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Platelet shape change is an extremely rapid process mediated by both the calcium-sensitive and p160^{ROCK} pathways. The present study examines how different features of shape change studied by scanning electron microscopy clearly correlate to changes in the pattern of light absorbance measured in an aggregometer. Platelets change shape from the initial 'disc' form by producing: membrane 'blebs', sphere formation (cell-rounding), filopodia extension, and surface membrane folding. The presentation of these features was dramatically slower in the absence of intracellular calcium mobilization. In the presence of the p160^{ROCK}-inhibitor, Y-27632, shape change was initially normal but platelets rapidly transformed back to smooth discs with extended filopodia. The reappearance of the disc shape is reflected by an increase in the amplitude of oscillations in the aggregometer shape change tracing. The kinetics of actin/cytoskeleton association correlated with filopodia formation but not with disc to sphere transformation. Changes in the level of tubulin polymerization correlated with changes from disc to sphere morphology. These experiments are consistent with a role for a RhoA/Rho kinase-regulated pathway in the maintenance of a spherical platelet shape after agonist-dependent activation. Continued disruption of the cytoskeletal microtubule ring, appears to be a Rho kinase-dependent event involved in the transformation of discoid platelets into spheres.

Benjamin Z.S. Paul, Carol Dangelmaier, James L. Daniel, Satya P. Kunapuli, Department of Pharmacology, Temple University School of Medicine, Philadelphia, PA 19140, USA; Soochong Kim, Jianguo Jin, Satya P. Kunapuli, Department of Physiology, Temple University School of Medicine, Philadelphia, PA 19140, USA; James L. Daniel, Satya P. Kunapuli, Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia, PA 19140, USA; John H. Hartwig, Division of Hematology, Brigham and Women's Hospital, Boston, MA 02115, USA; Chandrasekaran Nagaswami, John W. Weisel, Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA.

Correspondence to: Satya P. Kunapuli, Ph.D., Department of Physiology, Temple University School of Medicine, 3420 North Broad Street, Philadelphia, PA 19140, USA. Tel.: +1-215-707-4615; Fax: +1-215-707-4003;
E-mail: spk@temple.edu

Abbreviations

p160ROCK:	p160 Rho-associated coiled-coil containing kinase (Rho-kinase)
5,5′-dimethyl BAPTA:	5,5′-dimethyl-bis-(o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid
P2Y1:	ADP receptor coupled to phospholipase C
P2Y12:	platelet ADP receptor coupled to the inhibition of adenylyl cyclase
MLCK:	myosin light chain kinase
Rho:	small Ras homologous GTPase
MAPs:	microtubule-associated proteins

Introduction

The initial response of platelets to agonist-stimulation is a change in cell shape from a lentiform 'disc' to a spiculated 'sphere' (spheroechinocyte).[1,2] The morphology that presents both before and during platelet shape change has been investigated using scanning and transmission electron microscopy.[3–8] Platelet shape change is a dynamic process that includes both contractile events and regulation of a circumferential microtubule coil.[9–11] Actin filaments originating from within the cytoplasm lead to the extension of filopodia.[8,12,13] An actin–myosin contractile apparatus surrounds the newly centralized dense- and α-granules.[2,8,12,14] Secretion-stimulating agonists (e.g., thrombin, and thromboxane A_2) cause release of the granule contents. Further production of thromboxane A_2 induces more platelet activation and aggregation forming the primary hemostatic plug.[1,10]

Controversy exists over both the role of the circumferential microtubule coil in maintaining the

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resting discoid shape and what subsequent changes to the microtubule coil occur both before and after shape change begins. Early biochemical[15,16] and morphological studies[17–20] have reported that the microtubule coil disassembles upon agonist-stimulated shape change. Others argue that the circumferential ring does not disappear but becomes centrally constricted within a mass of actin and myosin microfilaments.[14,21] Furthermore, there is a debate over the role of the plasma membrane actin filament skeleton in the maintenance of the cell's resting discoid shape. This actin filament mesh is anchored to glycoproteins by either actin-binding protein or through spectrin and ankyrin.[2] Hartwig and coworkers[13,22] have provided evidence that the discoid morphology is maintained by a spectrin-reinforced surface membrane cytoskeleton. In contrast, White and Rao[11] have found that cold-induced sphere formation was dependent on reversible changes to the microtubule coil.

Nishimura *et al.*[23] have shown in rat hepatoma cells that RhoA/p160^{ROCK} (Rho-associated coiled-coil containing kinase, also referred to as 'Rho-kinase') mediated signaling (also referred to as the 'Rho/Rho-kinase pathway') is involved in the regulation of both cytoskeletal actin filaments and microtubule organization. The small GTPase Rho mediates regulation of the cell cytoskeleton in such events as: cell motility, rearrangement of stress fiber formation, stable microtubule orientation, contractility, and tumor cell progression.[24–31] There is a growing body of evidence for the role of G-protein α subunits,[32] phosphatases,[33] and kinases in the regulation of microtubule polymerization and disassembly (for reviews, see Refs. [34–36]). Finally, a number of investigations have shown that microtubule disruption increases RhoA-mediated myosin light chain kinase (MLCK) activity and inhibition of myosin specific protein phosphatase activity resulting in increased contractility in both endothelial cells and fibroblasts.[31,37–39] However, the molecular signaling events that regulate microtubule stability during platelet shape change have never been investigated.

