treated with dimethyl-BAPTA (15 μmol/L) underwent normal shape change without \([\text{Ca}^{2+}]_i\) elevation; 7) cytochalasin D (10 μmol/L) inhibited both shape change and \([\text{Ca}^{2+}]_i\) elevation; 8) colchicine (1 mmol/L) was ineffective in both regards. We conclude that although platelet adhesion-associated shape changes may be accompanied with heterogeneous \([\text{Ca}^{2+}]_i\) changes that are microfilament-dependent, \([\text{Ca}^{2+}]_i\) changes do not happen immediately after platelet-surface contact and they are not required for adherent platelets to undergo postcontact morphological changes.

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RESTING PLATELETS are smooth discs with intracellular free calcium concentration, \([\text{Ca}^{2+}]_i\), normally close to or less than 100 nmol/L. On activation at sites of vascular injury they undergo rapid activation, becoming spherical, extending pseudopods and spreading, and recruiting other incoming platelets from the blood stream to form a hemostatic plug at the injured site. Many of these activation-associated processes, such as shape change, aggregation and release reaction, can be mimicked by the addition of agonists to a platelet suspension. From such studies, \([\text{Ca}^{2+}]_i\) is believed to serve as an important second messenger in agonist-induced platelet activation processes.²,³

However, the very first step of platelet activation during hemostasis, i.e., platelet adhesion, has not been extensively studied, perhaps because of difficulties in research technique. Platelet adhesion in vitro is a rapid and dynamic process, as evidenced by our blood flow studies using fluorescence video microscopy.³,⁴ Some adherent platelets on the protein-coated surfaces relocate or detach under arterial blood flow conditions, whereas others remain at the same location almost permanently. In the absence of any exogenous agonist, these adherent platelets can undergo extensive shape changes as those occurring in vivo. Adherent platelets are nonoverlapping on fibrinogen-coated surfaces, but they rapidly form large clumps on a collagen-coated surface. Whether adhesion of platelets triggers or is accompanied with \([\text{Ca}^{2+}]_i\) changes is basically unknown. In this study, we used a flow chamber mounted on an epifluorescence microscope equipped with a digital ratio-imaging setup to examine the \([\text{Ca}^{2+}]_i\) changes during the adhesion of fura-2-loaded platelets on the fibrinogen-coated surface. Moreover, adherent platelets were individually identified under a scanning electron microscope to establish the relationship between adhesion-associated shape changes and \([\text{Ca}^{2+}]_i\) changes. To detect possible rapid changes of \([\text{Ca}^{2+}]_i\) at the moment of platelet-surface contact, some flow experiments were also monitored through a fluorescence video microscope attached to a dual-wavelength spectrofluorimeter.

MATERIALS AND METHODS

Materials. Fura-2/AM and dimethyl-BAPTA/AM were purchased from Molecular Probes (Eugene, OR); oryaprase (grade V), bovine albumin, colchicine, and cytochalasin D were from Sigma (St Louis, MO); human fibrinogen was from Kabi (Stockholm, Sweden).

Calpeptin, a membrane permeable calpain inhibitor,¹ was a generous gift from Dr Jun-ichi Kambayashi (Osaka University, Japan).

Platelet preparation and fluorescence labeling. Blood was drawn from healthy donors who had not taken any medication for 2 weeks before blood collection. One-tenth volume of sodium citrate (0.12 mol/L, pH6.0) was added to 1 volume of blood to serve as the anticoagulant. Platelet-rich-plasma (PRP) was prepared by centrifugation at 180g for 10 minutes. Platelets were washed once by a further centrifugation (700g, 10 minutes) onto a 40% albumin cushion and resuspended in HEPEs buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L MgCl₂, 3 mmol/L NaH₂PO₄, 5.5 mmol/L glucose, 0.35% albumin, 2.5 mmol/L N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid, pH 7.2) that contained 3 U/mL apyrase (about 1 U/mL adenosine diphosphatase [ADPase]) and 4 μmol/L fura-2 acetoxymethyl ester. The labeling process was performed by incubating platelets with the fluorescent dye for 45 minutes at 37°C. After labeling, the platelets were centrifuged again on the albumin cushion and resuspended in HEPEs buffer. The remaining blood sample was clotted by recalcification (10 mmol/L CaCl₂). After the clot was removed, the red cells were washed four times in HEPEs buffer. Appropriate amounts of labeled platelets and washed erythrocytes were mixed together to form a suspension containing 1 × 10⁹ platelets/mL and 35% hematocrit.

