Mechanisms of Cold-induced Platelet Actin Assembly*

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Various agonists but also chilling cause blood platelets to increase cytosolic calcium, polymerize actin, and change shape. We report that cold increases barbed end nucleation sites in octyl glucoside-permeabilized platelets by 3-fold, enabling analysis of the intermediates of this response. Although chilling does not change polyphosphoinositide (ppI) levels, a ppI-binding peptide completely inhibits cold-induced nucleation. The C terminus of N-WASP, which inhibits the Arp2/3 complex, blocks nucleation by 40%; GDPγS, N17Rac and N17Cdc42 have no effects. Some gelsolin translocates to the detergent-insoluble cytoskeleton after cooling. Chilled platelets from gelsolin-deficient mice have ~50% fewer new actin nuclei compared with platelets from wild-type mice. EGTA completely inhibits gelsolin translocation into the cytoskeleton, and the small amount of gelsolin initially there becomes soluble. Chilling releases adducin from the detergent-resistant cytoskeleton. We conclude that platelet actin filament assembly induced by cooling involves ppI-mediated actin filament barbed end uncapping and de novo nucleation independently of surface receptors or downstream signaling intermediates besides calcium. The actin-related changes occur in platelets at temperatures below 37 °C, suggesting that the platelet may be more activatable at temperatures at the body surface than at core temperature, thereby favoring superficial hemostasis over internal thrombosis.

As the suspension medium temperature falls below 15 °C platelets abruptly change their shape from smooth discs into spiny spheres with irregular projections and aggregate (1–5). These events resemble platelet responses to thrombin, ADP, collagen, and other stimuli that operate optimally at 37 °C through energy-dependent signaling reactions resulting in actin remodeling. However, cooling slows these reactions and causes most cell types to round up. The response of human blood platelets to chilling has profound medical consequences. It limits the storage of over 90 million platelet units collected worldwide per year for transfusion to 5 days at room temperature, because longer storage leads to unacceptable amounts of microbial growth. Even 5-day storage of platelets without refrigeration results in occasional septic complications following transfusion (6, 7).

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Unactivated discoid platelets have a unique submembrane coil of microtubules. Some microtubules depolymerize at low temperatures, and the microtubule coils of platelets dissolve in the cold (1, 8). At reduced temperatures, cells also become less able to maintain energy-dependent low cytosolic calcium levels, and chilled platelets have increased cytosolic calcium concentrations (4). Since calcium-dependent severing of the actin scaffold that maintains the discoid shape of the resting platelet is an early step in normal platelet activation, this calcium rise is presumably a mediator of cold-induced platelet activation. In addition to these clues to possible mechanisms underlying cold activation of platelets, recent work has implicated phosphoinositide-mediated actin assembly (9, 10). Platelet stimulation through the PAR-1 receptor activates the Rho GTPase Rac leading to the synthesis of polyphosphoinositides (ppIs). Studies in permeabilized platelets indicated that these lipids induce actin assembly by producing actin nucleation sites equivalent to actin filament fast growing barbed ends. Since these ppI release barbed end capping proteins such as gelsolin from actin filaments in permeabilized platelets, uncapping of preexisting actin filaments is one pathway proposed from ppIs to actin assembly and is amplifiable by actin filament severing and capping, which increase the number of ends. A second pathway involves ppI and Cdc42-activated unfolding of Wiskott-Aldrich syndrome protein (WASp) family proteins, which then bind the Arp2/3 complex, resulting in branching barbed end nucleation at the cell cortex that leads to cell movement (11–16). Receptor tyrosine kinases, the Rho family GTPase Cdc42, and probably G-protein-coupled receptors transmit the signals to WASp-Arp2/3 (17–20) and link signaling pathways to cell motility.

Although ppI turnover would predictably diminish in the cold, if degradation declined more than synthesis, net ppI levels might increase, leading to actin assembly. A more plausible explanation is that temperature influences the structure of these lipids. The physical chemistry of lipid presentation affects lipid-protein interactions in general and gelsolin actin binding in particular (21). Tablin et al. (22) have proposed evidence for lipid phase changes at the critical temperature for cold-induced platelet activation.

