Mechanism of Shape Change in Chilled Human Platelets

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The so-called cold activation of platelets that precludes refrigeration of platelets for storage has long been recognized, but its mechanism has remained a mystery. Cooling of discord resting platelets to temperatures below 15°C causes shape distortions, and the chilled cells rewarmed to above 25°C are spheres rather than discs. As platelet shape change responsive to receptor activation at normal temperatures requires the remodeling of an actin scaffold (Hartwig JH, 1992, J Cell Biol 118:1421-1442), we examined the role of actin in the morphologic changes induced by cooling. The addition of actin monomers onto the fast-exchanging (barbed) ends of actin filaments accompanies the initial physiologic platelet shape changes, and a key control point in this growth is the removal of proteins (caps) from the filament ends. This uncapping of actin filament ends is mediated by polyphosphoinositide aggregates in vitro, suggesting that cold-induced phase changes in membrane lipids might uncap actin filaments and thereby account for actin assembly-mediated shape alterations during cooling. Consistent with this hypothesis, reversible inhibition of actin assembly with cytochalasin B prevented the distortions in shape, although cooled platelets had increased actin nucleation sites and became spherical. Another step in normal platelet shape changes requires the severing of actin filaments that maintain the resting platelet. The proteins that sever initially bind to the broken filament ends, and uncapping of these fragmented filaments provides numerous nucleation sites for growth of actin filaments to fill in spreading filopodia and lamellae. Actin filament fragmentation requires a rise in intracellular calcium, and we showed that chilling platelets from 37°C to 4°C increases free cytosolic calcium levels from 80 nmol/L to approximately 200 nmol/L in minutes, thus providing an explanation for the spherical shape of cooled, rewarmed platelets. Blocking the calcium transient with nanomolar concentrations of the permeant calcium chelators Quin-2 and Fura-2 prevented the increase in nucleation sites and the spherical, but not the other shape changes of chilled and rewarmed platelets. However, a combination of micromolar cytochalasin B and millimolar intracellular calcium chelators preserved the discoid shapes of chilled and rewarmed platelets. After removal of cytochalasin B and addition of sufficient extracellular calcium, these platelets responded with normal morphologic alterations to glass and thrombin activation.

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PLATELETS CIRCULATE in blood as thin discs with smooth surfaces. A variety of stimuli rapidly cause platelets to undergo irreversible shape changes with protrusion of spikes (filopodia), veils (lamellae), or both, to secrete bioactive products, and to aggregate. The shape change is driven by the destruction of an actin-dominated skeleton in the discoid resting platelet, followed by its remodeling into the new shapes found in the activated cell.1,2 This remodeling is accomplished by an increase in the fraction of total cellular actin in a polymeric state (F-actin).3 Eversion of an extensive array of internal membranes, the open canalicular system, and granule fusion during secretion allow the membrane to accommodate these architectural rearrangements.

Changes in platelet shape can also be caused by chilling cells to below 15°C in the absence of other stimuli, a process termed cold-activation. Platelets contain a peripheral microtubule coil that disappears in the cold,4 and disassembly of this microtubule coil has been blamed for releasing constraints holding cells in the discoid shape. However, depolymerization of microtubules with agents such as colchicine has little effect on the structure of resting platelets maintained at 37°C.5,6 In contrast, the actin polymer system of the platelet, which is responsible for agonist-induced shape changes at physiologic temperatures, has received surprisingly little attention in cold-activation. We report here that the cooling of platelets induces actin to assemble. This actin assembly is best explained by removal of the blockade (uncapping) of actin filament ends that controls, in part, the level of actin polymerization in resting platelets. The blocked ends where uncapping occurs are the fast-exchanging ends, identified as barbed by arrowheads of myosin subfragment 1 bound to the sides of actin filaments. Uncapped, barbed filament ends serve as nuclei for the addition of actin subunits, and the elongating actin filaments modify cell shape. This cold-induced nucleation of actin filaments does not require elevation of free cytosolic calcium, although calcium levels do rise during chilling. Increased cytosolic calcium does contribute to the cold-induced shape changes by promoting the severing of cortical actin filaments. The shape changes of platelets caused by cooling can be blocked by preventing the rise in cytosolic calcium and inhibiting the assembly of actin subunits onto cold-exposed actin nuclei.