Flow experiments. A device consisting of a parallel-plate flow chamber mounted on an epifluorescence microscope was used to allow direct visualization of platelet adhesion under blood flow conditions.² The dimension of the chamber cavity was 32.5 x 12.5 x 0.13 mm³ with a standard coverglass (0.16 mm thickness) as the light transmitting surface. At the beginning of an experiment, the coverglass was coated with a droplet of fibrinogen (1 mg/mL) and

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Fig 1. Fluorescence ratio images of platelets adhering under static conditions. Fura-2 labeled platelets were settling on the fibrinogen-coated coverglass and their fluorescence intensity ratios between 340 nm and 380 nm were processed by digital imaging analysis as described in Materials and Methods. The pseudocolor scale covered the fluorescence ratio range from 0.5 to 5.5. These four pictures were sequentially taken with 15 seconds apart from one to the next.

Fig 2. Fluorescence ratio changes of platelets adhered under flowing conditions. A mixed suspension of red cells and fura-2 labeled platelets was drawn through a flow chamber with platelets adhering to fibrinogen-coated surface under flow condition as described in Materials and Methods. The top panel represents a fluorescence ratio image taken at 4 minutes. The kinetics of fluorescence ratio changes in several individual platelets are shown in the bottom panel. Arrows indicate the moments of arrival for each marked adherent platelet.
up to automatically control the excitation filter wheel that alternated between two filters of 340 nm and 380 nm. The fluorescent emission light that passed through a 440-nm dichroic mirror (long-pass filter) and a 510-nm band-pass filter was finally collected on a low light level camera (C2400-08; Hamamatsu). The images were registered at 4-second intervals with every 16 frames averaged for readings at 340 nm and 380 nm; this left about 2.5 seconds in the dark to minimize photobleaching of the specimen. In certain experiments, the images were acquired at 2-second intervals to improve the temporal resolution. After the dark current image (16-frame average) had been corrected, these fluorescent images (256 × 256 pixels) were stored and processed off-line for 340/380 ratio images. Geometric regions matching individual platelets were analyzed for temporal changes in fluorescence intensity ratio. Because of the minute amounts of fura-2 in each cell and the inevitable quenching of signal, the calibration of fluorescence ratios to standardized [Ca^{2+}] was unsuccessful. Results are, therefore, represented as fluorescence ratio changes instead of absolute [Ca^{2+}] values. 

Fluorimetric ratio measurements. To gain access to the early kinetics of fluorescence ratio changes during platelet adhesion, the microscope was attached to a dual-wavelength spectrofluorimeter (CM11T11; Spex, Edison, NJ). The spectrofluorimeter served as both the light source and the signal processor. The excitation light was switching between 340 nm and 380 nm at 400 Hz by a beam chopper. The emission signal was monitored at 510 nm through a photomultiplier and was synchronized with a microprocessor-operated data acquisition system. An aperture was installed in the optical path to ensure that the signal was only collected from an area of about 50 μm² on the fibrinogen-coated surface. This area size was selected because it gave a reasonable possibility of spotting one single adherent platelet within the experimental time period of a few minutes and a satisfactory signal-to-background noise ratio. The video signal of the platelet adhesion process was simultaneously monitored through a silicon-intensified camera connected in parallel to the photomultiplier.