We previously showed that chilled platelets assemble actin from barbed end nuclei (4). In this paper, we document that chilling activates ppI-induced barbed end assembly independently of phospholipid synthesis and GTPase activation. We show that ppIs work through both actin filament barbed end

1 The abbreviations used are: ppI, polyphosphoinositide; WASp, Wiskott-Aldrich syndrome protein; OG, m-ocetyl-p-β-D-glucopyranoside; Pef0, 1,4-piperazinediethanesulfonic acid; GTPγS, guanosine 5′-O-β-thiotriphosphate; GPDPβS, guanylyl-5′-yl thiophosphate; FITC, fluorescein isothiocyanate; PtdIns-4,5-P2, phosphatidylinositol 4,5-bisphosphate; HPLC, high pressure liquid chromatography; AM, acetoxymethylester; GST, glutathione S-transferase; TRAP, thrombin receptor anchoring peptide.
uncapping and Arp2/3-mediated nucleation in chilled platelets. We also provide direct evidence that chilling of platelets activates gelsolin to amplify the actin remodeling response.

EXPERIMENTAL PROCEDURES

Materials—Chemical reagents were purchased from Sigma. Rabbit skeletal muscle actin was isolated and labeled with pyrene as previously described (23). The QRLFQVKGRG 10-mer polyphosphoinositide-binding peptide was synthesized based on residues 160–169 of gelsolin (24). The Arp3 component of the Arp2/3 complex was detected using affinity-purified anti-Arp3 antibody provided by Dr. Matt Welch (University of California, Berkeley, CA). Rabbit polyclonal anti-adducin antibody was kindly provided by Dr. Vann Bennett (Duke University Medical Center, Durham, NC). The monoclonal anti-gelsolin antibody (2C4) was previously described (25).

GST Fusion Proteins—Vectors encoding GST-CA (aa 450–505 of N-WASP) and GST-VCA (aa 392–505 of N-WASP) constructs were derived from the C-terminal end of N-WASP, were kindly provided by Drs. Rajat Rohatgi and Marc Kirschner (Department of Cell Biology, Harvard Medical School, Boston, MA). The GST-RacN17 and -Cdc42N17 expression constructs were kindly provided by Dr. G. Bokoch (Scirrps Research Institute, La Jolla, CA). GST proteins were expressed in Escherichia coli, purified on glutathione-agarose beads, and stored at −80 °C. Purified GST-CA and GST-VCA were thawed at 37 °C and diluted immediately before use. Purity was checked following SDS-polyacrylamide gel electrophoresis through 15% gels and staining with Coomassie Brilliant Blue.

Preparation of Human and Mouse Platelets—Blood was drawn from normal human volunteers by venipuncture into 0.1 volume of Aster-Jandl citrate-based anticoagulant as previously described (26). Platelet-rich plasma was prepared from the plasma proteins by gel filtration (26) through a small Sepharose 2B column. Blood was obtained from wild-type and gelsolin−/− (27) mice by cardiac puncture into 0.1 volume of Aster-Jandl anticoagulant. Mouse platelet-rich plasma was separated from the red blood cells by centrifugation at 100,000 × g for 6 min, followed by centrifugation of the supernatant and the buffy coat again at 100,000 × g for 20 min. The supernatant and the buffy coat were resuspended in 140 mM NaCl, 3 mM KCl, 0.5 mM MgCl2, 5 mM NaHCO3, 10 mM glucose, 12.5 mM sucrose, pH 6, by centrifugation of the blood at 100,000 × g for 6 min, followed by centrifugation of the supernatant and the buffy coat again at 100,000 × g for 20 min. The supernatant and the buffy coat were resuspended in 140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, 12.5 mM sucrose, pH 6, by centrifugation at 1,100 × g for 12 min. This washing procedure was repeated, and the platelets were resuspended in 140 mM NaCl, 3 mM KCl, 0.5 mM MgCl2, 5 mM NaHCO3, 10 mM glucose, and 10 mM Hepes, pH 7.4. The concentration of human and mouse platelets was adjusted to 3 × 10^9/ml, and platelets were allowed to rest for 30 min at 37 °C before use.

