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CHANGES IN INTRACELLULAR Ca^{2+} AND STRUCTURE IN PLATELETS CONTACTING SYNTHETIC SUBSTRATES

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Abstract

Platelet structural changes such as spreading and cytoskeletal reorganization that occur upon contact with synthetic surfaces have been well documented. Structural changes such as spreading will vary depending on the substrate involved. Although platelet structural changes following adhesion have been recorded, the cellular mechanisms including changes in intracellular calcium flux underlying these platelet responses are less well understood. In this study, video microscopy was used to image platelet adhesion and spreading while simultaneously imaging intracellular free Ca^{2+} levels in individual Fura-2 loaded human platelets. This was accomplished using fluorescence video microscopy coupled with video enhanced asymmetric illumination contrast (AIC) to image Ca^{2+} transients and correlative electron microscopy to image associated structural changes in individual platelets contacting glass and polyvinyl formal, formvar, substrates. Three main points were concluded from this study: **1)** Intracellular Ca^{2+} levels increase with surface-induced activation on either formvar or glass. **2)** In partially spread or fully spread platelets, intracellular Ca^{2+} decreases to a steady state value. **3)** Platelet-platelet contact leads to intracellular Ca^{2+} transients.

Key Words: Platelets, calcium, activation, fura-2, biomaterials.

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Introduction

Understanding platelet interactions with natural and artificial surfaces at the cellular level is critical to determining material biocompatibility. Platelet structural changes occurring on contact with a variety of surfaces have been previously investigated [2, 3, 7, 14, 18]. In general, when circulating platelets contact a foreign surface they adhere, form pseudopods and spread [3]. The extent of these structural changes is related to the nature of the substrate surface. Release of granule substances during adhesion and spreading can cause further platelet activation and aggregation [10]. These platelet secreted substances include ADP, serotonin, and Ca^{2+} from dense granules and thrombospondin, fibronectin, and fibrinogen from alpha granules. During activation and spreading, the platelet cytoskeleton rearranges to form distinct zones within the platelet [14].

Increases in cytoplasmic free Ca^{2+} levels have been implicated in mediation of platelet activation [10, 15]. Ca^{2+} is an important intracellular regulator and plays a role in platelet activation, structural changes, aggregation, secretion, clot retraction, and microtubular disassembly [10]. In particular, Ca^{2+} is integrally involved in platelet shape change via the polymerization of microfilaments and disassembly of microtubules, membrane receptor organization, and fusion events between complementary areas of the plasma membrane and membranes of secretory granules. The concentration of intracellular free Ca^{2+} in resting platelets is approximately 0.1 μM whereas the total intracellular Ca^{2+} concentration is approximately 70-100 μM [15]. This large pool of Ca^{2+} is stored in the inner leaflet of the plasma membrane, the dense tubular system (DTS), dense granules, and mitochondria. Intracellular Ca^{2+} transients observed with platelet activation are possibly due to Ca^{2+} release from the DTS via the action of inositol triphosphate. Release of Ca^{2+} from the DTS is most likely the result of a receptor-ligand interaction at the platelet surface which triggers the production of 1,2-diacylglycerol and inositol triphosphate via inositol-phospholipid transduction pathway [1, 15]. Ca^{2+} can also enter the platelet via Ca^{2+} channels such as GP IIb-IIIa (integrin $\alpha_{IIb}\beta_3$) [10, 15].

The intracellular probe, Fura-2, has been used to measure intracellular free Ca^{2+} in platelet suspensions [20, 21, 23]. Binding of Fura-2 to Ca^{2+} occurs in a 1:1 ratio and causes a shift in the required excitation wavelength from 380 to 340 nm. Using an intensity ratio of the emission derived from excitations at 340 and 380 nm (340/380) calibrated to known Ca^{2+} concentrations provides a quantitative measure of Ca^{2+} concentrations essentially independent of the unbound Fura-2 in the cells, the path length, or instrument sensitivity [9].

Using Fura-2 or other Ca^{2+} sensitive fluorescent probes, it has been demonstrated that intracellular Ca^{2+} levels in platelet suspensions rise upon addition of agonists such as ADP, thrombin, platelet activating factor, epinephrine, and arachidonate [20, 23, 25]. These studies were conducted using bulk suspensions of platelets. The increases of intracellular free Ca^{2+} have been attributed to the influx of Ca^{2+} possibly through the GP IIb/IIIa receptor and from release of internal stores of calcium. Studies investigating Ca^{2+} transients in individual cells using digital imaging microscopy have been reported [19, 26]. Due to the larger size of the nucleated cells, such as smooth muscle cells, the investigators were able to distinguish differences in intracellular free Ca^{2+} levels within different cellular regions. Some investigators have employed digital imaging microscopy to study intracellular free Ca^{2+} levels in individual surface-adherent platelets [11, 17, 24]. Most of these investigations involved adding agonists, such as serotonin or thrombin, to fully spread platelets to measure their effect on intracellular Ca^{2+} levels.

The aim of our study was to simultaneously image Ca^{2+} levels and platelet structural changes in adherent platelets. This work represents preliminary investigations on two thrombogenic materials, glass and formvar. These two materials were chosen for initial characterization of Ca^{2+} levels during platelet spreading. Platelets usually achieve a fully spread state on formvar and glass. Therefore, by using these materials we hoped to measure Ca^{2+} levels throughout platelet spreading. Using fluorescence ratio imaging, the Ca^{2+} transients in individual platelets were calculated and simultaneously correlated to platelet structural changes using transmitted light video microscopy. The same platelets were then further studied using low voltage high resolution scanning electron microscopy (LVSEM), thus enabling direct correlation between Ca^{2+} transients and platelet morphology produced in response to synthetic substrates.

