Micropipette Aspiration of Human Platelets after Exposure to Aggregating Agents

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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, \(4 \times 10^{-2}\) 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 \(35 \times 10^{-2}\) 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 \(35 \times 10^{-2}\) dynes (−7.5 cm H2O).


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 antimitic 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 \(\times 10^{-2}\) 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.\(^4\)\(^-\)\(^6\) 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.\(^4\)\(^-\)\(^6\) 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.\(^7\) One volume of the C-PRP mixture was then added to 9 volumes of Ca\(^{++}\)/Mg\(^{++}\)-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 $10^{-5}$ 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 µm segment of the pipette. Internal diameters of the pipettes were measured on the video monitor that was standardized with a 10 µm 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 (Defon-brune, 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 platelet. 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 µm by the radius of the pipette ($R_p$), also in µm. A tension stress parameter ($P - R_p$) 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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\[ x_c = A(P \cdot R_p) + x_0 \]  

where A is the slope and \( x_0 \) the intercept. The cell extension produced by the lowest stress used in the study was termed \( x_c \). The \( x_c \)'s of the control platelets was 2.16 ± 0.12 and A was 11.23 ± 0.99 (range, 10.75 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^-7 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. 3 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
activation are virtually unknown. Deformability is clearly important in platelet vessel-wall interaction, but the phenomenon has not been evaluated by appropriate techniques. 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 pathophysiology in the circulation, we have begun a series of investigations to define mechanical properties of the cell.

The results of our present investigation have demonstrated that the resistance of platelets to deformation in micropipettes is significantly reduced after exposure to aggregating agents. Adenosine diphosphate, thrombin, and the calcium ionophore A23187, at concentrations that produced 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 extension lengths aspirated from the surfaces of control and activated platelets. At first this result was surprising, since fundamental changes in biochemistry and physical organization develop rapidly in platelet membranes after exposure to aggregating agents. Diglycerides and fatty acids are cleaved from the cell wall, and phospholipids undergo significant reorientation. The alterations might be expected to change the intrinsic membrane properties, resulting 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 significant role in platelet deformability.

The submembrane zone lying just under the cell surface, however, may provide as formidable a barrier to aspiration of activated platelets as control cells. Recent investigations using simultaneous fixation and detergent extraction in the presence of lysine and phalloidin to protect actin filaments have resulted in excellent preservation of membranes and organelles. The submembrane zone in resting cells prepared in this manner is a fine amorphous layer with some actin filaments. Actin is more prominent in the submembrane zone of activated platelets, but the layer is just as imposing as in resting cells. Platelets require 10 times the negative pressure necessary to draw red cells into micropipettes, and the submembrane zone may provide the major barrier. Since the submembrane zone of activated platelets appears about as dense as that of discoid cells, it is probable that both cells would offer the same degree of resistance to deformation.

Following exposure to further increments in negative pressure, the extension lengths on stimulated platelets became progressively longer than those on control cells until both plateaued at different maxima on exposure to tensions above 35 × 10^{-2} dyne/cm. The stress-response relationship was linear between 4.0 and 35 × 10^{-2} dyne/cm for both control and activated platelets, but activation doubled the slope of the linear response. Maximum extensions were longer on activated platelets than on control cells, but occurred at the same deforming stress. The larger maximum extension length of activated platelets was due solely to the doubling of the slope of the linear portion of the stress response relationship.

The increased deformability following activation was less marked and qualitatively different than the increased micropipette extensibility observed after chilling. Exposure of platelets to 2° to 4° C for 15 to 30 minutes completely dissolved the circumferential band of microtubules in platelets. Aspiration of chilled platelets revealed a dramatic increase in extension lengths at each increment throughout the full range of pressures. This linear increase in extension lengths, unlike that of control or activated platelets, continued to increase at high deforming stresses, revealing only a slight reduction in slope. Absence of the extension length plateau at higher pressures in chilled cells was due to near passage into the pipette as evidenced by diminution in size of the cell portion remaining outside.

The structural basis for the increased deformability of activated platelets may be related to changes in the size and organization of the circumferential band of microtubules. Control platelets did not pass through micropipettes and reached maximum cell extension length aspirations without diminution in the size of the cell body remaining outside the pipette. The importance of an intact coil of microtubules for this mechanical behavior was shown by the chilling studies where removal of the coil allowed partial cell passage. Aggregating agents trigger a process of internal contraction resulting in constriction of the coils into tight rings around centrally concentrated organelles. The internal transformation is also associated with a concentration of the actin filaments at the cell periphery and around the constricted microtubule rings in the cell centers. Reduction in the number of organelles and filaments in the cytoplasm between centrally constricted elements and the submembrane zone may be a principal reason for the decreased resistance of activated platelets on aspiration at intermediate negative pressures.

Recent ultrastructural morphometric studies have shown that activation of resting platelets by thrombin and ADP reduced the diameters of microtubule coils up to 50%. Since the circumferential microtubule is the major structure resisting aspiration of resting platelets, reduction 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 micropipette extension segments twice those observed on control 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 deformation.

It is possible, however, that the increased length of segments drawn from activated platelets was due to extrusion of channels from the surface connected open canalicular system. Morgenstern et al. have suggested that activa-
tion and formation of pseudopodia result in an approximate doubling of the platelet surface with a corresponding re-
duction in platelet endomembrane systems. If, as suggest-
ed, the rapid increase of platelet surface was due to evag-
nination 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.23 Examination
of bovine platelets by techniques of freeze fracture and elec-
tron-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.
Ex-
posure 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 de-
velopment of the plateau at higher negative pressures was
demonstrated in our previous investigations.1 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 relation-
ship with a slope identical to that produced by chilling.3 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 signifi-
cant changes in the resistance of platelet membranes to
deformation in micropipettes after exposure to aggregating
agents. At the lowest negative tension, 4 \times 10^{-2} dynes/cm
(\sim 1 \text{ cm } H_2O), 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 plate-
lets. Lengths of aspirated segments on resting and stimu-
lated cells plateaued at a negative pressure of 35 \times 10^{-2}
dynes/cm (\sim 7.5 \text{ cm } H_2O). The findings suggest that con-
striction 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 ag-
gregating agents provides a barrier so that further in-
creases in negative pressure result in a plateau in mem-
brane segment lengths. Thus, the circumferential micro-
tubule appears to be a major factor in platelet resistance to
deformation before as well as after activation.

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