Permeabilization of Platelets with n-Octyl-p-nitrophenylphosphate (OG)—Resting platelets in suspension (90 u M containing 1.5 × 10^9 platelets) were permeabilized at 37 °C by the addition of one-ninth volume of 1% Triton X-100 in PHEM buffer. Permeabilization was evaluated and washed from platelet-rich plasma by two sequential centrifugations at 80 °C in concentrations of 33% of the original volume of 4°C. The purified platelets (10^9/ml) were incubated for 1 hour at 37 °C with 2 mCi/ml of [32P]orthophosphoric acid. 32P in the medium was separated from the platelets by gel filtration over a Sepharose 2B column. The platelets were resuspended at ice bath temperatures for 10, 20, 30, or 40 min, and total phospholipids were extracted using two combined washes of chloroform/methanol/HCl. ppIs were decayed and analyzed by HPLC as described (9, 30, 31).

Measurement of Filament End Numbers—Intact platelets (90 μl containing 1.5 × 10^9 platelets), incubated at ice bath temperatures for 5 min or treated with 1 unit/ml thrombin for 60 s, were permeabilized with one-ninth volume of 1% Triton X-100 in PHEM buffer. To assess filament end numbers in a temperature-dependent manner, intact platelets (90 μl containing 1.5 × 10^9 platelets) were incubated for 5 min at 37, 25, 20, 15, 10, 5, or 0 °C with or without the addition of 25 μM TRAP for 60 s and were permeabilized as described above. To assay filament barbed ends, 185 μl of a solution containing 100 nM RCI, 2 mM EGTA, 2 mM MgCl2, 0.5 mM ATP, 0.1 mM dithiothreitol, and 7 μl of 37 °C with 2 mCi/ml of [32P]orthophosphoric acid. TRAP was added to 100 μl of the Triton X-100 or OG-lysate. The actin polymerization rate assay was started by the addition of 15 μl of 20 μM norepinephrine-lyabeled rabbit skeletal muscle actin to a final concentration of 1 μM. In this assay, the fluorescence increase is proportional to actin assembled into filaments. Fluorescence was recorded in a LS50 spectrophotometer (PerkinElmer Life Sciences) using excitation and emission wavelengths of 566 and 586 nm, respectively. Actin assembly inhibited by 2 μM cytochalasin B is defined as occurring at the barbed end of the actin filament. Activity not inhibited by cytochalasin B is defined as pointed end assembly. The number of free actin filament barbed ends was calculated from the known assembly rates of actin and the cytochalasin sensitivity of this assembly as previously described (22). The number of pointed and barbed ends and addition rates are 10 and 1 monomer s^−1, respectively (32). Immunoblot Analysis of Platelet Cytoskeletal Proteins—Resting and cold-activated platelets (1, 2, 5, and 30 min at ice bath temperatures) or platelets loaded with 40 μM EGTA-AM for 30 min at 37 °C and then chilled (1, 2, 5, and 30 min at ice bath temperatures) were lysed using a final Triton X-100 concentration of 0.1% in PHEM buffer. The lysates were examined by SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon-P membrane (Millipore Corp.). Membranes were blocked using 1% bovine serum albumin in 100 mM NaCl, 20 mM Tris/HCl, pH 7.4, and then probed with specific antibodies and appropriate peroxidase-tagged secondary antibodies. Detection was performed with an enhanced chemiluminescence system (Pierce).

RESULTS

Actin Assembly in OG-permeabilized Platelets Induced by Chilling—Fig. 1 shows that most platelets remain discoid at 37 °C under permeabilization with OG (Fig. 1A), and that these permeabilized platelets change shape when chilled (Fig. 1B). Chilled OG-permeabilized platelets have blebs on their surfaces and elaborate filopodial-like structures that remain after rewarming (Fig. 1C). Rewarming is, however, required to detect actin filament barbed ends that nucleate the assembly of pyrene-actin, suggesting that OG-treated platelets reseal upon cooling but become permeable again when rewarmed (Fig. 1D).
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We verified this idea by extracting OG-permeabilized cells with Triton X-100 after cooling, followed or not by rewarming. After Triton X-100 treatment, 199 ± 30 barbed end nucleation sites were detectable in chilled platelets, an increase of 3-fold over cells maintained at 37 °C. Chilling of OG-permeabilized platelets for ≥5 min at 4 °C led to the production of 170 ± 10 barbed ends/cell, demonstrating that the permeabilized cells retain ~90% of their response to cold. Exposure of filament ends was restricted to barbed ends in OG-permeabilized platelets, since 2 μM cytochalasin B, an agent that binds to the barbed end and inhibits monomer assembly onto this end, abolished all detectable actin assembly from OG-permeabilized platelets (data not shown). In summary, cooling and rewarming of permeabilized platelets reports barbed end exposure and allows dissection of this process through the addition of inhibitory reagents normally unable to penetrate into platelets.