While aggregation in response to ADP is dependent upon the concomitant stimulation of the higher affinity G_q-coupled P2Y1 receptor and the lower affinity G_i-coupled P2Y12 receptor, shape change is solely dependent on ADP-stimulation of the P2Y1 receptor.[40,43–45] Previously, it has been shown that RhoA/p160^{ROCK} signaling coupled to the P2Y1 receptor produces shape change in human platelets.[46] Furthermore, a calcium-sensitive signaling mechanism is required for a faster rate of platelet shape change induced by ADP, U46619 (a thromboxane A_2 mimetic), and thrombin.[46,47] Calcium-sensitive signaling mechanisms can be investigated using the intracellular calcium chelator, 5,5′-dimethyl BAPTA. The calcium-insensitive aspect of platelet shape change is mediated by p160^{ROCK} (Rho-kinase) when stimulated by U46619,[48] ADP,[46] and thrombin or the thrombin receptor-activating peptide, YFLLRN.[47] RhoA, a member of the Ras homologous small GTPase family, is present in platelets and following stimulation by its guanidine nucleotide exchange factor, p115^{RhoGEF}, it will associate with and activate p160^{ROCK} (Rho-kinase).[49–52] The RhoA/p160^{ROCK} (Rho-kinase) pathway is involved in the actin–myosin contractile response of smooth muscle cells, fibroblasts, endothelial cells, and neuronal cell lines.[53–60] Human platelets contain myosin phosphatase, which is phosphorylated and inactivated by the RhoA/p160^{ROCK} pathway.[49,61] P160^{ROCK} can be selectively inhibited by the two structurally different compounds Y-27632 and HA 1077.[56]

Platelet shape change includes several morphological changes including filopodia extension, plasma membrane folding, and sphere formation. Although shape change is known to be regulated independently by both calcium-sensitive and P160^{ROCK}-sensitive pathways, it is not clear how these pathways regulate different morphological features of shape change. In this study we investigated the regulation of cytoskeletal actin and myosin by the RhoA/p160^{ROCK} pathway and the involvement of actin and tubulin in the different aspects of platelet shape change. Here we demonstrate that actin polymerization and filopodia formation can be mediated through either a calcium-dependent pathway or the RhoA/p160^{ROCK} pathway. Our results show that an increase in cytosolic calcium will initiate the disruption of the microtubule coil. In contrast, the RhoA/p160^{ROCK} pathway both initiates and maintains microtubule coil disruption during platelet transformation from disc to spherical morphology.

Materials and methods

Platelet preparation

Human blood was collected from an informed group of volunteers derived from the students and staff at Temple University School of Medicine. Blood was collected into a one-sixth volume of ACD (85 mM sodium citrate, 111 mM glucose, 71.4 mM citric acid). Platelets were treated with acetylsalicylate and resuspended in calcium-free Hepes-buffered Tyrode's solution as previously described.[46,62] The platelet count was adjusted to 2×10^8 cells/ml. 5,5′-Dimethyl-BAPTA-loaded platelets were prepared from platelet-rich plasma which was incubated with 1 mM acetylsalicylic acid and 50 μM 5,5′-dimethyl BAPTA-acetoxymethylester as previously described.[46] Platelet p160^{ROCK} (Rho-kinase) was inhibited by incubating the platelets with stirring (900 rpm) for 3 min at 37°C with 10 μM Y-27632.[46]

Platelet aggregation and shape change

Platelet aggregation and shape change were measured as previously described.[40,46,62,63] In brief, platelet shape change was followed after the addition of 1 μM SC-57101 before agonist-stimulation. SC-57101 blocks fibrinogen binding to its receptor and is a known inhibitor of platelet aggregation.[64] All experiments were performed in the presence of 2 mM $CaCl_2$ and were repeated at least three times using platelets from different donors.

Scanning electron microscopy

Platelets resuspended in calcium-free Hepes-buffered Tyrode's solution were activated with 10 μM ADP following the addition of 2 mM $CaCl_2$ and 1 μM SC-57101. Other conditions are as indicated. At specified times the platelets were fixed by the addition of an equal volume of 2.5% glutaraldehyde buffered at pH 7.2 with 100 mM phosphate. Incubation with the fixative continued for at least 30 min in a 37°C water bath. Collected specimens were then gently suction-filtered onto Nucleopore polycarbonate filters (0.2 μm) that had been preincubated with 0.01 mg/ml polylysine for 12 h and then washed in phosphate-buffered saline (pH 7.4). Filters with collected platelets were stored in 1.25% glutaraldehyde at room temperature until further processing. As previously described,[65] specimens were washed in 50 mM cacodylate buffer (pH 7.4) and then fixed with 1% osmium tetroxide for 15 min at room temperature. The samples were dehydrated in a graded series of ethanol concentrations through 100% over a period of 1.5 h. The specimens were critical point dried with CO_2 in a Denton apparatus. Finally, specimens were mounted and coated with ~12.5 nm of gold–palladium (60 : 40), observed at 10 kV, and photographed digitally using a Philips XL20 scanning electron microscope (Philips Electron Optics, Eindhoven, The Netherlands). Conditions were repeated on three separate occasions using cells from different donors. Representative samples are shown.