MATERIALS AND METHODS

Preparation of resting platelets. Human blood from healthy volunteers, drawn into 0.1 vol of a citrate-based anticoagulant (Aster-Jandl), was centrifuged at 110g for 10 minutes. The platelet-rich plasma was gel-filtered through a Sepharose 2B column equilibrated and eluted with a solution containing 145 mmol/L NaCl, 10 mmol/L HEPES, 10 mmol/L glucose, 0.5 mmol/L NaH2PO4, 5 mmol/L KCl, 2 mmol/L MgCl2, and 0.3% bovine serum albumin, pH 7.4 (platelet buffer). The cells were left standing for 30 minutes at 37°C to insure a resting state.9 Platelets in suspension at 2 × 10^10/mL were chilled to 4°C by placing them in an ice-water slurry.

In experiments designed to stabilize platelets against chilling, platelets were loaded for 30 minutes at 37°C with 40 μmol/L Quin-
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2AM or Fura-2AM from a 6-mmol/L stock solution in 50% dimethyl sulfoxide (DMSO). A final concentration of 2 μmol/L cytochalasin B from a 10-mmol/L stock solution in 100% DMSO was then added for 2 minutes, and then the cells were chilled. To restore cells to a resting state, cells were returned to 37°C. Cytochalasin B was removed from cells incubated at 4°C by sedimenting the cells at 250g for 5 minutes, thereby decreasing the amount of buffer containing cytochalasin B by approximately 1,000-fold. The cell pellet was then resuspended in platelet buffer containing 1 mmol/L CaCl2 to overwhelm the calcium chelator.

Measurement of actin filament content and nucleation activity. The polymerized actin (F-actin) content of resting and chilled platelets was determined by quantitating fluorescein isothiocyanate (FITC)-phallolidin binding to paraformaldehyde-fixed and detergent-permeabilized cells. Platelets were fixed after chilling for various times by the addition of an equal volume of ice-cold 3.7% paraformaldehyde for 30 minutes. The fixed cells were permeabilized by adding 0.1 vol of 1.0% Triton X-100 containing 100 μmol/L FITC-phallolidin.8,10 The mean fluorescence of 10,000 cells was quantitated in a flow cytometer (Becton-Dickinson FACScan, Mansfield, PA) after a minimum of 60 minutes of exposure to the FITC-phallolidin. The platelets were gated by forward- and side-scattering.

The nuclei content in detergent lysates from resting and chilled platelets was determined by assaying the effect of the cell lysates on the rate and extent of pyrene-labeled rabbit skeletal muscle actin polymerization, as described previously.11 Suspensions of resting or chilled cells at concentrations of 2 × 108/mL were permeabilized by the addition of 0.1 vol of 60 mmol/L Pipes, 25 mmol/L HEPES, 10 mmol/L EGTA, 2 mmol/L MgCl2, 1.0% Triton and 210 mmol/L leupeptin, 658 μmol/L benzamidine, and 15.3 μmol/L aprotonin to inhibit proteases (10 × PHEM-Triton buffer)11 and 5 μmol/L phallacidin to stabilize actin filaments against depolymerization by dilution. One hundred microliters of each detergent lysate was added to 190 μL of 100 mmol/L KCl, 2 mmol/L MgCl2, 0.5 mmol/L adenosine triphosphate (ATP) 0.1 mmol/L EGTA, 0.5 mmol/L dithiothreitol, and 10 mmol/L Tris, pH 7.0. The polymerization rate assay was started by the addition of monomeric pyrene-labeled rabbit skeletal muscle actin to a final concentration of 1 μmol/L. In this assay, pyrene-labeled actin monomers are added to cell lysates to detect exposed actin filament ends contained in them. The spontaneous aggregation of actin monomers into trimer nuclei is relatively slow. Therefore, if exposed actin filament ends (or their equivalent) are present in platelet cytoskeletons, actin assembly occurs more rapidly. As the rate of monomer addition is 10-fold higher at the barbed end than the pointed, filaments grow most rapidly in the barbed direction. The relative contribution of pointed versus barbed filament ends to nucleation is determined by assaying the effect of detergent-extracted platelet cytoskeletons on the rate of actin assembly in the presence of 2 μmol/L cytochalasin B. Cytochalasin B inhibits assembly of actin at the barbed filament end under these conditions, and pyrene actin assembly in the presence of cytochalasin B is ascribed to nucleation off the pointed ends of filaments. Cytochalasin B-inhibitable activity is defined as barbed-end assembly.1