A ppI-binding Peptide Inhibits Actin Filament Barbed End Exposure in Response to Cold—The addition of a 10-mer peptide derived from the ppI-binding site of gelsolin to OG-permeabilized platelets diminished barbed actin filament end exposure when permeabilized platelets were chilled for 5 min. 2 μM peptide decreased detectable barbed ends by ~65%, and 20–30 μM completely inhibited barbed end exposure following chilling (Fig. 2a). Resting platelets have 44 ± 16 exposed actin filaments barbed ends. This result is consistent with previous studies showing that 25 μM of the 10-mer peptide inhibits all barbed end nucleation in permeabilized PAR-1-activated platelets and formyl-methionine-leucine-phenylalanine-activated neutrophils (9, 34). Incubation with 25 μM of the 10-mer peptide prevented the growth of protrusions from the surface of chilled permeabilized platelets (Fig. 2b). The addition of PT-DIns-4,5-P₂ to OG-permeabilized, peptide-treated platelets rescued actin nucleation in a dose-dependent manner, whereas phosphatidylserine (PS) was without effect (Fig. 2c). These data show that ppIs are intimately involved in the actin assembly reaction that distorts the shape of the platelet in the cold. To determine whether the ppI content changes during the chilling process, platelets, loaded with 32P to label the phospholipid pool, were incubated at ice bath temperatures for 5–40 min. Fig. 3 shows that cooling did not alter the content of D3- and D4-containing ppIs in platelet membranes at time points when actin assembly and shape change are maximal.

Formation of Barbed End Nucleation Sites is Temperature-dependent—To determine the temperature dependence of cold-induced actin assembly, platelets were incubated at decreasing temperatures for 5 min. As the temperature decreased, the barbed end number per platelet increased from 55 ± 9 at 37 °C to maximal values at or below 10 °C of 254 ± 21 barbed ends per cell. The platelet F-actin content increased maximally by ~25% at temperatures of ≤15 °C. Both barbed end exposure and F-actin content begin to increase when the temperature decreases to ≤20 °C. Tablin et al. (22) have shown that membrane phase transitions and platelet shape changes begin at this temperature. Platelets lose their responsiveness as their temperatures decrease and do not respond to TRAP at ≤5 °C (Fig. 4a).

GDPS8 and Dominant Negative GTPases Do Not Inhibit the Cold-mediated Actin Filament Barbed End Exposure—When platelets are activated at 37 °C by ligation of the PAR-1 receptor, GTPases are upstream of ppI production (9, 10). As reported, GTPase antagonists, GDPβS or dominant negative GTPases, are potent inhibitors of PAR-1-mediated signaling to actin in platelets (9). Fig. 5 shows that actin assembly is not coupled to GTPases in chilled OG-permeabilized platelets. The nonhydrolyzable GDPβS and GTPβS analogs fail to affect the number of barbed ends exposed after chilling of OG-permeabilized platelets. In accordance with this finding, the dominant negative GTPases, 1.5 μM GST-N17Rac1 or 3 μM GST-N17Cdc42, do not affect the number of barbed filament ends produced in chilled OG-permeabilized platelets (Fig. 5). These data, combined with a lack of new ppI synthesis in the cold and the temperature dependence of barbed end exposure, support the notion that lipid rearrangements, uncoupled from receptors and GTPases, induce actin assembly and shape change in cooled platelets.