Measurement of actin association with the Triton-insoluble cytoskeletal fraction

Platelet samples were treated with 1% Triton X-100, 50 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM EDTA, 2 mM PMSF, 2 mM NaF, 1 mM sodium orthovanadate and 10 μg/ml leupeptin. After 10 min at 0°C, the cytoskeletons were pelleted by centrifugation at $7000 \times g$. Samples were dissolved in SDS sample buffer and electrophoresed on 10% polyacrylamide gels. Gels were stained with GelCode blue and resulting gels were scanned and analyzed for intensity of staining using NIH image 1.61. Experimental conditions were repeated three times using cells from different donors.

Isolation of polymerized tubulin fraction and determination of β-tubulin level

Aspirin-treated platelets were prepared as previously described and resuspended in calcium-free Hepes-Tyrode's buffer at $2–3 \times 10^8$ cells/ml. Cells were allowed to rest at 37°C for 45 min. Incubations and stimulations were performed in the presence of 1 μM SC-57101 in a Chronolog Aggregometer at 37°C with stirring (900 rpm). Five hundred-μl volumes of platelet preparation were incubated with either 10 μM Y-27632 for 3 min or vehicle (control) and 10 μM taxol was added 10–30 s prior to the addition of 10 μM ADP. The platelets were lysed at indicated time points by the addition of an equal volume of calcium-free Hepes-Tyrode's buffer containing 0.8% Triton X-100, 12 mM EGTA, and 20 μg leupeptin at room temperature. Microtubules were pelleted by centrifugation at $100\,000 \times g$ for 30 min at 25°C. Samples were solubilized in SDS sample buffer and boiled for 10 min. Samples were resolved on 10% SDS–PAGE. Proteins were transferred to Immobilon-P (polyvinylidene difluoride membrane), blocked with 5% nonfat dried milk and probed for β-tubulin using a mouse monoclonal IgG_{2b} (Santa Cruz Biotechnology, Santa Cruz, CA) raised against the common amino acid residues 210–444. This sequence is found in the human β-tubulin isotypes: Mβ1 to Mβ5 and Mβ7. The Mβ1 isotype is expressed only in hematopoietic cells (megakaryocytes, platelets, and nucleated fetal erythroblasts).[66] The antibody is used at a 1 : 1000 dilution for 60 min at room temperature. A secondary HRP-conjugated goat ant-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used at a dilution of 1 : 10 000 for 60 min at room temp. Bands were detected by chemiluminescence (Pierce Supersignal Pierce Technology, Iselin, NJ) and analyzed using an Intelligent Dark Box II (Fuji Systems USA, Stanford, CT). The experiment was repeated three times using cells from different donors.

Results

The rate and extent of ADP-induced shape change is affected by inhibition of $p160^{ROCK}$ (Rho-kinase) with Y-27632 and prevention of an increase in intracellular calcium concentration with 5,5′-dimethyl BAPTA

A platelet aggregometer measures changes in ultraviolet light scattering. The tracing reflects changes in optical density as the cells change shape and aggregate. Shape change is known to correlate with an increase in optical density (greater scattering of light). ADP-induced shape change is altered in a

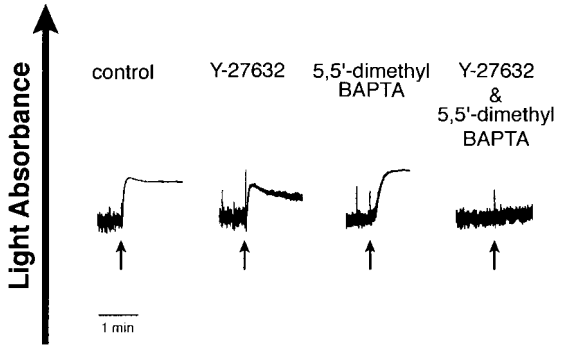

Figure 1. Inhibition of $p160^{ROCK}$ (Rho-kinase) by Y-27632 and preventing an increase in cytosolic calcium concentration by 5,5′-dimethyl BAPTA have different effects on both the extent and rate of ADP-induced shape change. Representative shape change tracings (three experiments using cells from three different donors) as measured in an aggregometer. Arrows indicate the addition of agonist. Platelets were all stimulated in the presence 2 mM $CaCl_2$ and 1 μM SC57101 under the conditions as indicated: 10 μM Y-27632 preincubation and/or 50 μM 5,5′-dimethyl BAPTA treatment.

time-dependent manner by Y-27632 treatment as compared to control platelets. In control and 5,5′-dimethyl BAPTA-treated platelets, pre-stimulatory oscillations in the tracings disappear following addition of agonist (Figure 1). When platelets are treated with 10 μM Y-27632 (the maximal concentration for selective inhibition of $p160^{ROCK}$ (Rho-kinase)[56]) the oscillations diminish but then steadily increase in amplitude following an initial ADP-induced increase in light scattering. The increase in oscillation amplitude suggests that the platelets had partially reverted to a 'resting' shape. This experiment suggests that the RhoA/$p160^{ROCK}$ (Rho-kinase) pathway may be required to maintain certain morphological features of platelet shape change initiated by ADP-stimulation at the P2Y1 receptor. On the other hand, the aggregometer tracing suggests that 5,5′-dimethyl BAPTA-treated platelets undergo shape change that is morphologically normal while differing from control only in rate of onset and completion.