Measurement of intracellular Ca2+ concentration. The resting levels of cytosolic calcium in platelets maintained at 37°C or chilled to 4°C in the presence of 0.1 mmol/L extracellular CaCl2 were determined fluorometrically. To measure the intracellular free calcium concentrations, platelets suspended to 2 × 108/mL were loaded with the calcium-sensitive dye Fura-2 by incubating them with 2 μmol/L Fura-2AM for 30 minutes at 37°C. Excess Fura-2AM was removed from the platelets by centrifuging the platelet suspension at 1,000g for 10 minutes in the presence of 3 mmol/L EDTA. The resultant cell pellet was resuspended at 108/mL in platelet buffer containing 3 mmol/L EDTA. The platelets were then diluted further to the original concentration of 2 × 108/mL with platelet buffer lacking EDTA, and 0.6 mmol/L MgCl2 was added to the cell suspension. Because of the temperature sensitivity of Fura-2 fluorescence, a fluorescence maximal in 0.1 mmol/L CaCl2 and minimal in 5 mmol/L EGTA was determined at both 4°C and 37°C temperatures in the presence of 10 μmol/L ionomycin. Calcium concentrations were calculated for each temperature using these values. Measurements were performed at excitation wavelengths of 340 nm and 380 nm for bound and unbound probe, respectively, using an emission wavelength of 510 nm in a Perkin-Elmer Fluorimeter LS-50 (Norwalk, CT).13

Calcium clamping. To maintain cytosolic calcium at or below its normal resting levels during cooling or thrombin receptor-activating peptide (TRAP) activation, cells were loaded with 40 μmol/L Fura-2AM or Quin-2AM for 30 minutes at 37°C as described above. The total Fura-2 content loaded into the cells was determined by releasing sequestered Fura-2 with 0.1% Triton X-100, and total fluorescence was quantitated in the presence of 1 mmol/L added calcium. A standard Quin-2 or Fura-2 concentration curve was generated using the free acids of each. Fura-2 content in cells was calculated using a cell volume of 7 fL.14

Morphological studies: Light microscopy. Resting, activated, and chilled platelets were fixed for 10 minutes in suspension by the addition of 1 vol of 2% glutaraldehyde. After fixation, the cells were pelleted onto the surface of a polylysine-coated coverslip by centrifugation at 250g for 5 minutes. Coverslips with adherent cells were washed with water, dried, mounted on microscope slides using aquapoly-mount (Polysciences, Inc., Warrington, PA), and photographed using disseminated intravascular coagulation (DIC) optics in a Zeiss IM-35 inverted microscope (Thornwood, NY). To quantitate morphologic changes, platelets were categorized and counted as either resting (discs) or having changed shape (lamellae, filopodia, and/or blebs) in each condition.

Electron microscopy: Rapid freezing and freeze-drying. Platelets were chilled for 30 minutes, and 250 μL of cells suspended to a concentration of 5 × 108/mL were placed in wells of a 96-well microtiter plate, each containing a polylysine-coated coverslip. The plate was centrifuged at 1,460g for 5 minutes at 4°C. Replicas of chilled cytoskeletons were prepared by permeabilizing the cells with PHEM-0.75% Triton X-114 buffer containing 5 μmol/L phallacidin and protease inhibitors for 2 minutes. The cytoskeletons were washed in PHEM buffer containing 0.1 μmol/L phallacidin and fixed in PHEM containing 1% glutaraldehyde for 10 minutes. Coverslips containing cytoskeletons were extensively washed with distilled water, rapidly frozen, freeze-dried, and rotary-coated with 1.4 nm of tantalum-tungsten and 2.5 nm of carbon without rotation in a Cressington CPE-50 apparatus (Cressington, Waford, UK).