Methods and Materials

Platelets

Platelets were obtained from normal, healthy, adult volunteers (5 males ages 21 to 37) who had not taken aspirin within the previous week. Blood, 10 ml, was obtained by venipuncture, placed in polypropylene tubes, and anticoagulated 1:9 with acid citrate dextrose. Platelet rich plasma, PRP, was separated by centrifugation of the whole blood at 180 g for 10 minutes at room temperature. A 2% w/v (weight/volume) solution of pluronic (F-127, Molecular Probes, Eugene, OR) in dimethylsulfoxide, DMSO (Sigma Chemical Co., St. Louis, MO) was heated for 20 minutes at 40 °C. Pluronic is a non-ionic surfactant that is useful for solubilizing water-insoluble dyes and other materials in physiological media. The pluronic-DMSO solution was combined with a 2 mM Fura-2 AM (Molecular Probes, Eugene, OR)-DMSO solution, and fetal calf serum (FCS). The pluronic-DMSO-Fura2AM-FCS solution was then combined with PRP and gently agitated for 45 minutes. A final concentration of 3.2 μM Fura-2 AM and less than 0.002% pluronic was achieved in the loading solution. This protocol is a modification of the procedure used by Poenie *et al* [19]. After incubation, the platelets were separated from plasma proteins and any Fura-2 AM not loaded into the platelets by passing through a Sepharose CL-2B column, 40 ml bed volume, preequilibrated at room temperature with Lagas-Tyrodes Ca^{2+} free buffer, pH 7.35, containing 2 g/l albumin. Lagas-Tyrodes buffer is composed of 136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4 H_2O, 12 mM $NaHCO_3$, 2 mM $MgCl_2$ $6H_2O$, and 5.5 mM dextrose. Fura-2 AM which had not been incorporated into the platelets was removed during column washing [21].

Controls were also included to verify that the pluronic and DMSO did not deleteriously affect platelet function. PRP, 2% w/v pluronic in DMSO, and fetal calf serum were combined and agitated as indicated above. The platelets were then added to formvar coated electron microscopy (EM) grids and allowed to spread for 25 minutes at 37 °C. After incubation platelet morphology was fixed by adding 1% glutaraldehyde in 0.1 M HEPES to the grid for 30 minutes. Another control was included to verify that agitation did not affect platelet behavior. In this control, PRP was agitated for 1 hour, added to EM grids, incubated at 37 °C for 25 minutes, and then fixed in glutaraldehyde as above. Two series of each control were conducted in separate experiments. The platelets in both controls were examined using LVSEM and high voltage electron microscopy (HVEM).

Platelet/Material Interactions

Glass cover slips and polyvinyl formal coated electron microscopy finder grids were exposed to Fura-2 column washed platelets using a specially constructed chamber [8]. The precleaned glass cover slips were wiped with lens paper to remove dust from the surface. Glass and formvar were used in this study because platelet spreading on these substrates has been well characterized [2, 7, 8, 14, 18]. We wished to record platelet intracellular Ca^{2+} transients from discoid to fully spread platelets and therefore, selected two materials on which platelets would achieve a fully spread state.

The interaction of platelets with the artificial surfaces was simultaneously viewed by light microscopy using both epifluorescence and asymmetric illumination contrast (AIC) [12]. Simultaneous images of both Ca^{2+}/Fura-2 fluorescence via epifluorescence and platelet

structural changes via AIC were imaged with a Nikon Diaphot inverted microscope using a Nikon Dual Optical Path Tube connected to Dage MTI ISIT and Newvicon cameras as shown in Figure 1. A Colorado Video video processor was used for analog image enhancement of the AIC signal. Real time AIC platelet morphological changes were captured on video tape for later image analysis. Image analysis of both fluorescence and AIC images was performed using Image 1 hardware and software [8].

Ca^{2+} transients were recorded and correlated to structural changes for each sample. Fluorescence data was typically collected over a 3 second period at intervals of 20 or 30 seconds. At each interval, a fluorescence ratio pair was obtained by rapidly alternating the incident illumination between 340 and 380 nm. Each image at 340 and 380 nm was obtained by averaging 16 video frames collected over a 1.5 second time period per wavelength. The ratioed, 340/380, signal was used to produce an intensity map of Ca^{2+} distribution within the platelets. At least 3 series of platelet events on glass and formvar were collected. The resulting fluorescent images and AIC recorded images were analyzed to correlate morphological events with Ca^{2+} levels.

Electron microscopy preparation

Following video-microscopic observation, formvar-adherent platelet samples were prepared for EM. Platelet morphology was preserved by fixation with 1% glutaraldehyde in 0.1 M HEPES buffer [14]. These samples were then dehydrated in a series of 30-100% ethanol and dried by the critical point procedure. Electron microscopy was conducted at the Integrated Microscopy Resource (IMR) at the University of Wisconsin-Madison using the Hitachi S-900 LVSEM.

Results

Representative series of platelet morphological changes and Ca^{2+} transients taken from 3 sets of experiments on both glass and polyvinyl formal, formvar, are presented. Data are displayed as fluorescent ratio images. The ratio values are not linear with respect to free Ca^{2+} concentration however, they represent a relative level of intracellular free Ca^{2+} in the adherent platelets and suffice to clearly demonstrate whether significant changes in intracellular Ca^{2+} levels occurred.