ADP-induced shape change morphology is affected by inhibition of $p160^{ROCK}$ (Rho-kinase) and prevention of an increase in cytosolic calcium concentration

Platelet shape change is known to involve cell rounding and filopodia formation. To investigate the morphological changes associated with the inhibition of $p160^{ROCK}$ (Rho-kinase), platelets were examined by scanning electron microscopy 1 min after agonist-stimulation. The initial data indicated that either the RhoA/$p160^{ROCK}$ (Rho-kinase) pathway or the calcium/calmodulin myosin light chain kinase (MLCK) pathway might mediate either cell rounding or filopodia extension. Unstimulated platelets treated with either 5,5′-dimethyl BAPTA, Y-27632, or both appear the same as unstimulated controls (data not shown).

In order to understand the role of calcium-sensitive and -insensitive pathways in the formation of specific morphological features of shape change (e.g., filopodia, sphere formation and membrane folding), the time course of ADP-induced shape change was followed by scanning electron microscopy in the absence and presence of specific inhibitors. Each of the images shown is representative of hundreds of similar micrographs from at least three experiments. Control platelets undergoing ADP-induced shape change produce three morphological characteristics at distinct periods in time (Figure 2). Initially, at 2 s, the platelet membrane is rippled and protrusions designated as 'blebs' begin to form. At 5 s, sphere formation (cell rounding) is present with greater 'bleb' formation and initial filopodia extension. By 15 s shape change is complete and the cells have become spheres with folded membranes and filopodia. The stable morphological characteristics of platelet shape change (a sphere-shaped cell body, a folded membrane surface, and filopodia) are characteristics of the 'spheroechinocyte' that are maintained up to 2 min following ADP-stimulation (Figure 2). Measuring the change in light absorbance over time, we[46] and other investigators[47] have shown that preventing increases in intracellular Ca^{2+} slows the rate of shape change. These scanning electron microscopy data reveal that when platelets that contain the intracellular Ca^{2+} buffer 5,5′-dimethyl BAPTA change shape there are delays in both the onset and occurrence of specific morphological features (Figure 3). Membrane ripples do not appear until 5 s; furthermore, initiation of membrane 'bleb' formation, cell rounding, and filopodia extension all occur between 5 and 15 s and are not complete until 30 s. As observed for normal ADP-induced shape change (Figure 2), the changes in morphology for Ca^{2+} buffer-treated platelets were maintained at 1 and 2 min following agonist-stimulation (Figure 3). The aggregometer shape change tracing induced by ADP in the presence of 10 μM Y-27632 suggests the presence of different morphological characteristics as compared to the corresponding control (Figure 1). Following an initial increase in light absorbance with a decrease in oscillation amplitude, there is a steady and dramatic increase in the oscillation amplitude by 1 min. Platelets treated with 10 μM Y-27632 and fixed at 2, 5, 15 and 30 s, and at both 1 and 2 min following the addition of ADP were examined by scanning electron microscopy (Figure 4). In these platelets the early steps of shape change that occur during the first 15 s are normal; however, by 30 s the sphere shape due to cell rounding begins to reverse, and at 1–2 min the platelets are shaped like flattened discs with the continued presence of filopodia. Thus, ADP-induced 'bleb' formation, membrane folding, cell rounding,

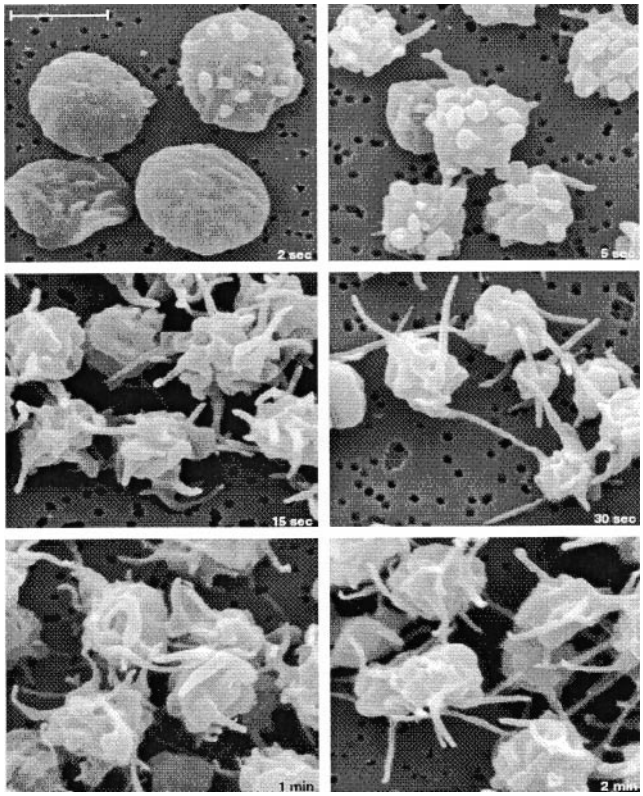

Figure 2. Morphological features are presented differently during ADP-induced shape change as compared to ADP-induced shape change with either the absence of an increase in cytosolic calcium concentration or the inhibition of $p160^{ROCK}$ (Rho-kinase). All samples were stimulated in the presence of both 2 mM $CaCl_2$ and 1 μM SC57101 as described in Materials and Methods and fixed at indicated times following the addition of 10 μM ADP. Bar represents 2 μm length. The data with untreated platelets are shown at different time points after stimulation. This experiment is representative of three similar experiments.