RESULTS

Morphology of chilled platelets and of cytoskeletons from chilled platelets. Cooling of platelets to 4°C results in alterations of their shapes. To examine the morphology of cooled cells and the structure of their underlying actin cytoskeletons, it was necessary to attach the cold-activated platelets to glass coverslips. For light microscopy, the chilled cells were first fixed and then applied to the coverslips. However, to study the underlying cytoskeletons, the cells could not be permeabilized and fixed until after surface attachment. While surface contact activates platelets maintained at 37°C in similar fashion to thrombin, we are confident that chilled cells are not responsive to glass activation or thrombin, because chilled platelets in suspension were completely unresponsive to thrombin, a more potent activator than glass, as determined in F-actin content and nucleation assays (data not

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shown). As shown in Fig 1, platelets exposed to cold temperatures for 60 minutes convert from discoid shapes (Fig 1a) by extending small lamellipodia, surface blebs, and longer filopodia (Fig 1b). When shape change was quantitated from light micrographs, 91% of the cells (n = 702) altered their morphology as described in the cold. These structural changes have previously been observed in the light microscope and in thin sections of chilled and embedded cells viewed in the electron microscope.

Replicas from frozen and freeze-dried cytoskeletons provide more detailed information on structural rearrangements of actin that occur after chilling. Figure 1C shows the organization of cytoskeletons prepared from cells cooled on ice for 30 minutes. The cytoskeletons from two chilled cells are shown, one of which formed filopods (left) and the other which spread circumferentially (right). A bundle of actin filaments fills each filopod (left). Filament bundles are also observed around the cytoskeletal periphery (arrowheads). The residue of a membrane skeleton can be seen in the background of the actin fibers. We have previously described the detailed structure of this spectrin-based membrane skeleton in the resting platelet. In the resting cell, the membrane skeleton is a dense planar network stabilized by underlying actin filaments. In the cytoskeleton of the spread cell, actin filaments are arranged into two different types of structures.

The cytoskeletal periphery, corresponding to the spread region of the cell, is composed of short, interconnecting filaments. Long filaments are found in the center of the cytoskeleton. These filaments spiral out from the cytoskeletal center. Platelets permeabilized in the cold with Triton X-114 retain a central mass of membranous material that is detergent-resistant.

Chilling of human blood platelets causes actin assembly. Figure 2 shows that when human platelets are chilled to ice-bath temperature for as little as 5 minutes or as long as 120 minutes, their filamentous actin content increases relative to resting cells maintained at 37°C. Actin assembly in cells was maximal at 5 minutes after exposure to the cold, whereas the cellular F-actin content reached 60% of the total. It then decreased slightly and stabilized at a new elevated level relative to resting cells in the warm. This overshoot may be caused by the cooling process, while the subsequent values of 50% to 56% represent the new 4°C F-G actin equilibrium. When compared with cells activated with 1 U/mL of thrombin or 10 μmol/L TRAP at 37°C, the total amount of F-actin assembled in chilled cells was considerably less (54% ± 80%; Fig 6). Addition of 2 μmol/L cytochalasin B18,19 to the cells before chilling inhibited the increase in actin filament content with chilling (data not shown). Filament assembly induced by chilling is, therefore, restricted to monomer addition to the barbed ends of filaments in the cells.
Exposure of the barbed ends of actin filaments permits the transfer of actin subunits bound to sequestering proteins, such as β4-thymosin, to the barbed ends of actin filaments.1,39 Therefore, we determined the effect of chilling on the ability of platelet lysates to nucleate actin assembly in vitro. Platelets chilled on ice for various time intervals were lysed with detergent, and the lysates were assayed for their ability to increase the rate of pyrene-actin assembly. Figure 3 shows that in detergent lysates from cells chilled for time intervals that generate maximal actin filament content the number of exposed barbed-end nuclei increases twofold to fivefold (resting cells contain 50 to 80 free barbed filament ends). Addition of cytochalasin B to the in vitro assembly assay inhibited the increase in the actin assembly rate, confirming that growth occurs selectively from the barbed ends of nuclei in the chilled cells. Based on the monomer addition rate to the barbed ends of actin filaments,161 chilling exposed 160 to 240 new barbed ends per platelet relative to resting cells. 