The first series of representative platelet events was recorded on a glass substrate. This series contains the intracellular Ca^{2+} transients of 4 individual platelets. Data collection commenced several minutes after platelet A adhered to the glass substrate. Platelet B adhered 1.7 minutes and platelet C 3.6 minutes after the start of data collection. The first AIC image presented in this series, Figure 2a, was recorded just after platelet C had adhered to the glass. Note that platelet A is almost fully spread at this time, as seen in Figure 2a. The corresponding fluorescence image for Figure 2a is shown in Figure 2b. The fluorescence image represents a ratio of the fluorescent signals obtained at 340 and 380 nm at

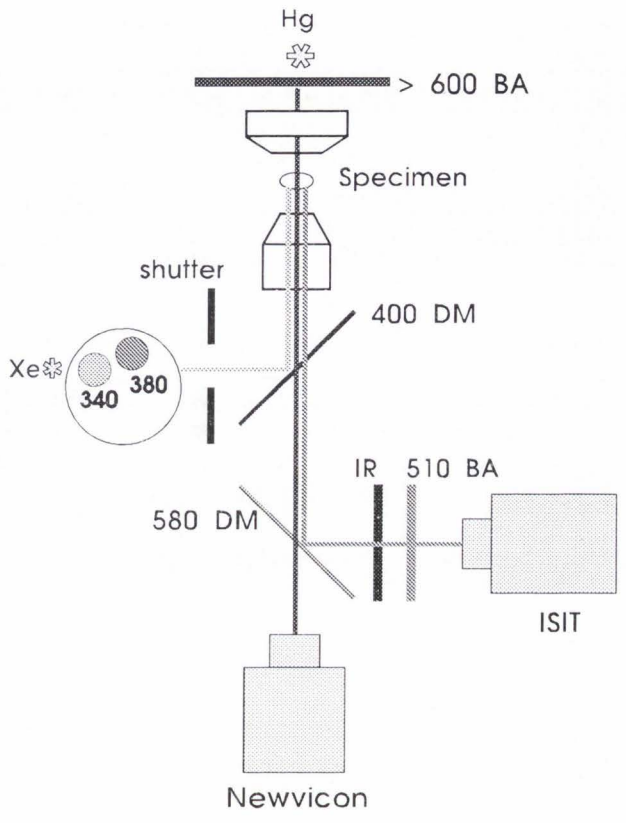

Figure 1. The AIC image was produced using a mercury lamp source passed through a 600 nm filter. The 600 nm filter passes wavelengths 600 nm and above. The fluorescence signal was produced using a xenon lamp source and a computer driven Sigma Line filter wheel which selected excitation wavelengths of 340 and 380 nm. The Dual Optical Path Tube is equipped with an infrared filter and a dichroic filter cube which transmits 580 nm and above to the Newvicon camera for AIC imaging and reflects 580 nm and below to the ISIT camera for fluorescence imaging. The ISIT camera is also equipped with a 510 nm barrier filter which selects for the optimal emission wavelength of Fura-2.

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this particular time point. The scale shown in Figure 2b extends from black, ratio = 0.0, to white, ratio = 3.0, and corresponds to the ratio of Ca^{2+} bound Fura to unbound Fura within the platelets. A ratio of 3 represents a highly elevated level of intracellular Ca^{2+}. Platelet C shows an intracellular Ca^{2+} level much greater than background fluorescence as it attached to the substrate, Figure 2b. A similar Ca^{2+} increase occurred when platelet B attached to the substrate (not shown). The intracellular Ca^{2+} in platelet B remained elevated 2 minutes after adherence, as shown in Figure 2b. Platelet A which adhered and partially spread prior to the start of data collection exhibits an overall lower intracellular Ca^{2+} level as compared to platelets B and C, Figure 2b.

The next pair of images, Figures 2c and d, were recorded at 4 minutes into the series (t = 4 minutes) or