RESULTS

As a preliminary experiment, we used the digital ratio imaging setup to monitor platelet adhesion under static conditions, ie, fura-2 labeled platelets were allowed to settle on the fibrinogen-coated surface under gravity. Individual platelets close to the surface could be identified from ratio images (Fig 1). As individual platelets approached the surface, they first appeared as faint blue (indicating low [Ca^{2+}]) objects against mix-colored noisy background. Although their exact time for surface contact could not be determined precisely, the boundary of these blue objects became sharper gradually and remained unchanged later on, indicating their settling on surface. Not every platelet behaved similarly in terms of its postcontact fluorescence ratio changes. Most of these adherent platelets showed fluorescence ratio changes that could last several minutes, but the onset of these changes seemed to vary considerably among individual platelets.

When a cell suspension containing red cells and fluorescence-labeled platelets was drawn through the flow chamber, scattered bright objects representing adherent platelets suddenly appeared in the prefocused field and gradually accumulated. Although platelets flowing by were also fluorescently labeled, they only contributed to the background of the frame because 1) they were out of focus under the 100 × objective lens, and 2) they did not stay in the same location long enough to form clear images. Therefore, we could accurately monitor the events of platelet adhesion, not only its onset and pattern of fluorescence ratio changes, but also the precise moment of surface contact (Fig 2). From detailed analysis of such results, most of the observations from static platelet adhesion experiments were confirmed: (1) platelet arrived at the fibrinogen-coated surface with low [Ca^{2+}]; (2) there seemed to be a lag time, ranging from a few seconds

![Fig 3. The correlation between fluorescence ratio change and lag time of individual adherent platelets.](image)

![Fig 4. The fluorescence intensity changes for a single adherent platelet as recorded by fluorimetric measurements.](image)
Fig 6. The correlation between fluorescence ratio change and morphology of individual adherent platelets. The morphology of adherent platelets was classified as following (please see ref 4 for detailed morphology): type 0 represents round cells; type P1 represents cells with fewer than 4 pseudopods; type P2 represents cells with 5 or more pseudopods; type PS represents cells with pseudopods and with web-like mildly spread structure; type SI represents moderately spread cells; type S2 represents severely spread cells. Fluorescence ratio change patterns were classified as in Fig 3. Results were taken from six experiments.

The above-mentioned fluorescence microscopic studies provided details of platelet adhesion records along with [Ca2+]i change patterns in the single platelet level. However, it offers little information regarding the detailed morphology of the cell. Scanning electron microscopy (SEM) was then employed to study the morphological changes of adherent platelets. We combined these two approaches by mapping fluorescence images with corresponding SEM pictures taken from the end-point sample of the same flow experiment. The vast majority of adherent platelets could withstand this additional sample handling procedure and be individually identified from the corresponding SEM picture (Fig 5). Remaining red cells were occasionally found in SEM pictures, but being unlabeled would not appear in fluorescence images. SEM pictures, especially at higher magnifications, clearly showed postcontact morphological changes of adherent platelets.

Fig 7. The correlation between morphology and [Ca2+]i elevation time intervals of individual adherent platelets. The platelet morphology was classified as in Fig 6. The lag time was defined as in Fig 3. The activation time was defined as the time interval between the onset of fluorescence ratio change and the arrival of glutaraldehyde containing buffer at the testing site. Results were taken from six experiments.