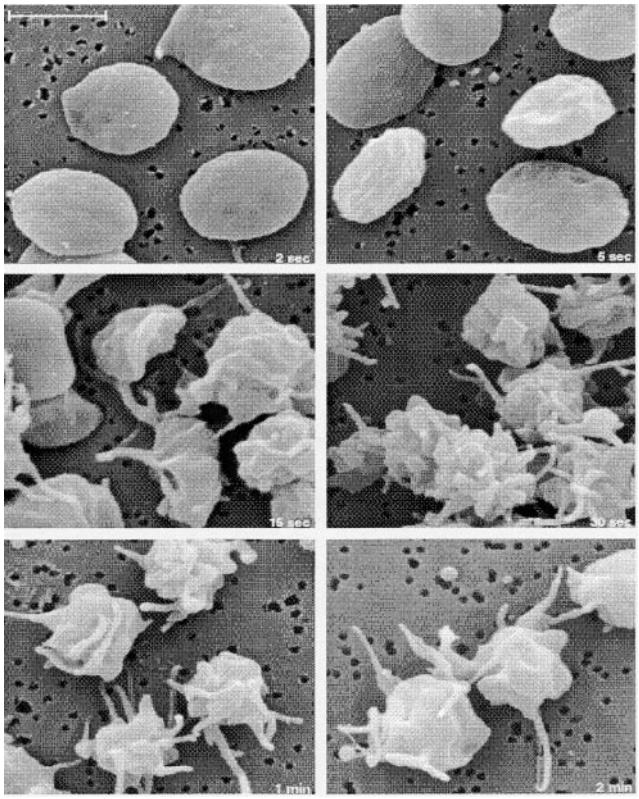

Figure 3. Morphological features are presented differently during ADP-induced shape change as compared to ADP-induced shape change with either the absence of an increase in cytosolic calcium concentration or the inhibition of $p160^{ROCK}$ (Rho-kinase). All samples were stimulated in the presence of both 2 mM $CaCl_2$ and 1 μM SC57101 as described in Materials and Methods and fixed at indicated times following the addition of 10 μM ADP. Bar represents 2 μm length. The data with 5,5′-dimethyl BAPTA-treated platelets are shown at different time points after stimulation. This experiment is representative of three similar experiments.

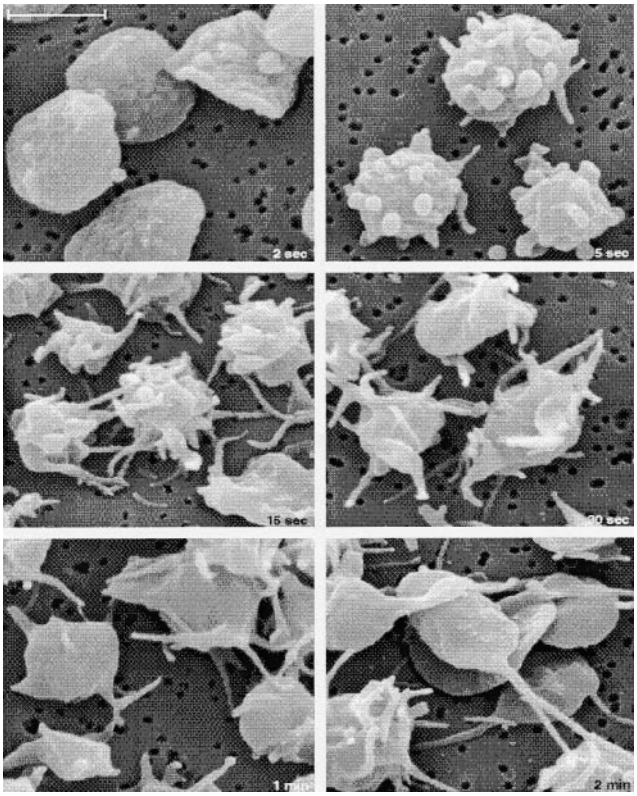

Figure 4. Morphological features are presented differently during ADP-induced shape change as compared to ADP-induced shape change with either the absence of an increase in cytosolic calcium concentration or the inhibition of $p160^{ROCK}$ (Rho-kinase). All samples were stimulated in the presence of both 2 mM $CaCl_2$ and 1 μM SC57101 as described in Materials and Methods and fixed at indicated times following the addition of 10 μM ADP. Bar represents 2 μm length. The data with Y-27632-treated platelets are shown at different time points after stimulation. This experiment is representative of three similar experiments.

and filopodia extension all occur in the absence of the RhoA/$p160^{ROCK}$ pathway. However, the maintenance of ADP-induced cell rounding and membrane folding is dependent on the RhoA/$p160^{ROCK}$ pathway. The combination of Y-27632 and 5,5′-dimethyl BAPTA completely prevents all the characteristics of shape change (e.g., filopodia, sphere formation, and membrane folding) and instead the platelets remained as smooth discoid cells that are no different from non-stimulated control cells (not shown). These results indicate that calcium/calmodulin MLCK-dependent events are not required for either ADP-induced filopodia extension or formation of the sphere-shaped morphology. In contrast, $p160^{ROCK}$ activity appears to play a role in ADP-induced cell rounding and plasma membrane folding.