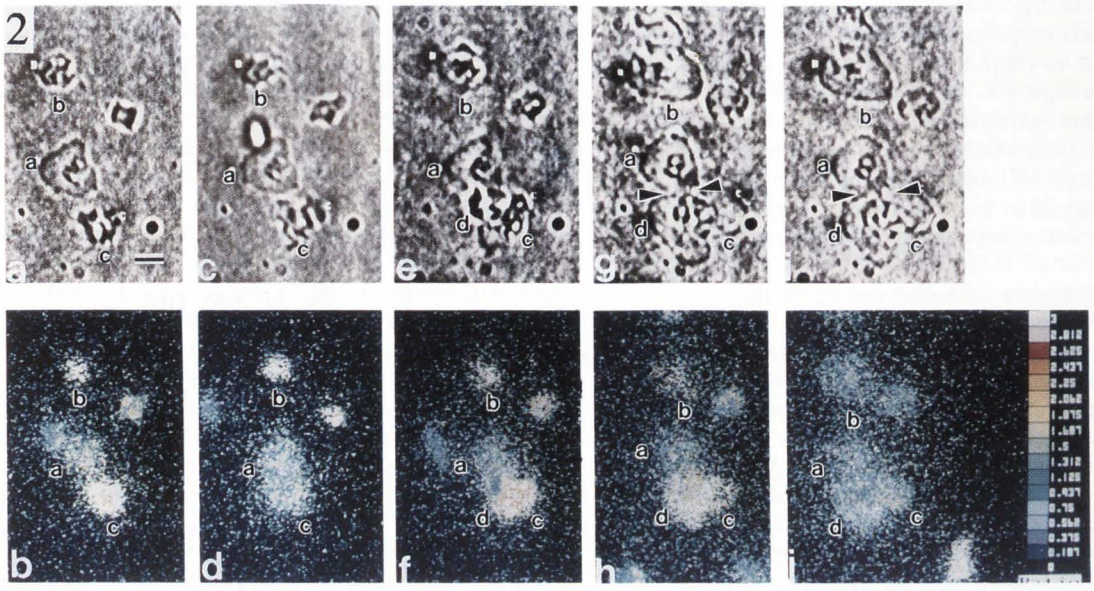

Figure 2. Transmitted light and intracellular Ca^{2+} images of adherent and spreading platelets on a glass substrate. Figure 2 a, c, e, g, and i are the transmitted light images of platelet morphology on the glass substrate. Figures 2 b, d, f, h, and j are the corresponding fluorescence ratio images indicating relative intracellular Ca^{2+} levels. Both the transmitted light and fluorescence images were recorded simultaneously. Ratio scale representing the relative intracellular free Ca^{2+} is given in Figure 2j; scale extends from black (ratio = 0.0) to white (ratio = 3.0). The images are paired as 2a and 2b, 2c and 2d, etc. Bar = 2 μm.

2a & 2b) AIC and fluorescent images for adherence of platelet C to the glass substrate. Associated Ca^{2+} burst is observed with attachment of platelet C.

2c & 2d) AIC and fluorescent images 20 seconds after platelet C attached to the glass substrate. Intracellular Ca^{2+} level of platelet C has decreased.

2e & 2f) AIC and fluorescent images of adherence of platelet D to the glass substrate. Both platelets C and D exhibit a intracellular Ca^{2+} increase as platelet D contacted both the substrate and platelet C.

2g & 2h) AIC and fluorescent images of both platelets C and D contacting platelet A. Contact between platelets C-D and A causes an intracellular Ca^{2+} increase in platelets C and D. Contact point is indicated by the arrowheads in Figure 2g.

2i & 2j) AIC and fluorescent images of both platelets C and D 30 seconds after contacting platelet A. Intracellular Ca^{2+} in platelets C and D has decreased to a steady state level as the separation between platelets C and D and platelet A increased. Increased separation is indicated by the arrowheads in Figure 2i.

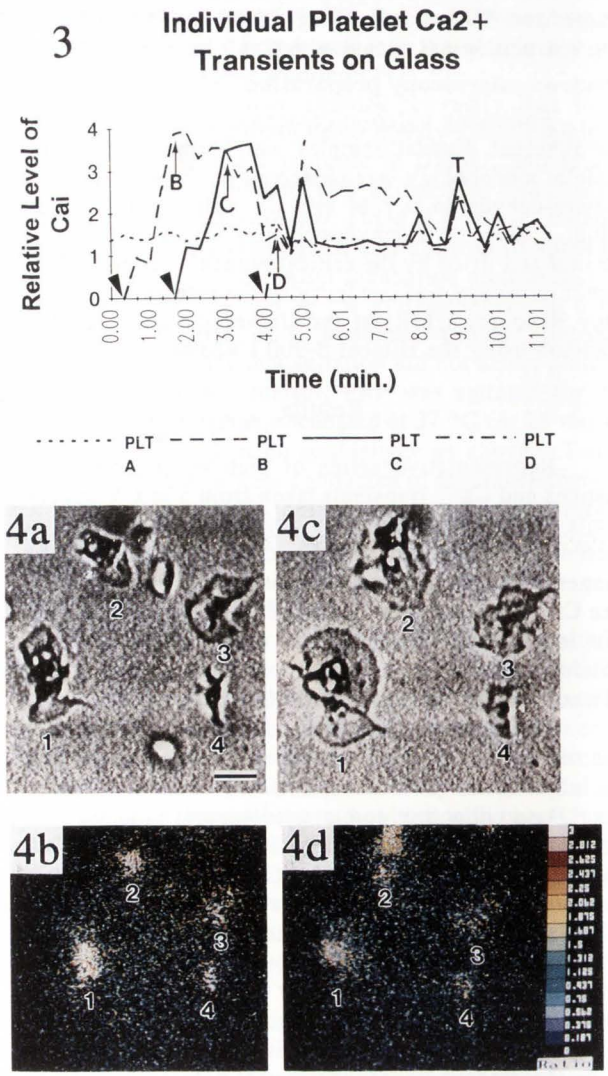

Figure 3 (on facing page 302). Relative level of intracellular Ca^{2+} versus time for four platelets seen in Figure 2. Larger arrowheads mark the adherence of platelets B-D to the substrate and lettered arrows indicate the corresponding transient rises of intracellular Ca^{2+}. Arrow D also indicates the Ca^{2+} increase in platelet C with attachment of platelet D. The series of platelet-platelet contact between platelets C and D and platelet A and corresponding increases and decreases in Ca^{2+} with contact and separation is indicated by the letter T.

Figure 4 (on facing page 302). AIC and intracellular Ca^{2+} images of platelets on a formvar substrate. The platelet morphology is shown in 4a and 4c and the corresponding Ca^{2+} fluorescence images are shown in 4b and 4d. Ratio scale representing the relative intracellular free Ca^{2+} is given in Figure 4d; scale extends from black (ratio = 0.0) to white (ratio = 3.0). Four regions are indicated in the AIC images. Bar = 2 μm.