Barbed End Capping Proteins—Barbed end capping proteins such as gelsolin or adducin regulate platelet actin assembly. In the resting platelet, gelsolin is inactive, and most of it is not bound to actin and therefore extractable by detergents (35). Binding to actin by gelsolin is rapidly induced by the intracellular free calcium increase following PAR-1 ligation. A large fraction of the bound gelsolin subsequently dissociates from actin. Fig. 6a shows that gelsolin reversibly associates with the actin cytoskeleton fraction of chilled platelets. Gelsolin is ~80–90% detergent-extractable in resting platelets at 37 °C, but 20–30% becomes inextractable ≤5 min after chilling. In contrast, the small amount of gelsolin in the resting cytoskeleton (10%) dissociates from the cytoskeletal fraction of EGTA-AM-loaded and cooled platelets (Fig. 6a). As we previously reported, platelet F-actin content increased following cooling (4). The
F-actin content increases from 40 to −60% after chilling for 30 min, as determined by FITC-phalloidin binding by flow cytometry (data not shown) (4). The inset in Fig. 6a shows the gelsolin/F-actin ratio in cooled untreated platelets and in platelets preloaded with 40 μM EGTA-AM. The ratio increases from 1:300 in resting cells to 1:180 after 5 min of cooling and then decreases to 1:250 after 30 min of chilling. In contrast, the ratio decreased to 1:1000 within the first minute of cooling in platelets loaded with EGTA-AM. Direct evidence that gelsolin is involved in the actin response induced by chilling comes from experiments on platelets from gelsolin−/− mice. Fig. 6b shows that gelsolin−/− platelets undergo only small distortions from their discoid shape when cooled compared with wild-type mouse platelets. The most prominent shape change is the elongation of the discs into barbell shapes, although a few filopodia and blebs are observable. Wild-type mouse platelets lose their discoid shapes and protrude filopodia and blebs in similar fashion to human platelets when chilled. Gelsolin−/− platelets expose 50% less barbed ends upon chilling compared with gelsolin+/+ platelets. Fig. 6c reports that cold activation stimulates a ∼3-fold increase of barbed end exposure in wild-type mouse platelets, whereas gelsolin−/− platelets have only a ∼1.5-fold increase in barbed ends. Exposure of filament ends is restricted to the barbed ends in the Triton X-100 permeabilized platelets, since 2 μM cytochalasin B abolished all detectable pyrene actin assembly (data not shown). Murine platelets activated with 1 unit/ml thrombin for 1 min at 37 °C increased the number of actin nuclei by ∼5-fold in wild-type and ∼2.8-fold in the gelsolin null platelets.

Chilling also dissociates the barbed end capping protein adducin from the platelet actin cytoskeleton (Fig. 7). In resting platelets, −70–80% of the total adducin is bound to the cytoskeletal fraction. Adducin begins to dissociate from the platelet actin cytoskeleton 2 min after chilling, and the dissociation becomes maximal after 30 min (Fig. 7). The inset in Fig. 7 shows the adducin/F-actin ratio in the cytoskeleton of cold-activated platelets. The amount of adducin bound to the cytoskeletal fraction decreases during the cold activation 1:100 in resting cells to 1:333 after 30 min. The distribution of CapZ, a barbed end capping protein that terminates actin filament assembly by capping the ends of elongating filaments (36–38), did not change following cooling of platelets (data not shown).

Role of the Arp2/3 Complex in the Platelet Actin Assembly Induced by Chilling—To determine whether the Arp2/3 complex participates in cold-induced actin assembly of OG-permeabilized platelets, we used constructs derived from N-WASp that inhibit Arp2/3 complex-mediated actin nucleation. The C-terminal CA domain of N-WASp (amino acids 450–505) binds to the Arp2/3 complex, inhibiting its function. In contrast, N-WASp VCA domain (amino acids 392–505) binds to...
actin monomers and the Arp2/3 complex and leads to an enhancement of actin nucleation (20). GST-CA added to the OG-permeabilized platelets before chilling inhibits the barbed end number induced by chilling in a concentration-dependent manner (Fig. 8). The addition of 0.1 mM of GST-CA to the OG-permeabilized platelets diminished the number of barbed ends measurable after chilling by 40% (Fig. 8), indicating that Arp2/3 activation contributes about half the nuclei used in the actin assembly reaction of chilled platelets.