The level of ADP-induced actin filament reorganization and myosin association is affected by either inhibition of $p160^{ROCK}$ (Rho-kinase) or prevention of an increase in cytosolic calcium concentration

Agonist-induced platelet shape change involves the polymerization of actin and its subsequent association with myosin. The actin–myosin contractile apparatus appears to centralize the dense- and α-granules into the center of the platelet.[2,12,33,67] The association of platelet myosin and actin is dependent upon phosphorylation of the regulatory myosin light chain by calcium/calmodulin MLCK[68,69] with concurrent inhibition of platelet myosin phosphatase PP1Mδ.[49,70] Furthermore, previous reports have shown that a strong correlation exits between the level of phosphorylated myosin light chain and the initiation of shape change.[71] Actin filament reorganization can be measured as the formation of the $7000 \times g$ Triton-insoluble cytoskeleton fraction, which also demonstrates the level of actin–myosin association. In control platelets, 10 μM ADP causes a rapid increase in the association of actin and myosin that peaks at roughly 10 s (Figure 5). In control platelets the level of actin in association with myosin remains elevated up to 2 min following stimulation. In the presence of Y-2763, the level of cytoskeletal actin–myosin peaks at 10 s (Figure 5) as compared to the level in control platelets, which quickly declines. Platelets pretreated with 5,5′-dimethyl BAPTA show a much slower and diminished increase in the cytoskeletal formation with a peak occurring roughly around 20 s following ADP-stimulation. In both Y-27632 treated platelets and 5,5′-dimethyl BAPTA-treated platelets the level of actin–myosin

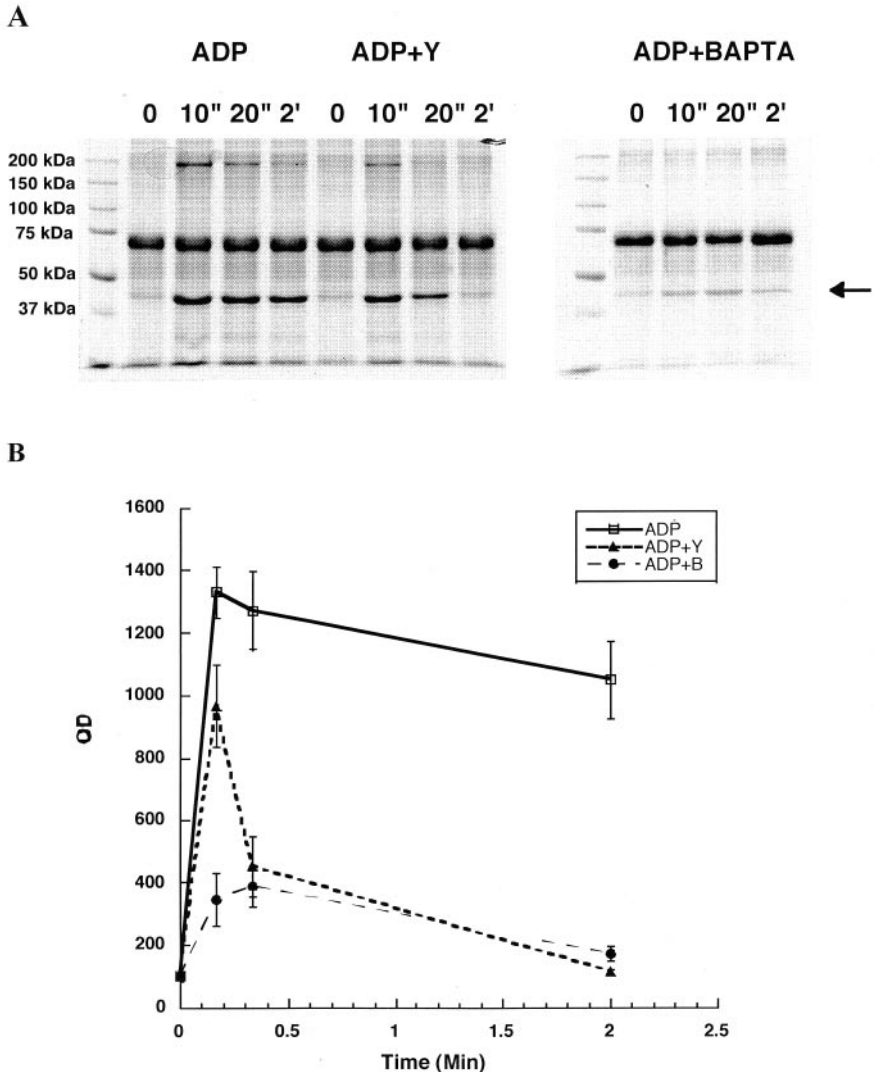

Figure 5. ADP-induced actin filament reorganization and myosin association is affected by inhibition of $p160^{ROCK}$ (Rho-kinase) and the absence of an increase in cytosolic calcium concentration. Triton-insoluble cytoskeletons were prepared from platelets stimulated with 10 μM ADP for indicated times. An equal volume of 1% Triton X-100 solution was added to the cell suspension to stop each reaction. Samples were analyzed as described in the Methods. (A) Gel Code Blue-stained SDS–PAGE. Actin band is 40 kDa and is indicated by arrow. (B) Densitometry of the actin band from the gels such as shown in (A). Each point represents the average and standard deviation of three determinations. This experiment is representative of three similar experiments.

association falls and reaches a similar level by 2 min (Figure 5).

