Micropipette Aspiration of Human Platelets after Exposure to Aggregating Agents

Steven M. Burris, Clark M. Smith II, David T. Tukey, Carlyle C. Clawson, and James G. White

The present study examined the influence of activation on platelet deformability. Aspiration of cells after exposure to thrombin, adenosine 5'-diphosphate, or the calcium ionophore A23187 at concentrations producing shape change and stickiness revealed significant changes from control cells. At the lowest negative pressure, 4 × 10⁻² dynes/cm (−1 cm H₂O), there were no differences in lengths of membrane segments aspirated from control and activated platelets. Each subsequent increase in negative pressure up to 35 × 10⁻² dynes/cm (−7.5 cm H₂O) resulted in significantly longer aspirated segments on activated cells compared to control cells. Greater negative pressures did not cause further increases in lengths of membrane segments drawn into the pipette. Thus, activation, which results in constriction of the circumferential microtubule, makes more membrane available for aspiration as negative pressure is increased. In both control and activated platelets, the microtubule coils served as a barrier to further lengthening of aspirated membrane segments as negative pressure was increased beyond 35 × 10⁻² dynes (−7.5 cm H₂O). (Arteriosclerosis 6:321–325, May/June 1986)

Recent reports from our laboratory have shown that the technique of micropipette aspiration can be used to gain new information concerning the factors influencing platelet deformability.¹ ³ The mechanical behavior of the platelets was shown to strongly depend on the state of organization of microtubules and microfilaments.¹ Depolymerization of microtubule coils by low temperature or antimitotic agents such as vincristine or colchicine, prevention of microfilament assembly by cytochalasin B, or a combination of the two caused platelets to become much less resistant to aspiration. Recently, we extended the micropipette studies by characterizing the stress response of platelets over a more complete range of deforming stresses ranging from 4.0 to 41.0 × 10⁻² dynes/cm (−1 to −10 cm H₂O). Application of the expanded micropipette evaluation to study microtubule reassembly during rewarming of chilled platelets has demonstrated that closely associated microtubule coils are essential for normal resistance to aspiration.³

After exposure to aggregating agents, resting platelets lose their discoid form, become irregular with multiple pseudopods, and undergo internal contraction.⁴ ⁵ Organelles become concentrated in cell centers enclosed by close-fitting coils of microtubules. The present study has used micropipette aspiration to assess the alterations in platelet deformability after activation by a variety of agents. The results demonstrate that activation reduces platelet resistance to deformation but that the change is markedly different from the increased deformability caused by chilling platelets to remove microtubules.

Methods

General

Blood was obtained after informed consent from healthy adult donors whose platelets had been evaluated in many previous studies from our laboratory.⁶ ⁷ Samples obtained by venipuncture were mixed immediately with citrate-citric (C) acid, pH 6.5 (93 mM sodium citrate, 70 mM citric acid, and 140 mM dextrose), in a ratio of 9 parts blood to 1 part anticoagulant. Platelet-rich plasma (PRP) was separated from whole blood by centrifugation at 100 g for 10 minutes at 23°C and was maintained at room temperature until used in specific experiments.

Preparation of Platelets

Samples of PRP were mixed with equal volumes of the C anticoagulant.⁸ One volume of the C-PRP mixture was then added to 9 volumes of Ca²⁺/Mg²⁺-free Hanks’ balanced salt solution (HBSS) containing 0.1% bovine serum albumin. The diluted PRP was maintained at room temperature until used in specific experiments.
**Exposure to Agonists**

Adenosine diphosphate (ADP), thrombin, and the calcium ionophore A23187 were chosen for the present study because all are potent aggregating agents that produce rapid shape change in stimulated cells. Each agent was tested in a stirred diluted platelet suspension. Platelet samples were then combined with $10^{-5}$ M ADP, $10^{-5}$ M A23187, or thrombin at a final concentration of 0.2 U/ml, inverted once, and allowed to stand at room temperature for 5 minutes. Preparation of similar samples for electron microscopy, diluted 1:2 rather than 1:9 in HBSS, revealed development of shape change and formation of small aggregates.\(^4,7\) Vincristine (Vincristine sulfate, Eli Lilly, Indianapolis, Indiana) was dissolved in phosphate-buffered saline as described previously,\(^7\) and was combined with some platelet samples at a final concentration of $10^{-8}$ M for 30 minutes before dilution. Examination of vincristine-treated samples in the electron microscope revealed disappearance of circumferential microtubules. After dilution, the samples were combined with 0.2 U/ml of thrombin and were evaluated for deformability in the micropipette.