**DISCUSSION**

Our results show that actin assembly observed during cold activation of platelets results from membrane lipid rearrangements without activation of GTPases and synthesis of ppIs. The mechanics of cold activation involve known regulatory processes of actin assembly. First is the activation of gelsolin to sever actin filaments through increased intracellular calcium (23, 35). Cooling of platelets leads to a slow rise in free cytosolic calcium to levels of 200–300 nM (4, 39), which is not surprising, because cold decreases the activity of calcium pumps that function to extrude calcium (40). In chilled platelets, gelsolin, the major actin filament-severing protein of platelets, transiently associates with the actin cytoskeleton and becomes maximally bound after 5 min. Translocation into the cytoskeleton is prevented if platelets are loaded with EGTA. We previously demonstrated barbed end exposure to be inhibited by 50% in Quin2-loaded platelets, consistent with the loss of calcium-activated gelsolin severing activity (4). Second is the exposure of actin filament barbed ends that induce new actin assembly by ppIs (9). ppIs are known to be intimately involved in the barbed end-based nucleation and actin assembly of PAR-1-activated platelets (9). Unlike PAR-1-mediated activation, barbed end exposure/nucleation is uncoupled in the cold from GTPases and ppI synthesis. The addition of GDPbS or the negative dominant Rho family GTPases N17Cdc42 or N17Rac...
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Effects of bacterially expressed GST-N17Rac1 (1.5 μM) on the number of barbed filament ends in OG-permeabilized chilled platelets. Values are mean ± S.D. for five individual experiments.

To investigate whether the cold-induced actin assembly observed in platelets involves ppI-mediated uncapping of filaments, we applied a permeabilization scheme used to define the mechanisms of the PAR-1-mediated actin assembly in platelets and formyl-methionine-leucine-phenylalanine-induced actin assembly in polymorphonuclear leukocytes (9, 34). Platelets, briefly permeabilized with OG, were chilled to 4 °C, and shape change and actin assembly were monitored. After chilling, blebs and protrusions developed on the surfaces of OG-permeabilized and chilled platelets. This demonstrates that ppIs are involved in the reactions that lead to barbed end exposure/uncapping of actin filament barbed ends and the induction of actin assembly. The temperature dependence of barbed end exposure correlates with that of the membrane phase transition and would change lipid packing, causing ppI clustering (22). Aggregation of ppIs would potentiate the activity of the phosphoinositol-lipid head groups in the platelet plasma membrane as has been shown for ppIs in mixed lipid vesicles in vitro (41). The aggregation process would also be predicted to occur independently of the activity of GTPases, as observed.

Targets of ppIs—Targets of these phospholipid clusters include barbed end capping proteins and the Arp2/3 complex. The uncapping of actin filament barbed ends was the first pathway defined to be involved in ppI-activated actin assembly, and evidence has been provided by us that barbed end capping proteins are released from actin filaments during cell activation by the addition of ppIs to permeabilized platelets (36). Our data are consistent with the idea that 50–60% of the barbed end-based nucleation activity in chilled platelets derives from actin filaments severed by gelsolin and subsequently uncapped. The small amount of gelsolin associated with the resting cytoskeleton dissociates when EGF-AM-loaded platelets are chilled for 5 min, indicating that signals (ppIs) that dissociate gelsolin from actin remain intact in these cells. A similar dissociation from the cytoskeletal fraction was observed after 30 min in untreated cooled platelets. In addition gelsolin-deficient platelets were found to be less responsive to chilling and to produce only 50% of barbed end nucleation sites of normal platelets.

Adducin is a second platelet protein that may contribute to the actin nucleation induced by ppIs. Adducin was first identified as a barbed end capping protein in the membrane cytoskeleton of red blood cells (42, 43). Adducin possesses a myristoylated alanine-rich protein kinase C substrate-related domain that is required for its actin filament barbed end capping activity (44). This 25-amino acid basic domain is phosphorylated at multiple serines by protein kinase C and also binds to calmodulin or PtdIns-4,5-P2 to regulate its interaction with actin (45, 46). In the resting platelet, 70–80% of the total adducin is associated with the actin cytoskeleton. Chilling dissociates adducin from the cytoskeleton. Adducin’s dissociation could be due to the calcium activation of calmodulin in chilled platelets or, since the gelsolin-derived ppI-binding peptide completely inhibits cold-induced barbed end exposure, by phospholipid binding within this myristoylated alanine-rich protein kinase C substrate domain. It is not clear yet which mechanism is involved, but dissociation of adducin from actin filaments is expected to contribute to barbed end exposure.