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approximately 20 seconds after platelet C adhered. The intracellular Ca^{2+} level has decreased in platelet C as evidenced by the blue-yellow fluorescence image. At 4.5 minutes a fourth platelet, D, adhered to platelet C, Figures 2e and f. Adherence of platelet D lead to a rapid increase in intracellular Ca^{2+} in platelet C. This rapid increase dissipated in approximately 30 seconds.

After adherence, platelet D and platelet C continued to spread. Both platelets C and D which were in close apposition were initially separate from platelet A. However, at approximately 9 minutes, platelets C and D contacted platelet A. This platelet-platelet contact was accompanied by increased intracellular Ca^{2+} in both platelets C and D, as seen in Figures 2g and h. Approximately 30 seconds after this contact, the Ca^{2+} level in platelets C and D returned to the steady state value, Figures 2i and j.

The intracellular Ca^{2+} levels of the platelets in Figure 2 are plotted in Figure 3. A relatively constant Ca^{2+} level was observed in platelet A throughout. Attachment of platelets B, C, and D to the substrate were accompanied by corresponding increases their Ca^{2+} levels as indicated in Figure 3. Furthermore, the Ca^{2+} increases in platelet C with attachment of platelet D and in the platelet-platelet contact of C-D with A are observed as rapid increases, occurring within seconds, in intracellular Ca^{2+}. Two other similar Ca^{2+} increases in platelets C-D, the result of apparent contact with platelet A, are also indicated on the graph. After approximately 11 minutes the intracellular Ca^{2+} level in all four platelets decreased to a similar steady state value.

The changes in intracellular Ca^{2+} levels for formvar adherent platelets are shown in Figure 4. The platelets were exposed to the formvar for a short period of time before measurements were collected. At the beginning of observation, four regions were designated for monitoring Ca^{2+} levels, as seen in Figure 4a. Region 1 contained two platelets and regions 2-4 contained 1 platelet each. The corresponding Ca^{2+} color fluorescence image is shown in Figure 4b. A comparison of Figures 4a and 4b readily illustrates that the platelet dense areas in regions 1-4 correspond to the concentrated Ca^{2+} regions observed in Figure 4b. In particular, a higher Ca^{2+} level is observed in the platelets in regions 1 and 2 since platelets in these regions had recently adhered to the substrate. All the platelets in Figure 4b contain higher Ca^{2+} levels than the surrounding extracellular media.

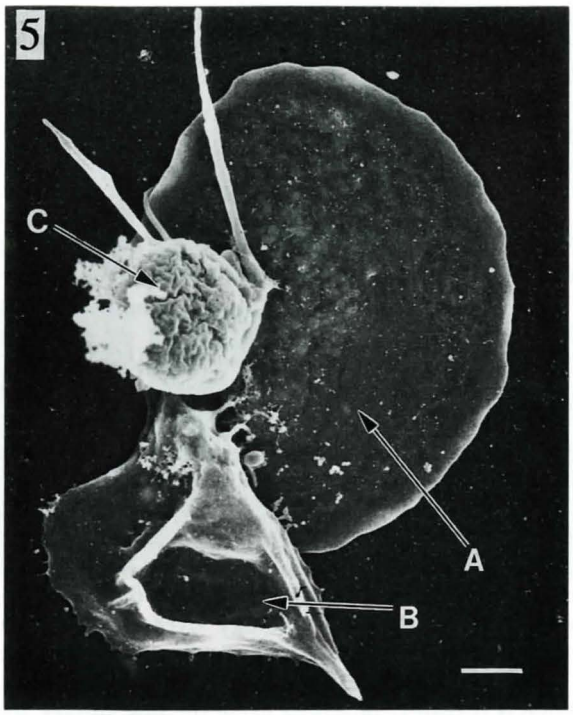
The AIC and corresponding fluorescence image of the same platelets 16 minutes later, Figures 4c and 4d, shows a range of platelet spreading. The platelets in regions 3 and 4 never completely reached a fully spread state whereas the platelets in regions 1 and 2 spread to a fuller extent. In addition, a second and third platelet on regions 2 and 1 respectively, have attached to the fully spread platelets in these areas. Throughout this series, the platelets in regions 3 and 4 did not significantly change their morphology over the 16 minute analysis period, at which time the fixative was added.

The spatial relationships and morphology of these platelets is readily observed using the low voltage scanning electron microscope. Scanning electron micrographs of regions 1 and 3 are shown in Figures 5 and 6. These micrographs display the final extent of platelet spreading achieved in these regions. In region 1, Figure 5, the last adherent platelet is still partially discoid in shape and has just attached pseudopods to both a partially spread and a fully spread platelet in this region. In region 3, Figure 6, this platelet achieved a partially spread morphology. This morphology did not significantly differ from the morphology observed during the course of data collection.

A graph of the relative intracellular Ca^{2+} level versus time for the platelets in Figures 4 and 5 is shown in Figure 7, with regions 1-4 corresponding to the areas plotted. Only regions 1 and 2 are displayed throughout the entire time period since the area of detectable signal in regions 3 and 4 became too small to measure. All four regions were initially at higher Ca^{2+} levels and then slowly decreased to a relatively low steady state value throughout the next 8 minutes of observation. In general, after a platelet has reached its final spread morphology the Ca^{2+} signal levels off to a steady state value. The result observed in regions 3 and 4 is representative of this decrease.

Controls

The controls to determine if pluronic or gentle agitation of the platelets during incubation was detrimental to platelet adhesion, activation, and spreading on the substrates were negative. No effect on platelet spreading, morphology, or cytoskeleton was observed. The small amount of pluronic and rocking did not negatively affect the platelets ability to adhere, activate, and spread on formvar or glass.