**Preparation of Micropipettes**

Boron silicate glass capillaries (1 mm in external diameter) were heated and pulled on a micropipette puller (Narishige PN3, Narishige Scientific Instrument Laboratory, Tokyo, Japan) to form micropipettes with an internal diameter range of 0.7 to 0.8 \(\mu\)m segment of the pipette. Internal diameters of the pipettes were measured on the video monitor that was standardized with a 10 \(\mu\)m grid described below and in previous reports.\(^1,3\) The tips of the pipettes were filled with phosphate-buffered saline by immersion for 15 minutes at ambient pressure, and the large diameter portions were filled with a syringe equipped with 30 gauge wire tubing.

**Micropipette Video Microscope Apparatus**

The pipette was placed in a micromanipulator (Defonbrune, Moe Scientific, St. Louis, Missouri) connected via saline-filled tubing to a pressure transducer and pressure generating system previously described in detail.\(^1,3\) The microscope apparatus has also been reported in detail.\(^1,3\)

**Experimental Method**

The tip of the pipette was advanced through the platelet suspension under direct vision, while a constant negative pressure of \(4 \times 10^{-2}\) dynes/cm (1.0 cm H\(_2\)O) was maintained. When a platelet was contacted, a portion of the cell was pulled into the pipette (Figure 1). Subsequent increase in the aspiration pressure by 1 cm increments to final negative pressures of 41.0 to 82.0 \(\times 10^{-2}\) dynes/cm (10.0 to 20 cm of H\(_2\)O) resulted in progressively larger extension lengths into the pipette. The lengths of the aspirated platelet segments were recorded on videotape for analysis at a later time.\(^1,3\) Control cells were studied with the same pipette used to evaluate chilled and taxol-treated platelets. Minor differences in pipette internal diameter were also accounted for in the data analysis as described below.

**Data Analysis**

The changes in extension lengths were measured by using a Hi-pad Digitizer (Bausch and Lomb, Houston, Texas) interfaced with a Terak 8510 Graphic Computer (Terak Corporation, Scottsdale, Arizona).\(^1,3\) The behavior of the platelet was analyzed by graphing steady-state extension deformations against a corresponding negative pressure. In a manner similar to previous erythrocyte and white cell micropipette studies,\(^8-10\) a dimensionless extension parameter \((X_e)\) was obtained by dividing the platelet extension length in \(\mu\)m by the radius of the pipette \((R_p)\), also in \(\mu\)m. A tension stress parameter \((P \times R_p)\) was defined by multiplying the aspiration pressure \((P)\) by the pipette radius.

Linear regression analysis was performed on the data by using a multiple regression interactive computer program developed by Weisberg.\(^11\) By plotting the residuals of the linear regressions, it was possible to assess linearity through the full range of pressures and to detect any deviation from a linear function. The statistical significance of the differences between individual linear regressions were determined by fitting the data to four separate discriminant models: intercept and slope different; different intercept but same slope; same intercept but different slope, same intercept and slope. The resulting sum of squares, residuals, F ratio, and coefficient of determination \((R^2)\) were used to assess the validity of the models, and \(p\) values were determined from the ratio.

**Results**

**Control Resting Platelets**

The deformability of control platelets was evaluated in the micropipette by measuring the change in extension length (portion of the platelet drawn into the pipette) at increasing pressure increments.\(^1,3\) As shown in Figure 2, the stress-strain relationship of control platelets was characterized by a linear increase in cell extension length with
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Figure 2. Effects of ADP, A23187, and thrombin on the stress-response of normal platelets. The lines represent the computerized linear regression fits showing eventual plateauing in both treated and untreated platelets.

 increments in negative tension between 4.0 and 35.0 \times 10^{-2} \text{ dyne/cm}. Cell extension plateaued with a further increase in stress up to 41 \times 10^{-2} \text{ dyne/cm}. Extensions produced in the study reached a maximum steady-state length in 2 seconds. The portion of the cell remaining outside the pipette during aspiration retained its discoid shape, probably because even the longest segments aspirated represented less than 8% of the cell surface area. On release of pressure, the extension returned to the cell body and the unaltered cell form was restored within 1 minute after expulsion. The linear portion of the stress-strain relationship was described by the equation:

\[ x_c = A(P - \text{Rr}) + x_0 \]  

where A is the slope and x₀ the intercept. The cell extension produced by the lowest stress used in the study was termed \( x_c \). The \( x_c \) of the control platelets was 2.16 ± 0.12 and A was 11.23 ± 0.99 (range, 10.76 to 12.39), as shown in Table 1.