Cooling increases cytosolic calcium concentrations in platelets. As actin assembly in agonist-treated cells is mediated by calcium-dependent and independent pathways, we determined if cooling alters platelet calcium metabolism. In agreement with earlier work,22,23 resting cells maintained at 37°C had intracellular ionized calcium concentrations of 81 ± 12 nmol/L (n = 3) at 37°C. Intracellular calcium was, however, markedly increased by cooling and reached a 174 ± 16-nmol/L concentration (n = 3) 5 minutes after exposing the cells to the cold. Once the cells had equilibrated to the ice bath temperature, intracellular ionized calcium stabilized at an elevated level of approximately 200 nmol/L concentration: intracellular ionized calcium concentrations were 189 ± 23 nmol/L (n = 3) and 199 ± 18 nmol/L (n = 3) after 10 and 60 minutes in the cold, respectively. These data suggested that calcium could be responsible, in part, for the changes in cell shape and the actin assembly that occur after cooling the platelets.

Effect of calcium chelation on cold-induced shape change. Platelets were loaded with high amounts of the intracellular calcium chelator Quin-2AM to determine if preventing the rise in intracellular calcium would modify the cellular responses to cooling. After loading for 30 minutes in 40 μmol/L Quin-2AM, cells accumulated 3.8 to 6 nmol/L of Quin-2. Although this concentration of Quin-2 completely prevented the calcium increases observed after cooling, it did not abolish the increases in F-actin and nuclei content in cooled cells. Chelation of calcium did, however, reduce the amount of actin assembled into filaments and the number of nucleation sites exposed in response to cooling of the cells (Fig 2 and 3).

As peripheral actin filaments in platelets are rapidly fragmented when cytosolic calcium increases transiently during activation,1 the effect of cytoplasmic calcium chelation on the morphology and cytoskeletal organization of chilled cells was also investigated. Figure 4a shows that platelets loaded with 40 μmol/L Quin-2AM for 30 minutes extended blebs and filopodia but not lamellipodia when cooled for 60 minutes. Visual quantitation of shape change showed that 98%
of platelets (n = 1,364) had distorted from discs under these conditions. The filopodia in chilled cells differed from those in thrombin- or glass-activated cells at 37°C, in that their distal ends were enlarged. In this respect, they are similar to filopodia extended by calcium-chelated, glass-activated cells at 37°C. Cytoskeletons prepared from the cold-activated chelated platelets show only long peripheral filaments (Fig 4c, arrows). The filaments run parallel to the cell margins and into filopods extended from the cell margins. The organization of actin filaments within these abnormal filopodia is, therefore, similar, to actin filaments filling filopodia of agonist-activated, warm cells loaded with Quin-2AM.1

Although chilling platelets begins a cascade of events that leads to irreversible shape change and aggregation, a partial reversal of the shape change occurs if the cells are rewarmed shortly after chilling. However, the rewarmed cells do not return to the discoid shapes of the resting cell, but instead become spherical (Fig 5b). Although Quin-2AM does not inhibit cell shape change with cooling, it effectively blocks the rounding of rewarmed cells, and long filopodia remain extended from the cell surfaces (Fig 5a). This finding suggests that changes in cytosolic calcium are required for the pseudorecovery of chilled and rewarmed platelets.

Prevention of chilling- and rewareming-induced shape changes. One prediction of the hypothesis that shape distortions induced by cooling are due to uncapping of actin filaments ends is that treatment with cytochalasin B, a reagent that binds to and caps the barbed end of actin filaments, should inhibit these changes. As shown in Fig 5b, cytochalasin B prevents gross alterations in the shape of chilled cells, as has been reported previously. However, it does not preserve cells in the resting discoid shape, and after treatment with cytochalasin B and cooling, the cells are round. Cytochalasin B in conjunction with Quin-2AM, however, stabilizes chilled cells as discs (Fig 4b). When rewarmed, these cells retain the ability to spread lamellipodia and form filopods when the cytochalasin B is removed and sufficient calcium (1 mmol/L) is added to the extracellular medium to replete free calcium to functional levels in the chelated cells (Fig 5c).