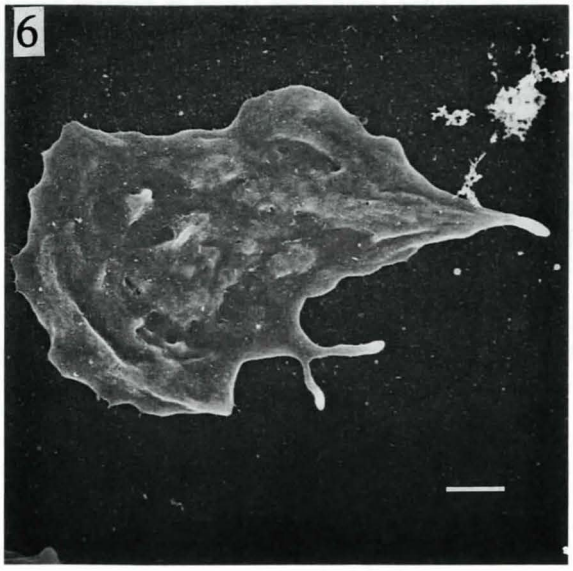


Figure 5. Low voltage scanning electron micrograph of a fully spread (A), partially spread (B), and pseudopodial platelet (C) on a formvar substrate. Platelets correspond to region 1 in Figure 4c and further clarify the spatial relationship and degree of spreading of these three platelets previously shown with AIC. Bar = 1 μm.

Figure 6. Low voltage scanning electron micrograph of a partially spread adherent platelet on a formvar substrate. Platelet corresponds to the platelet in region 3 in Figure 4c. Bar = 1 μm.

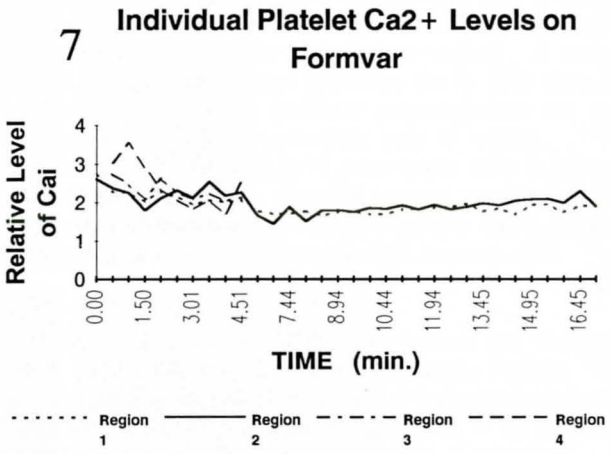


Figure 7. Relative level of intracellular Ca^{2+} versus time for the platelet regions shown in Figure 4. All platelets are initially at higher intracellular Ca^{2+} levels which correspond to initial attachment of platelets or platelet-platelet contact with attachment. Relative intracellular Ca^{2+} levels decrease to steady state value after several minutes of platelet adherence to the formvar substrate.

Discussion

The two series presented are representative of the dynamic changes in platelet intracellular free Ca^{2+} levels on formvar and glass. Three main points can be concluded from these experiments. First, upon adherence to a substrate, platelet intracellular Ca^{2+} levels increase. This increase is coincident with surface-induced activation of the platelet and is characteristically much higher than the background Ca^{2+} signal in our system. The intracellular Ca^{2+} level in a platelet which has just adhered can remain elevated for up to 2 minutes or can decrease in less than 30 seconds. Second, the intracellular Ca^{2+} level in fully or partially spread platelets eventually decreases to a steady state level. The steady state level is usually achieved within 10 minutes after initial platelet adherence and appears to be associated with the final spread form of the platelet. At this point, the platelet may be in a metabolic "resting" state and may have already released its granular contents. Third, intracellular Ca^{2+} levels transiently increase upon platelet-platelet contact. These platelet-platelet Ca^{2+} transients have been observed both when adherent, spreading platelets contact one another and when platelets in suspension attach to adherent platelets. The increase in intracellular free Ca^{2+} upon platelet-platelet contact can be due to increased mobilization of internal Ca^{2+} stores as well as influx of external Ca^{2+} since this contact is mediated in part via fibrinogen binding to the GP IIb-IIIa receptor, a putative Ca^{2+} channel in the platelet [4].

Other sources of Ca^{2+} may also be involved, such as the ADP-evoked receptor-operated channel, perhaps via ADP provided through dense granule release [16].

Measurements of Ca^{2+} levels in adherent platelets were collected over a 3 second period at intervals of 20 or 30 seconds. This means of acquisition produced the point-to-point appearance of the graphs seen in Figures 5 and 7. It is important to cycle the acquisition of fluorescence data to avoid bleaching of the Fura-2 intracellular dye. Other means to avoid unnecessary bleaching of the dye were also employed [5]. Intracellular Ca^{2+} transients shorter than the 20 second interval were not recorded. To avoid the loss of detecting very short transients, many platelet events were studied. Thus even though all transients might not have been detected, the likelihood of detection was extremely high since many events were recorded.