**Activated Platelets**

Resistance of platelets to deformation in micropipettes after exposure to aggregating agents was quantitively different from that of resting cells. The stress-response relationship of activated platelets, as in resting cells, was characterized by a linear increase in cell extension length with each increment in stress, followed by a plateau at greater tensions (Figure 2). The \( x_c \)'s of ADP, thrombin, and A23187 activated platelets were identical to control cells. However, activation doubled the length of cell extensions produced by the subsequent increases in aspiration tension. The tension at which the cell extension lengths on activated platelets began to plateau was similar to control cells. Maximum extension lengths observed on activated cells were twice those of control platelets. The part of the cell body that remained outside the pipette retained an irregular spherical shape during aspiration that did not change recognizably in size or shape. After expulsion from the pipettes, the extensions of the activated platelets returned to the cell bodies within 2 minutes. Essentially identical effects on deformability were produced by all three agonists (Table 1).

**Vincristine-Treated Platelets**

Samples of C-PRP incubated with 10^{-8} M vincristine for 30 minutes before dilution were devoid of circumferential microtubules when examined in the electron microscope. Resistance of vincristine-treated platelets to deformation in the micropipette was markedly reduced in all negative pressures, yielding a linear stress-strain relationship with a slope identical to that of chilled platelets. The addition of ADP, thrombin, or A23187 to platelets after incubation with vincristine did not further increase the altered deformability of these cells. The slope of the linear stress-strain relationship was identical to that of vincristine-treated platelets that had not been combined with ADP, thrombin, or A23187.

**Discussion**

Platelets in circulating blood resemble the flattened discus thrown by athletes. The small size of the cell and its shape favor location at the periphery of the column of blood flowing in the arterial system. Any injury to the endothelial lining of blood vessels results in immediate adhesion of platelets to the damaged site. Adherent platelets lose their discoid shape, extend long pseudopods and spread out on the exposed subendothelial surface. Products secreted by the surface-activated cells bring other platelets to the site, and these react with the damaged vessel and with each other to establish a hemostatic plug.

Much has been learned about the biochemistry and morphology of the hemostatic response, but little is known about the biorheology of the platelet. Measurements of the forces and factors involved in maintaining the discoid shape of discoid platelets and alterations that occur after

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**Table 1. Initial Cell Extension Length and Slope of the Linear Portion of the Stress-Response Relationship of Untreated, ADP-, Thrombin-, and A23187-Treated Platelets**

<table>
<thead>
<tr>
<th>Platelet treatment</th>
<th>Slope P.Rp (10^{-2})^{-1}</th>
<th>Initial cell extension (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>11.23 ± 0.99</td>
<td>2.16 ± 0.12</td>
</tr>
<tr>
<td>Adenosine diphosphate</td>
<td>27.86 ± 1.45*</td>
<td>2.08 ± 0.13</td>
</tr>
<tr>
<td>Thrombin</td>
<td>25.54 ± 1.21*</td>
<td>2.02 ± 0.09</td>
</tr>
<tr>
<td>Calcium ionophore A23187</td>
<td>25.52 ± 0.80*</td>
<td>2.19 ± 0.14</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation. *p < 0.001; comparison with untreated platelet.

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activation are virtually unknown. Deformability is clearly
important in platelet vessel-wall interaction, but the phe-
omenon has not been evaluated by appropriate tech-
niques. The precise mode of action by which many agents
currently in use block the platelet contribution to thrombotic
disease is unknown. It is possible that some of the agents
produce their effects by interfering with the mechanical
properties and behavior of the platelet in flowing blood. To
develop a new perspective on platelet function and patho-
physiology in the circulation, we have begun a series of
investigations to define mechanical properties of the cell.

The results of our present investigation have dem-
strated that the resistance of platelets to deformation in
micropipettes is significantly reduced after exposure to ag-
gregating agents. Adenosine diphosphate, thrombin, and
the calcium ionophore A23187, at concentrations that pro-
duced shape change and aggregation in parallel samples,
all caused similar increases in platelet extensibility. The
differences between activated and control platelets were
revealed by examining the stress-response relationship
over a range of deforming stresses from 4.0 to 41.0 × 10⁻²
dyne/cm (−1 to −10 cm H₂O). At the lowest negative
pressure evaluated (X), there were no differences in ex-
tension lengths aspirated from the surfaces of control and
activated platelets. At first this result was surprising, since
fundamental changes in biochemistry and physical organi-
ization develop rapidly in platelet membranes after expo-
sure to aggregating agents.¹¹ Diglycerides and fatty acids
are cleaved from the cell wall,¹³ and phospholipids under-
go significant reorientation.¹⁴ The alterations might be ex-
pected to change the intrinsic membrane properties, re-
sulting in less resistance to deformation in the
micropipette. Failure to demonstrate any differences in
lengths of segments aspirated on control and activated
platelets at the lowest negative pressure suggests that
intrinsic properties of the lipid bilayer may not play a signifi-
cant role in platelet deformability.