Because the temporal resolution for digital ratio imaging system operated at low light levels was at best 1 or 2 seconds, we decided to use the dual-wavelength spectrofluorimeter, which had a temporal resolution better than 0.1 seconds, to see if a subsecond transient [Ca2+]i elevation existed upon platelet contact on the fibrinogen-coated surface. Figure 4 shows a typical curve from this type of high-resolution kinetic study on a single-platelet adhesion event. At the moment when a platelet arrived, both fluorescence intensity curves jumped up together, while there was a concomitant reduction of the ratio value from noisy background values. This steady ratio value remained low for 119 s before it took off, indicating a delayed [Ca2+]i elevation. Detectable transient ratio change was observed in none of the 25 single adherent platelets carefully tested.
CHANGES IN \([Ca^{2+}]_i\)

**Fig 8.** The correlation between fluorescence ratio change and morphology of individual adherent platelets treated with dimethyl-BAPTA. Platelets were incubated in HEPES buffer containing both fura-2 AM (4 \(\mu\)mol/L) and dimethyl-BAPTA AM (15 \(\mu\)mol/L) for 45 minutes. After washing, these platelets were mixed with washed erythrocytes to form the cell suspension for flow experiments. The classifications of cell morphology and fluorescence ratio change were the same as defined in the legend of Fig 6. Results were taken from four experiments.

**Fig 9.** The correlation between fluorescence ratio change and morphology of individual adherent platelets treated with cytochalasin D. Cytochalasin D was added to the final cell suspension 5 minutes before the flow experiment took place. Results were taken from four experiments at either concentration of cytochalasin D.

**Fig 10.** The correlation between fluorescence ratio change and morphology of individual adherent platelets treated with either calpeptin or colchicine. Calpeptin (30 \(\mu\)mol/L) or colchicine (1 \(\mu\)mol/L) was added to the final cell suspension 5 minutes before the flow experiment took place. Results were taken from four experiments in either treatment.

Ent platelets, confirming results of our previous study in which acridine red-labeled platelets in whole blood were observed. According to their shape, these adherent platelets were classified into round cells (type 0), cells with pseudopods (type P1 for those having 4 or less pseudopods, type P2 for those having 5 or more pseudopods), mildly spread cells with pseudopods (type PS), and spread cells (type S1 for moderately spread ones, type S2 for extensively spread ones).

We were thus capable of measuring three platelet adhesion related parameters, ie, the lag time between platelet attachment and its subsequent onset of fluorescence ratio change, the pattern of its fluorescence ratio change, and the morphological type. Apparently the pattern of fluorescence ratio change correlated with morphological changes. For example, mild fluorescence ratio changes corresponded to mild shape changes, and strong fluorescence ratio changes to severe shape changes (Fig 6). More than 90% of all spread platelets showed significant fluorescence ratio changes, even the PS ones did so as well. Significant fluorescence ratio changes were observed in about half of P2 cells, but not in more than two thirds of type O or P1 cells. Adherent platelets with elevated \([Ca^{2+}]_i\) were to determine the possible correlation between their morphological type and their time intervals either before or after \([Ca^{2+}]_i\) elevation (Fig 7).

To determine whether \([Ca^{2+}]_i\) elevation was necessary for these postcontact morphological changes, platelets were incubated with dimethyl-BAPTA acetoxymethyl ester (15 \(\mu\)mol/L) during fura-2 loading. This treatment completely blocked \([Ca^{2+}]_i\) elevation, but it had little influence on shape changes (Fig 8). Moreover, the adhesion density of these BAPTA-treated platelets was similar to control platelets as well. When 1 \(\mu\)mol/L EGTA was substituted for 1 \(\mu\)mol/L CaCl2 to remove extracellular calcium ions in the cell suspension, platelet adhesion under flow was completely prevented.

Because cellular shape changes are likely to involve rearrangements of cytoskeletal structures, we also examined the roles of microfilaments or microtubules in these adhesion-associated phenomena. A high concentration (10 \(\mu\)mol/L) of cytochalasin D, a microfilament dissociating reagent, inhibited both shape changes and \([Ca^{2+}]_i\) elevation (Fig 9). Almost all adherent platelets were round cells and none showed...
detectable fluorescence ratio changes. Because round cells tended to be less adhesive and easily flushed away by the flow, the density of adherent platelets from these experiments was usually lower than control experiments. At a much lower concentration (0.1 μmol/L), cytochalasin D largely prevented platelet pseudopod formation without affecting cytoplasmic spreading. Under these circumstances, fluorescence ratio changes went largely unaffected. Neither 30 μmol/L calpeptin (a membrane permeable calpain inhibitor) nor 1 mmol/L colchicine (a microtubule dissociating reagent) was effective in either regard (Fig 10).