Previous studies conducted with platelet suspensions have analyzed platelet activation in response to various agonists by measuring the intracellular Ca^{2+} concentration [23, 25]. Typically, intracellular Ca^{2+} levels rise with stimulation from an agonist such as ADP, collagen, or thrombin. The transient intracellular Ca^{2+} increase in platelets exposed to agonists is generally 1-10 μM depending on the fluorescent probe employed [23, 25]. Other work has used ratio imaging to determine intracellular Ca^{2+} levels in platelet suspensions treated with different agonists. These previous studies have measured intracellular Ca^{2+} levels in platelets adherent on glass, fibrinogen coated glass and poly-ethyleneimine coated glass [11, 17, 24]. The major emphasis in these studies was to measure the effect of various agonists on individual surface adherent platelets rather than study the Ca^{2+} fluctuations from platelets adhering on different surfaces. Other studies have utilized epifluorescence microscopy of Fura-2 to investigate intracellular Ca^{2+} levels in much larger cells such as eosinophils, smooth muscle cells, and PtK_2 cells [6, 19, 26]. These studies are comparable to our experimental design except that the cells investigated are over 20x the volume of platelets and thus allow study of the intracellular distribution of Ca^{2+}. Using simultaneous video enhanced, interference based microscopy coupled with video enhanced epifluorescence microscopy, we are now able to study platelet-surface specific activation events in individual platelets.

Some reports of intracellular Ca^{2+} transients of platelet suspensions in contact with polymers have been reported [22, 27]. One group investigated Ca^{2+} levels in rabbit platelets in contact with polystyrene latex beads coated or uncoated with albumin [27]. Another study investigated Ca^{2+} transients from platelets passed through commercially available mini dialyzer columns [22]. These studies were conducted by exposing bulk platelet suspensions to the materials then measuring intracellular Ca^{2+} of the platelet suspension using a spectrofluorometer. In both these studies platelet intracellular Ca^{2+} rose when platelets contacted the artificial substrates. Some differences in the level of the transients were observed depending on the materials studied. Although we also observed increases in intracellular Ca^{2+}, we also coupled Ca^{2+} levels with simultaneous imaging of the platelet morphology. We observed that the surface-induced Ca^{2+} increases appear to correspond to the active platelet spreading response. Platelet morphology, specifically platelet shape change, granule release, or platelet-platelet contact were not delineated in the previous bulk platelet suspension studies because morphology could not be observed [22, 27].

Using correlative procedures to simultaneously monitor platelet morphology and Ca^{2+} transients permits the analysis platelet morphological changes throughout shape change and spreading, and in platelet-platelet interactions. We observed that increases in intracellular free Ca^{2+} occurred upon platelet adherence to previously spread substrate adherent platelets, and when two or more adherent platelets contacted each other. Platelet-platelet contact did not always elicit a Ca^{2+} increase in both platelets, with more fully spread platelets often not responding with a significant increase in intracellular Ca^{2+} when contacted by other platelets. This was observed in Figures 2f and 2j where, in both cases, platelet A did not exhibit a Ca^{2+} influx upon adherence of platelet D or with contact to the combination of platelets C-D. It is possible that lower levels of Ca^{2+} dependent activity are present in fully spread platelets. It is also possible that internal stores of Ca^{2+} may be depleted in these fully spread platelets.

Due to the semi-quantitative nature of fluorescence ratio video microscopy imaging, this technique can be further employed to study the relative level of platelet responsiveness on different synthetic surfaces, and in platelet-platelet, platelet-ligand, and platelet-nucleated cell interactions. Many different surface chemistries have been implemented to decrease the thrombogenicity of artificial surfaces *in vivo*. Studies to determine the effectiveness of these various chemistries, such as SO_3 incorporation in polyurethanes, have probed the platelet response to the surfaces via morphological changes alone [13]. The coupled techniques of Fura-2 Ca^{2+} measurements and platelet morphological changes observed with AIC light microscopy could provide insight into the biochemical changes and associated morphological changes occurring in platelets as they contact and spread on different substrates.

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Discussion with Reviewers

K. Park: The calcium ion level is high in the contact adherent platelets, but decreased as platelets spread. It appears that the distribution of calcium ions in the spread platelets is rather homogeneous and no unique distribution was observed. Is it something expected by the authors? It may be expected that calcium ions can be localized around the cytoskeletons such as microfilament bundles.

Authors: The areas of adherent platelets, even when observed in video enhanced light microscopy, are small. Due to this small size and consequently due to the limits of resolution and detection we are unable to precisely localize the intracellular Ca^{2+} distribution, except perhaps as general regions within a cell (left edge, center, etc.). In particular we used an ISIT camera for the fluorescence imaging. The ISIT is designed for improved signal detection in low light level systems. However, it also intensifies the signal thus producing some nonlinearity in spatial location in the signal.

K. Park: Calcium levels were measured only for 11 minutes after adhesion of platelets. Since platelets continue to change their shape to spread, it would be interesting to continue to measure the calcium level longer than 11 minutes.
Authors: We have observed Ca^{2+} levels in fully spread platelets for up to 25 minutes. The obtained relative steady state levels of intracellular Ca^{2+} were continuously observed once the platelet its spread morphology. No major changes in intracellular Ca^{2+} are observed as the platelet slowly continues to increase its spread area.

G. Pasquinelli: Is it possible that the burst of intracellular Ca^{2+} caused by platelet-platelet contacts may reflect the delivery of dense bodies? With regard to this latter point, did you find any differences between platelets contacting glass and those contacting formvar?
Authors: It is possible that the intracellular Ca^{2+} burst seen during platelet-platelet contact could coincidentally be the secretion of the dense granules, however, we would then assume that some platelet-platelet contact events would not solicit Ca^{2+} rises if granular release was the sole mechanism driving Ca^{2+} increases. In all the platelet-platelet contact events observed, we have seen simultaneous increases in Ca^{2+}. With regard to your second question, our continued studies have shown no major difference between glass and formvar. However, the individuality of a platelet response does produce a lot of variability in the magnitude and length of Ca^{2+} transients. Therefore, although platelets adhering and spreading on glass and formvar do have similar responses, a wide variability exists in the length and relative magnitude of those responses. Similar variability between Ca^{2+} levels in adherent platelets has been observed in a related study [11].