$P160^{ROCK}$ (Rho-kinase)-inhibition affects tubulin depolymerization in ADP-stimulated platelets

The change of disc-shaped platelets to spiculated spheres appears to involve depolymerization of microtubules potentially by G-protein activation of RhoA with subsequent increased activity of $p160^{ROCK}$. If true, then during this process the amount of tubulin found in the pellet by high-speed centrifugation ($100\,000 \times g$) would decrease due to conversion of microtubules to soluble tubulin. The analysis of microtubules in the cytoskeleton was performed as described by Kenney and Linck[3] in a manner that quantitatively preserves the assembled microtubules from the cytoskeleton without causing any polymerization of soluble platelet tubulin. Figure 6 provides the first clear evidence that, immediately following agonist-stimulation in control cells, there is a dramatic decrease in the amount of β-tubulin found in this pellet. The rate of this decrease appears greatest during the first 30 s. By 90 s the level of β-tubulin in the microtubules had decreased to 40% of the unstimulated level. To evaluate the contribution of $p160^{ROCK}$ (Rho-kinase) in this process, cells were pretreated with the selective inhibitor Y-27632. Over the course of shape change, the rate and level of β-tubulin in the microtubule pellet was similar over the first 10 s; however, by 30 s there is a significant difference. As shown in Figure 6, the β-tubulin content only reached a maximal decrease of ~65% by 30 s. From this time point on there is a steady increase in the level of β-tubulin in the isolated microtubule fraction. By 2 min the level of β-tubulin in the microtubule fraction is the same as in unstimulated, resting cells. These results clearly

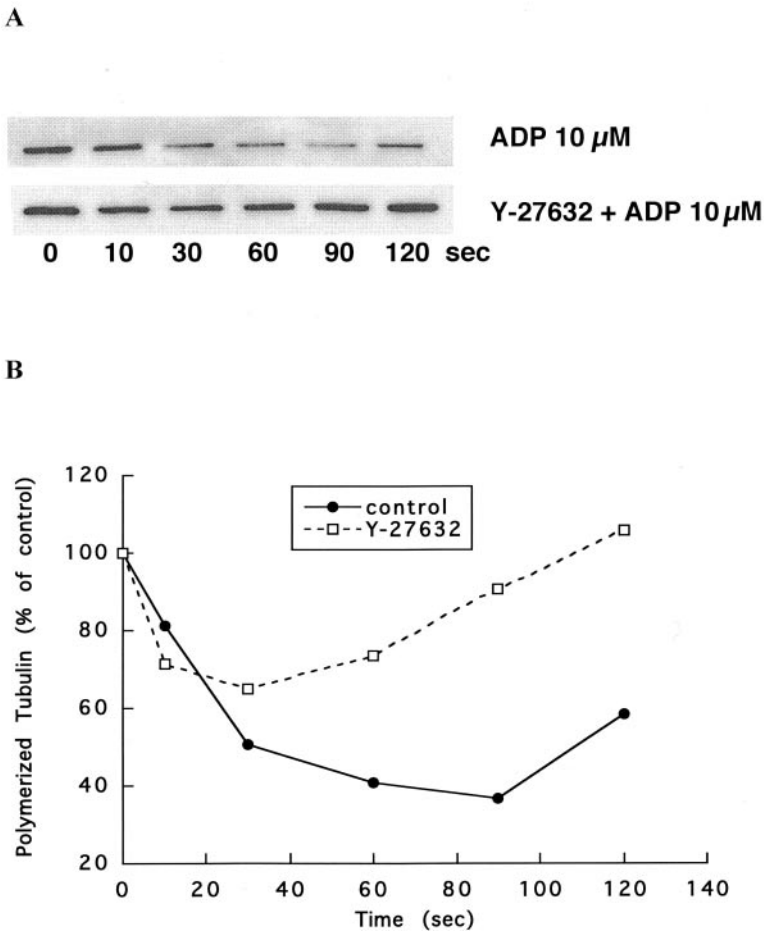

Figure 6. Tubulin polymerization in ADP-stimulated platelets is affected by the inhibition of $p160^{ROCK}$ (Rho-kinsase). Human platelets, untreated or treated with Y-27632, were stimulated with ADP for 10, 30, 60, 90 and 120 s. Triton-insoluble protein was obtained as described in the Methods. The proteins were electrophoresed, transferred to a membrane, and probed with anti-β-tubulin antibody. (A) Western blot and (B) densitometry. Experimental conditions were repeated three times using cells from different donors.

demonstrate for the first time that agonist-induced signaling events regulate tubulin depolymerization and repolymerization during platelet activation.