**DISCUSSION**

With the help of a video ratio-imaging setup and a dual-wavelength spectrofluorometer, we were able to monitor the platelet adhesion process along with its associated [Ca<sup>2+</sup>]<sub>i</sub> changes at the single-cell level and with good temporal resolution. The mapping of adherent platelets between fluorescence images and SEM micrographs at the end linked [Ca<sup>2+</sup>]<sub>i</sub> changes and cell shapes in each of these platelets. These features are of crucial importance for examining the complex steps involved in platelet adhesion under flow conditions. Results from this study revealed the following characteristics of platelet adhesion on fibrinogen-coated surface: (1) there was no immediate [Ca<sup>2+</sup>]<sub>i</sub> change on platelet-surface contact; (2) as a population of cells, platelets were very variable in terms of their kinetics and pattern of [Ca<sup>2+</sup>]<sub>i</sub> changes; (3) [Ca<sup>2+</sup>]<sub>i</sub> elevated in 90% platelets undergoing cytoplasmic spreading, but not in most nonspread platelets; (4) blocking the [Ca<sup>2+</sup>]<sub>i</sub> elevation did not prevent postcontact morphological changes; (5) platelets with disrupted microfilaments showed neither shape change nor [Ca<sup>2+</sup>]<sub>i</sub> elevation. In conclusion, although platelet adhesion-associated shape changes were accompanied with unpredictable microfilament-dependent [Ca<sup>2+</sup>]<sub>i</sub> changes, [Ca<sup>2+</sup>]<sub>i</sub> change itself was not required for either platelet adhesion or postcontact morphological changes.

Agonist-evoked [Ca<sup>2+</sup>]<sub>i</sub> elevation in platelets in suspensions usually happens rapidly, 1.6,11. The delay for ADP-induced Ca<sup>2+</sup> entry is as short as 10 milliseconds.1,8 Other agonists, such as thrombin, thromboxane A<sub>2</sub>, or platelet-activating factor, bind to platelet membrane receptors and induce a cascade of intracellular signal transduction events that eventually lead to the Ca<sup>2+</sup> release from intracellular stores. Even in these latter cases, the [Ca<sup>2+</sup>]<sub>i</sub> rises in a few seconds after agonist addition. For example, rises in [Ca<sup>2+</sup>]<sub>i</sub> with thrombin or thromboxane A<sub>2</sub> have been observed within 2 seconds.1,8,11 In contrast, our results showed that platelet adhesion on fibrinogen-coated surface under flow was not accompanied with a concomitant [Ca<sup>2+</sup>]<sub>i</sub> elevation. The lag time between platelet attachment and subsequent [Ca<sup>2+</sup>]<sub>i</sub> elevation could be as long as several minutes (Figs 2 through 4). Moreover, when flowing platelets became adherent to a collagen-coated surface, a lag time from a few seconds to 1.5 minutes was also observed (Jen and Lai, unpublished results). These results suggest that [Ca<sup>2+</sup>]<sub>i</sub> changes were not directly coupled to the platelet contact with the surface.