The stabilizing effect of a combination of cytochalasin B and Quin-2AM is also apparent from in vitro measurements of the actin nucleation (Fig 6) capacity of chilled cells. Resting platelets contain little barbed end-based nucleation activity, while thrombin-treated platelets increase the exposure of their barbed ends 10-fold. As shown in Figs 3 and 6, actin assembly in vitro is stimulated by lysates prepared from cooled cells. To restore the cells to a resting state, the cytochalasin B must be removed after the cells have been rewarmed to 37°C. Removal of cytochalasin B at 4°C causes
when cooled, which suggests that the cytoplasmic machinery supporting these structures relaxes. Platelets, however, distort from a discoidal shape when cooled to less than 15°C by extending filopodia and small lamellipodia. We report here that the driving force for these protrusions in the cold is a result of actin filament assembly after uncapping of a small number of actin filament barbed ends. Cooling also increases cytosolic calcium levels, and this increase in calcium contributes to the shape change. A calcium-dependent severing of actin filaments leads to swelling of chilled platelets after rewarming (Fig 7, step d).

Mechanism of actin assembly in chilled cells. Part of the stimulus for shape change in the cold-activated platelet appears to be the exposure of barbed filament ends. Lysates prepared from resting cells have fewer than 50 free barbed filament ends, out of a total of 2,000 that exist per cell, as

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Cold-induced protrusive activity of a cell is unusual. Most eukaryotic cells withdraw protrusions and become spherical

Fig 5. Reversal of the stabilizing effects of Quin-2AM and cytochalasin B on the morphology of chilled and rewarmed platelets. (a) Morphology of platelets preloaded with 40 μmol/L Quin-2AM for 30 minutes at 37°C, chilled to 4°C for 30 minutes, rewarmed for 30 minutes at 37°C, and then attached to coverslips and fixed; (b) morphology of platelets incubated with 2 μmol/L cytochalasin B for 5 minutes at 37°C and 30 minutes at 4°C and then rewarmed for 30 minutes in the presence of cytochalasin; (c) morphology of platelets treated with 2 μmol/L cytochalasin B and 40 μmol/L Quin-2AM, chilled to 4°C for 30 minutes, rewarmed to 37°C for 30 minutes, and the cytochalasin B removed by changing the bathing solution and plated on glass coverslips in medium containing 2 mmol/L CaCl₂. The bar is 5 μm.