The submembrane zone¹⁵ lying just under the cell sur-
face, however, may provide as formidable a barrier to aspi-
ration of activated platelets as control cells. Recent inves-
tigations using simultaneous fixation and detergent
extraction in the presence of lysine¹⁶ and phalloidin¹⁷ to
protect actin filaments have resulted in excellent preserva-
tion of activated platelets as control cells. Recent ultrastructural morphometric studies have
shown that activation of resting platelets by thrombin
and ADP reduced the diameters of microtubule coils up to
50%.¹⁷ Since the circumferential microtubule is the major
structure resisting aspiration of resting platelets,¹⁶⁻²⁰ reduc-
tion in the size of the coil would be expected to free up
more cell surface for entry into the pipette. In the present
study, activated platelets demonstrated a plateau of micro-
pipette extension segments twice those observed on con-
trol platelets. This finding that increased extension lengths
occur on activated platelets at the same deforming stress
as the shorter lengths on control cells further supports the
concept that greater extensibility of activated platelets is
related to increased availability of surface area for defor-
mation.

It is possible, however, that the increased length of seg-
ments drawn from activated platelets was due to extrusion
of channels from the surface connected open canalicular
system. Morgenstern et al.²² have suggested that activa-
tion and formation of pseudopodia result in an approximate doubling of the platelet surface with a corresponding reduction in platelet endomembrane systems. If, as suggested, the rapid increase of platelet surface was due to evagination of elements from the surface connected channel system, there would be no need for internal contraction and constriction of microtubule coils away from the surface to make more membrane available.

A reasonable answer to this objection was suggested in a recent study by Zucker-Franklin et al. Examination of bovine platelets by techniques of freeze fracture and electron-dense tracers failed to delineate a surface connected canalicular system in these cells. In preparation for the present study, we carried out a micropipette evaluation of bovine platelets. Discoid bovine platelets were identical to resting human cells in their resistance to deformation. Exposure to ADP, thrombin, and A23187 produced the same decreases in resistance to aspiration as noted for human platelets in the present investigation. Thus, the surface-connected canalicular system does not appear to be a major source of membrane for the increased length of segments aspirated from activated platelets.

The importance of the circumferential microtubule to development of the plateau at higher negative pressures was demonstrated in our previous investigations. Removal of the microtubule coils by chilling, vincristine, or colchicine resulted in aspiration of segments twice as long as those drawn from untreated platelets at a constant negative pressure of 10 cm H2O. In the present study, treatment with vincristine resulted in a linear stress-strain relationship with a slope identical to that produced by chilling. The addition of thrombin, ADP, or A23187 to vincristine-treated platelets had no further effect on deformability. Thus, the constricted microtubule coils appear just as important for development of the pressure plateau in activated platelets as in resting cells.

In summary, the present study has demonstrated significant changes in the resistance of platelet membranes to deformation in micropipettes after exposure to aggregating agents. At the lowest negative tension, 4 × 10^-2 dynes/cm (-7.5 cm H2O), there were no differences in segment lengths aspirated on control platelets and on platelets after exposure to ADP, thrombin, or A23187. At each subsequent increase in negative pressure, longer segments of membrane were drawn on activated than on control platelets. Lengths of aspirated segments on resting and stimulated cells plateaued at a negative pressure of 35 × 10^-2 dynes/cm (-7.5 cm H2O). The findings suggest that constriction of the circumferential microtubule in activated platelets makes more membrane available for aspiration in response to increasing negative pressures. The presence of the coiled microtubule before and after exposure to aggregating agents provides a barrier so that further increases in negative pressure result in a plateau in membrane segment lengths. Thus, the circumferential microtubule appears to be a major factor in platelet resistance to deformation before as well as after activation.

References


Index Terms: platelet deformability • platelet aggregation • activated cells • anticoagulant • citrate • thrombin • adenosine diphosphate • calcium ionophore A23187 • micropipette aspiration • stress response
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