The variability of the lag time among individual platelets, ranging from a few seconds to several minutes, could mean either that adherent platelets were intrinsically very heterogeneous in their reactivity, or that each individual adherent platelet might have encountered a very different microenvironment under flowing conditions. The former possibility is supported by the observation that platelets which adhered under static conditions also showed different [Ca<sup>2+</sup>]<sub>i</sub> (Fig 1). Moreover, there was a noticeable heterogeneity in [Ca<sup>2+</sup>]<sub>i</sub> oscillations among individual adherent platelets that had been treated with both aspirin and apyrase.6,7 Some of these adherent platelets on fibrinogen-surface showed repetitive spikes of [Ca<sup>2+</sup>]<sub>i</sub> without addition of agonists, whereas others remained at low and constant [Ca<sup>2+</sup>]<sub>i</sub>. The latter possibility was also plausible because at later phases of a flow experiment some adherent platelets had neighbors close by whereas others remained solitary (Figs 2 and 5). Adherent platelets in a small cluster seemed to be more apt to have elevated [Ca<sup>2+</sup>]<sub>i</sub> than those isolated ones. Our video records showed that when an incoming platelet bombarded a “silent” adherent platelet and landed next to it, this process frequently triggered [Ca<sup>2+</sup>]<sub>i</sub> elevation in both. Besides, [Ca<sup>2+</sup>]<sub>i</sub> elevations in singly adherent platelets could also be triggered by the bombardment of other flowing cells (platelets or red cells), which could not be clearly shown in the present video system.

The possible role of several physiological factors known to cause platelet [Ca<sup>2+</sup>]<sub>i</sub> elevation, such as thrombin, ADP or thromboxane A<sub>2</sub>, has also been examined. However, inhibitors/antagonists of these factors (80 μmol/L D-phenylalanyl-L-prolyl-L-arginyl chloromethylketone, 10 μmol/L adenosine triphosphate (ATP) or 100 μmol/L aspirin) were unable to prevent either [Ca<sup>2+</sup>]<sub>i</sub> elevation or shape changes of adherent platelets (Jen et al, unpublished results). Local thrombin generation has been shown to be the most important factor of this kind in mural thrombogenesis when heparinized blood flows through a similar chamber.12 Perhaps in our system, which mainly consisted of washed platelets and red cells, none of these factors could reach high enough local concentration to stimulate [Ca<sup>2+</sup>]<sub>i</sub> elevation in adherent platelets. In addition, the possible effect of flow in this system was found to be insignificant within a shear rate range of 100 to 300 second⁻¹, which was too low to show shear-induced platelet aggregation.13 Presently we do not know the precise cause of these adhesion-associated [Ca<sup>2+</sup>]<sub>i</sub> changes.

We have previously shown that adherent platelets on various protein-coated surfaces undergo rapid shape changes from round cells to cells with a few short pseudopods, ie, type P<sub>i</sub> cells.4 This first step of shape change occurs to almost every adherent platelet and can happen within 1 second of platelet-surface contact. After this initial morphological change, adherent platelets undergo further changes in an irregular manner; some form more pseudopods or spread on the surface within several seconds, whereas others remain in the P<sub>i</sub> stage for minutes. This heterogeneity among individual platelet was seen in this study not only in the patterns of their shape change, but also in those of their [Ca<sup>2+</sup>]<sub>i</sub> change. When morphological changes and [Ca<sup>2+</sup>]<sub>i</sub> changes were grouped together for individual adherent platelets (Fig 6), it seemed likely that the initial shape change might not be associated with [Ca<sup>2+</sup>]<sub>i</sub> elevation at all. [Ca<sup>2+</sup>]<sub>i</sub> elevation was mostly associated with more advanced shape changes, especially the cytoplasmic spreading.
Question may arise whether adherent platelets of various morphology can stand different flow rates. A tapered-flow chamber, which generates a linear gradient of shear stress under constant flow rate, has been used to investigate the flow-induced detachment of adherent platelets undergoing morphological changes on fibrinogen-coated surface. Most round cells and P2 cells are flushed away from the surface by low levels of shear stress (<10 dyne/cm², normal venous shear level). On the other hand, even mildly spread platelets (type PS) can withstand shear stress levels as high as 50 dyne/cm² (a higher shear level in arteries). Moreover, round cells sitting on top of the spread cells have been observed in the high-shear regions after flow flushing, indicating that the luminal side of the spread cells is very adhesive as well. Besides, the present study showed that [Ca²⁺]i elevated in the majority of spread platelets under control conditions (Fig 6). Taken together, such platelets may be regarded as “surface-activated” and they may serve as the initiation site for developing a hemostatic plug on a reactive surface.