Fig 6. Reversal of barbed-end nucleation activity after chilling platelets. (1) Actin nucleation activity present in detergent lysates from cells chilled for 90 minutes at 4°C. (2) Actin nucleation activity present in detergent lysates from cells preloaded with 40 μmol/L Quin-2AM for 30 minutes at 37°C, then 2 μmol/L cytochalasin B for 2 minutes, followed by chilling for 90 minutes at 4°C. The cytochalasin was then removed by centrifugation and replacing the bathing liquid. The cells were then lysed, and nucleation activity was assayed. This procedure did not modify the effect of cold on increasing nucleation activity in the cells. (3) The procedure used in (2) has been modified to restore nucleation activity to resting levels. Actin nucleation activity present in detergent lysates from cells loaded with 40 μmol/L Quin-2AM for 30 minutes at 37°C, then 2 μmol/L cytochalasin B for 2 minutes, followed by chilling for 90 minutes at 4°C. The cytochalasin was then removed by centrifugation and replacement of warm bathing liquid lacking cytochalasin. The cells were then lysed at 37°C, and nucleation activity was assayed. (4-6) Restoration of nucleation activity in chilled and rewarmed cells. (4) Cells were prepared as in (3), resuspended in platelet buffer lacking calcium, and treated with 1 U/mL of thrombin for 30 seconds. In the absence of external calcium, the cells remain unresponsive to thrombin. (5) Control warm cells exposed to thrombin for 30 seconds. In response to 1 U/mL of thrombin, cells dramatically increased their nucleation activity. (6) Restoration of responsiveness of chilled cells to the agonist thrombin. Cells were treated as in condition (4), except that they were resuspended in warm platelet buffer containing calcium before and after removal of the cytochalasin B.
Fig 7. Schematic summary of the cytoskeletal rearrangements that occur when platelets are chilled. (a) The resting cell. The plasma membrane of the resting human platelet is invested with a dense membrane skeleton. Major components of the membrane skeleton are glycoproteins, spectrin, actin-binding protein 280 (ABP), and actin filaments. Actin filaments in the cytoplasm are stabilized by ABP crosslinks and run to the membrane, where they end. All barbed actin filament ends are capped in the resting cell. (b) Shape change during cooling results from the severing of some of the membrane-associated portions of actin filaments. Severing of actin requires an increase in cytosolic calcium because it is abated if cells are preloaded with the permeant calcium chelator Quin-2AM. Actin filament assembly is potentiated when the barbed ends of severed and preexisting filaments become exposed. (c) Proof that exposed barbed ends drive the assembly of actin is derived from the use of cytochalasin B, which blocks the net assembly of actin in the cooled cell. However, despite the blocking of filament growth, cytochalasin B treatment alone does not maintain the discoid resting shape when cells are cooled. Stabilization of the discoid shape also requires chelation of cytoplasmic calcium. (d) Rewarming of cooled cells results in an irreversible conversion to spherical shapes. (e) Shape changes occur in chelated cells because endogenous actin filaments elongate when barbed ends become uncapped and form abnormal filopodia. Actin assembly occurs on the barbed ends of preexisting filaments. The cell body retains its discoid shape because filament severing is inhibited. This keeps the membrane skeleton in its dense, unsprung form. (f) If cells treated with both Quin-2AM and cytochalasin B are rewarmed, the cytochalasin is removed, and calcium is added back to the media, normal activation of the cells is possible.

determined by the enhancement of cytochalasin B-sensitive actin assembly. Platelets activated by thrombin yield lysates that contain 10-fold more free barbed ends, and lysates from chilled platelets increase their content of free barbed ends twofold relative to those from resting cells. The maintenance of a large monomeric actin pool and of a stable filament population in resting platelets requires the concerted efforts of proteins that cap barbed filament ends and that sequester actin monomers, preventing their spontaneous assembly into filaments. Based on our studies in platelet lysates and studies on the interaction of actin with monomer binding proteins and capping proteins in vitro, which are likely to be involved in the regulation of these events in platelets, the most critical step for initiation of actin assembly is the unveiling of barbed filament ends. We have hypothesized that gelsolin and capZ, proteins that can bind tightly to barbed filament ends in vitro and are abundant in platelets cap the filament ends in the resting cell (Fig 7, step e). Binding of gelsolin to actin requires micromolar calcium, but once gelsolin is bound to a filament, calcium chelation is insufficient to remove it from a filament end. Binding of capZ to actin filament ends is not known to be regulated. Selve and Wegner have shown that when greater than 95% of barbed actin filament ends are capped by gelsolin in vitro the critical concentration of the monomeric actin exchanging with actin filaments in solution increases to that determined by exchange with the pointed end alone. As resting platelets have approximately 98% of their barbed filament ends capped and all their pointed ends free, the free monomer concentration should be determined solely by the pointed filament ends. Under these conditions, the binding of β4-thymosin, the major actin sequestration protein of the platelet, to monomeric actin
is sufficiently tight to prevent spontaneous nucleation and filament assembly. Although the affinity of β4-thymosin for actin monomers can prevent spontaneous nucleation, it is not sufficiently high to block monomers from adding to barbed filament ends. As chilling increases the number of free ends in lysates twofold, this increase in uncovered barbed ends is adequate to dissociate actin monomers from β4-thymosin and to drive platelet actin assembly (Fig 7, step b).