H.J. Busscher: What is the mechanism by which platelets sense that they are at a surface and what stimulates the Ca^{2+} concentration changes?
Authors: Much speculation exists about how platelets sense and attach to surfaces, such as a biomaterial. Specific attachment may be mediated by various glycoprotein receptors that bind to various adsorbed ligands at the surface or bind directly (non-specifically) to the surface. Whatever the mechanism of attachment, there is most likely a subsequent transmembrane stimulation in the platelet which triggers the inositol triphosphate pathway to produce diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 causes release of Ca^{2+} from the dense tubular system. Another large pool of Ca^{2+} can also be released from the plasma membrane. It is also believed that a certain portion of the initial Ca^{2+} transient or possibly fluctuations above an initial baseline transient are due to extracellular Ca^{2+} influx possibly through the putative Ca^{2+} channel GP IIb/IIIa.

H.J. Busscher: Very short contact times for platelets on surfaces have been reported (Feuerstein IA, Buchan SM, Horbett TA, Hauch KD, Platelet adherence and detachment: A flow study with a series of hydroxyethyl methacrylate ethyl methacrylate copolymers using video microscopy, J. Biomed. Mater. Res. **25**, 185-198, 1991). Can the author speculate if and how the platelets respond so quickly in their Ca^{2+} concentration?
Authors: As related to your previous question, triggering of the inositol triphosphate pathway results in the quick response to surface-induced platelet activation. We have observed that simple contact of a platelet process with the surface does not usually trigger intracellular Ca^{2+} increases. Instead the platelet must adhere to the surface, i.e., all wandering movement ceases, and initiate pseudopod extension before the Ca^{2+} rise is observed. It is not surprising that the initial contact activation transient would simultaneously correspond to pseudopodial development since Ca^{2+} plays an integral role in microfilament polymerization via phosphorylation of myosin by myosin light chain kinase.

Y. Takemoto: The author indicated that the scale shown in Figure 2b extends from black, ratio = 0.0, to white, ratio = 3.0. I think that the ratio means an intensity ratio at 340 and 380 nm. Therefore, ratio = 0.0 is unreasonable. I think that in this system the ratio was not calculated and ratio was presented as 0.0 if the fluorescent intensity at 340 nm was much weak. Is this true?
Authors: Ratio = 0.0 in the Image I/FL system usually corresponds to a very low 380 nm intensity as well as a low 340 nm intensity. We have never observed ratio levels at 0.0 for adherent platelets. Ratio values of 0.0 usually only correspond to the background which usually has little or no intensity at either 340 nm or 380 nm.

Y. Takemoto: According to Tsien's formula 9 (text ref. 23) what are R, R_{max}, and R_{min}?
Authors: As stated in the text, the ratio values presented represent a relative level of intracellular free Ca^{2+}. The ratio values are not linear with respect to free Ca^{2+}, however, they clearly demonstrate whether significant changes in intracellular Ca^{2+} levels were measured. No R_{max} or R_{min} are available.

Reviewer VI: Previous studies have indicated that platelet Ca^{2+} signals are in the form of complex spikes or oscillations. This is the case in agonist-stimulated and contact-activated cells [11, 17]. Given this, is it

appropriate to monitor the Fura-2 fluorescence in the way the you have, averaging over a 3 second period every 20 or 30 seconds? This approach will fail to resolve any oscillatory behavior which may or may not be present. A snap shot of the cytosolic calcium concentration at infrequent intervals may not reflect accurately the complex changes which may be occurring. It is accepted that there will be difficulties with bleaching of Fura-2 if continuous records are made, but anything else will not achieve the aim of correlating the Ca^{2+} signal with morphologic changes.

Authors: We have measured intracellular Ca^{2+} levels in individual adherent platelets at intervals as rapid as 15 seconds and have not observed the large oscillatory behavior discussed in the references you cite. The oscillatory behavior discussed in these references seems to occur over 20 second periods. It appears that these oscillations are more frequent with the addition of platelet agonists such as ADP [11]. Furthermore, these Ca^{2+} oscillations in serotonin stimulated platelets were diminished when 1 mM extracellular Ca^{2+} was **not** added to the medium [17]. In our study, we did not specifically add agonists or extracellular Ca^{2+}. The only agonists and extracellular Ca^{2+} present would be that released from platelet alpha and dense granules. Since these oscillations potentially could appear, we will investigate the potential for oscillations further in our system by sampling more frequently when platelets initially adhere to the substrate.

Reviewer VI: The authors mention that controls were carried out to check that there were no adverse effects of pluronic. However, why was it necessary to use the detergent? Most groups load Fura-2 into platelets from the ester in platelet-rich plasma without the need for pluronic. It is also not clear why fetal calf serum was added to the platelet-rich plasma. Similarly, most groups avoid gel-filtration in favor of collection by centrifugation, in order to prepare cells with greater viability.

Authors: Molecular Probes recommends the use of pluronic for loading the Fura-2 into cells. We followed a modified protocol by Poenie *et al.* [19]. The amount of pluronic actually present in the loading solution is extremely low and causes no change in platelet behavior on formvar or glass. We column purify our platelets not only to separate plasma proteins but also to remove unbound Fura-2. We find this method superior to centrifugation, since centrifugation can artificially activate the platelets prior to contact with the experimental substrates.