A more recently discovered mechanism leading to cytoplasmic actin assembly is the de novo actin nucleation by the Arp2/3 complex (19, 20, 47–51). We investigated whether the Arp2/3 complex participates in cold-induced actin assembly by adding a negative dominant inhibitor of its function (GST-CA) to the permeabilized platelets. GST-CA inhibits the barbed end production induced through chilling by ~40% when added to OG-permeabilized platelets prior to chilling. The Arp2/3 complex nucleates actin downstream of WASp family proteins (WASp, N-WASP, Scar/WAVE), which require GTPases and ppIs to become active (20, 52, 53). PtdIns-4,5-P2 and the small GTPase Cdc42 co-activate N-WASP (20). N-WASP can be activated by PtdIns-4,5-P2 micelles alone, whereas GTPCdc42 is a poor activator of N-WASP in the absence of PtdIns-4,5-P2 (53).

Temperature Dependence of the Cold-induced Response—It is well established that platelets begin to change shape at temperatures of <25 °C and that the number of activated platelets increases with decreasing temperatures until 5 °C (22). We investigated the relation of the temperature to the barbed end nucleation activity in platelets and found that free barbed end...
FIG. 6. Role of gelsolin in barbed end exposure mediated by cooling. A, interaction of gelsolin with the actin cytoskeleton of untreated chilled platelets (closed circles) or platelets first incubated with 40 μM EGTA-AM for 30 min (open circles). Soluble gelsolin was separated from cytoskeletal bound gelsolin in Triton X-100 platelet lysates by centrifugation at 450,000 × g for 30 min. Gelsolin was detected by immunoblotting. The graphs quantify the movement of gelsolin into the platelet cytoskeleton. The values are the means ± S.D. for three individual experiments. The inset shows the gelsolin/F-actin ratio in the cytoskeleton of chilled platelets (closed squares) and platelets preloaded with EGTA-AM and then chilled (open squares). B, gelsolin −/− platelets have a diminished shape change response to chilling compared with wild-type mouse platelets. The panel compares the morphology of resting (Rest) and chilled (Cold) platelets of wild-type (Gsn +/+ ) and gelsolin −/− mice. C, gelsolin −/− platelets expose fewer barbed end nucleation sites when chilled compared with normal mouse platelets. Actin nucleation sites when quantified in Triton X-100 permeabilized gelsolin +/+ and gelsolin −/− platelets chilled for 5 min or stimulated with 1 unit/ml thrombin for 1 min. The resting barbed end numbers were 65 ± 4 in wild-type platelets and 83 ± 16 in gelsolin −/− platelets.
nucleation begins at temperatures of <25 °C, as has been reported for the membrane phase transitions that change the packing of membrane lipids (22). As platelets are activated by cold, they lose their ability to respond to receptor-mediated stimuli. Below 5 °C, platelets lack responsiveness to TRAP.

Although we expected barbed end nucleation in platelets induced by TRAP to be maximal at 37 °C, higher barbed end nucleation and actin assembly were found in platelets activated at 20–25 °C. These results imply that at 20–25 °C nucleation activity derives from the combined effects of temperature-induced nucleation and the normal receptor-coupled signaling events. It also suggests that the exquisite sensitivity of platelets to temperature may lead to a coupling of membrane dynamics and signaling events mediating a maximal physiological response at temperatures below 37 °C, such as those that exist in demis and at wound sites.

Our data show that platelet actin filament assembly induced by cooling requires membrane ppIs to mediate the uncapping of actin filaments by gelsolin and adducin and de novo actin nucleation by the Arp2/3 complex. However, the signaling pathways are uncoupled from receptors and small GTPases. The data lead to a better understanding of how cooling leads to platelet shape changes and actin assembly and shows their sensitivity to temperature changes. This knowledge may contribute to a better storage of platelets.

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