This mechanism leading to uncapping of the barbed actin filament ends in activated platelets is not established. However, only the polyphosphoinositides phosphatidylinositol 4-phosphate (PI₄P) and phosphatidylinositol 4,5-bisphosphate (PI₄,5P₂) have been shown to dissociate gelsolin, capZ, and other capping protein from the barbed end of actin filaments in vitro. The bulk levels of these two lipids are, however, reported to initially decrease with platelet activation. They are subsequently restored to levels equal to or exceeding those present in the resting cell. Phosphatidylinositol 3,4-bisphosphate (PI₃,4P₂) and phosphatidylinositol 3,4,5-trisphosphate (PI₃,4,5P₃), on the other hand, increase rapidly after addition of agonists, although these lipids have not been available in sufficient quantity to test for effects on actin. Whatever specific lipid is implicated in terms of shape change induced when platelets are cooled, the physical state of membrane lipids may be important. Previous studies have shown that the aggregation state of phosphoinositides influences their interaction with gelsolin in vitro. Cooling may generate phase transitions in membranes that expose or alter the aggregation of the polyphosphoinositides, providing the necessary signal for filament uncapping.

Formation of long actin filaments in chilled cells resembles effects in Quin-2AM–loaded cells stimulated at greater than 25°C. Our studies suggest that the subsequent lengths to which the cytoplasmic actin filaments grow in activated cells is inversely proportional to the number of nuclei exposed. Cytoskeletons in lysates from thrombin-treated platelets have an average of 500 barbed-end nuclei per cell. In the electron microscope, lamellipodia at the periphery of these cytoskeletons are composed of dense networks of short (approximately 0.5 μm) actin filaments. On the other hand, lysates containing cytoskeletons from Quin-2AM–loaded and thrombin-activated cells, or cooled cells, uncover 100 to 200 nuclei per cell. The periphery of these cytoskeletons is composed of fewer but longer actin fibers. This occurs despite the finding that the Quin-2AM–loaded and chilled cells assemble less actin than cells chilled without Quin-2AM.

Shape change when cooled platelets are rewarmed. Chilled platelets revert from spiny to ballooned shapes when returned to 37°C (Fig 7, step d). For this transition to occur, cytosolic calcium must transiently increase, as Quin-2AM–loaded cells did not make this transition. To transform to these spherical shapes after rewarming, cells are likely to rearrange their actin cytoskeleton. Cooling results in the assembly of long actin filament coils that fill the filopods extruded at the cell margins. The fragmentation mechanism used to amplify filament ends in thrombin-stimulated cells could be used to rearrange the cytoskeleton of the chilled cell, particularly after rewarming. The finding that calcium transients are required suggests that gelsolin, the most abundant calcium-activated severing protein of the platelet, may be involved in this remodeling.

The ability to cool and rewarm platelets without inducing shape changes indicates that long-term storage in cool temperatures may be possible (Fig 7, step c). Storage in the cold is desirable, because many of the metabolic changes that occur in cells stored at room temperature should be prevented or diminished. However, prevention of shape change without maintenance of platelet function would be of little value. Although our assays have been limited to actin assembly, nucleation, and cell morphology, these criteria platelets preserved using cytoplasmic calcium chelation and cytochalasin remain fully capable of spreading on glass surfaces and of assembling actin in response to thrombin treatment once the cytochalasin B was removed and sufficient Ca²⁺ was added to overcome the chelation effect.

Prevention of shape change in cooled platelets. We tested the effect of agents that (1) decrease the number of barbed ends and (2) block exposed barbed ends on the shape transition induced by cooling. Preincubation of resting platelets with cytochalasin B prevented some, but not all, of the shape distortion found with cooling. As we have previously observed in thrombin- and glass-treated cells, cytochalasin B treatment caused the discoid platelets to become spherical when cooled. Based on previous work, the cytochalasin B effect is suggestive of the stabilization of a population of short cortical actin filaments. Short filaments form during rewarming of platelets, most likely by fragmentation of the long filaments of the chilled cell, and are inhibited from elongating by cytochalasin B. Cooling could increase cytosolic calcium by inhibiting calcium efflux. As actin filament fragmentation in platelets requires transient changes in calcium concentrations, we reasoned that the combined effects of Quin-2AM loading and cytochalasin B should prevent this conversion from discs to spheres. Cells subjected to this treatment (equivalent to step e in Fig 7) did not change from their resting state. Therefore, cooling in the absence of filament growth can lead to actin filament fragmentation. This severing must release constraints preserving the discoid shape and, therefore, lead to the spherical cell shape.

REFERENCES


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