All agonists that induce platelet shape change in the suspension, ie, the change from smooth discoids to cells with spiny pseudopods, can increase [Ca²⁺]i, mostly by the mobilization of Ca²⁺ from intracellular stores. On the other hand, little information is available regarding the requirement of [Ca²⁺]i elevation for platelet shape changes on an attachment surface. A Ca²⁺ chelator, Quin-2, has been reported to inhibit the formation of lamellipodial networks (the cytoskeletal structure underneath the membrane of spread platelets) but not filopodial bundles (the cytoskeletal structure within pseudopods) after platelet-surface contact. This would indicate that [Ca²⁺]i elevation is required for platelet spreading but not pseudopod formation. However, [Ca²⁺]i changes in individual adherent platelets were not monitored in that study. In our hands, dimethyl-BAPTA, one of the strongest Ca²⁺ chelators available, was able to completely block [Ca²⁺]i elevation without preventing adherent platelets to become spread cells (Fig 8). Although a gross shape change may be initiated by local [Ca²⁺]i elevation, which could not be detected by our methods, our results clearly showed that even the most extensive shape change in adherent platelets did not require cell-wide [Ca²⁺]i changes.

There is no doubt that the integrin GPIIb-IIIa is involved in platelet adhesion to fibrinogen-coated surface. This point has been supported by our previous observation that RGDS (Arginyl-glycyl-aspartyl-serine) is able to partially block the platelet adhesion under similar flow conditions. In addition, the deposition of fibrinogen to surfaces alters its conformation with exposure of the RGDF (Arginyl-glycyl-aspartyl-phenylalanine) epitope. At the cytoplasmic side of platelet membrane, GPIIIb-IIIa are known to be associated with a membrane skeleton in resting platelets. The binding of GPIIIb-IIIa to fibrinogen during platelet activation induces cytoskeleton reorganization so that some membrane skeleton proteins become phosphorylated and associated with underlying cytoplasmic actin filaments. Presumably this type of ligand-receptor-cytoskeleton association also happens during platelet adhesion to fibrinogen-coated surfaces and eventually leads to postcontact morphological changes in adherent platelets. We have tested this hypothesis in our flow system by including 0.1 mmol/L RGDS in the cell suspension. However, very few flowing platelets adhered to fibrinogen-coated surface and this made the mapping of fluorescence images with SEM pictures very difficult.

Whether [Ca²⁺]i elevation in adherent platelets requires an intact cytoskeleton is an interesting question. Both IP₃ binding and IP₃-induced Ca²⁺ release from saponin-permeabilized platelets were significantly inhibited by cytochalasin D (a microfilament inhibitor) and colchicine (a microtubule inhibitor). This would suggest that a functionally intact cytoskeleton is required for [Ca²⁺]i elevation, at least from IP₃-sensitive intracellular stores. This point can be further supported by the fact that collagen-induced platelet [Ca²⁺]i elevation can be inhibited by cytochalasin D. In the present study we found that microfilaments, not microtubules, were important for [Ca²⁺]i elevation in platelets adherent to fibrinogen-coated surface (Figs 9 and 10). However, a partial disturbance of microfilaments, either by a low concentration of cytochalasin D to disturb pseudopods but not lamellipodia, or by calpeptin to prevent the hydrolysis of actin-binding protein during platelet activation, was ineffective in preventing [Ca²⁺]i changes. A largely intact platelet microfilament structure was apparently enough to support [Ca²⁺]i changes.

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Changes in cytosolic calcium concentrations and cell morphology in single platelets adhered to fibrinogen-coated surface under flow

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