Discussion

Stimulation of the P2Y1 receptor by ADP causes platelet shape change;[41,42,63] furthermore, all downstream signaling mediating shape change is initiated by the heterotrimeric G-protein, Gq.[72] Prior studies have shown that agonist-induced platelet shape change involves both calcium-dependent and calcium-independent mechanisms as measured by increased light scattering in an aggregometer.[46–48] We have used ADP to stimulate shape change and have investigated the regulation of morphology and cytoskeletal architecture. ADP-induced shape change provides a unique opportunity to identify the regulation and contribution of actin filaments and microtubules without obscuring events such as secretion or multiple receptor/G-protein signaling. The intracellular calcium chelating agent 5,5′-dimethyl BAPTA has been used to completely prevent the increase in intracellular calcium following stimulation by ADP, thereby preventing the activation of calcium dependent pathways such as calcium/calmodulin MLCK.[46] The pyridine derivative Y-27632 is selective for the inhibition $p160^{ROCK}$ in human platelets ($IC_{50} = 1\,\mu M$).[56]

The scanning electron microscopy data presented in this report provide a better understanding of the contribution made by calcium-sensitive and -insensitive mechanisms to the different features of shape change. Filopodia extension, plasma membrane folding, and sphere formation all require either an increase in intracellular calcium (Figure 3), or the activity of $p160^{ROCK}$ (Figure 4). The calcium-sensitive pathway and the calcium-insensitive $RhoA/p160^{ROCK}$ pathway independently induce all the features of shape change. Shape change mediated by the calcium-sensitive pathway proceeded at the same rate as in control platelets; however, plasma membrane folding and the spherical shape are not maintained in the absence of functional $p160^{ROCK}$ (Rho-kinase) (Figure 4). This is in contrast to shape change mediated by the $RhoA/p160^{ROCK}$ pathway that occurs at a much slower rate and is maintained (Figure 3). Interestingly, comparison of the aggregometry tracings and the cells examined by scanning electron microscopy reveals a novel correlation

between the amplitude of the aggregometry oscillations (Figure 1) and the presence of disc-shaped cells (Figure 2). The relative increase in amplitude of the oscillations indicates an increasing presence of disc shaped cells (Figures 1 and 4). In contrast, a decrease in amplitude indicates a diminishing presence of disc-shaped cells and an increase in sphere-shaped cells (Figures 1–3).

We investigated whether the spherical shape was dependent upon actin association with myosin in the cytoskeleton. Platelet myosin phosphatase is phosphorylated and inactivated by RhoA/p160^{ROCK}.[49,50] The maintenance of both ADP-induced membrane folding and the spherical shape could require continual inactivation of myosin phosphatase to preserve myosin light chain phosphorylation and the actin–myosin contractile response. However, there was no difference in the level of actin associated with the cytoskeleton by 2 min between Y-27632 disc-shaped cells and 5,5′-dimethyl BAPTA-treated sphere-shaped platelets (Figure 5). Furthermore, the levels of phosphorylated myosin light chain in either Y-27632 or 5,5′-dimethyl BAPTA-treated platelets was the same by 45 s following ADP-stimulation.[46] Therefore, such a mechanism for the maintenance of spherocyte morphology is unlikely. However, there is a clear correlation between the presentation of filopodia and the maximal association of actin and myosin in the cytoskeleton. In control and Y-27632 treated platelets (inhibition of p160^{ROCK}) the maximal level of association occurs at 10 s and maximal filopodia presentation occurs between 5 and 15 s. In the absence of calcium mobilization (5,5′-dimethyl BAPTA) the maximal level of actin and myosin association occurs at 20 s and maximal filopodia presentation occurs at a point between 15 and 30 s.

A connection between the maintenance of spherical morphology and RhoA/p160^{ROCK} activity is demonstrated in Figure 4. In the absence of p160^{ROCK} activity the platelets are unable to remain spherical and revert back towards the disc shape. The disruption of the microtubule coil leads to a decreased level of β-tubulin in the isolated microtubule fraction.[16] The kinetics of the decrease in β-tubulin in the 100 000×g pellet correlates with the time points at which disc-shaped cells change into sphere-shaped cells (Figures 2 and 6). The time frame for reverting back to the disc shape in Y-27632-treated cells correlates closely with the return of tubulin polymerization (Figures 4 and 6). Thus, p160^{ROCK} (Rho-kinase) initiates and maintains microtubule disassociation; whereas, the calcium-sensitive pathway contributes more to initiation of microtubule disassembly. Subsequent to activation, as the intracellular calcium level falls, the calcium-sensitive pathway fails to maintain the spherical shape. Investigations using Y-27632, toxin B, and C3 exotoxin have revealed a role for RhoA/p160^{ROCK} (Rho-kinase)-mediated microtubule disruption in endothelial cells,[37] cardiomyocytes[31] and an invasive tumor cell line.[23] The present investigation extends the early observations made by Behnke,[73,74] White and co-worker,[75,76] and Kenny and Linck[3] regarding the role of the microtubule coil in supporting the discoid shape. Furthermore, we demonstrate that the p160^{ROCK} (Rho-kinase) pathway regulates microtubule disruption in platelets and contributes to the maintenance of spherical morphology.

Further investigation into the mechanism utilized by platelets to regulate microtubule stability is required. The platelet microtubule coil may be maintained by microtubule-associated proteins (MAPs), which stabilize and bundle microtubules when they are in a non-phosphorylated state. When MAPs are phosphorylated they detach from microtubules and permit tubulin depolymerization because of inherent dynamic instability.[35,36] Perhaps MAPs are dephosphorylated by PP1Mδ, a phosphatase that is inactivated by p160^{ROCK}.[49,61] Just as likely, p160^{ROCK} phosphorylates and inactivates an unidentified phosphatase in addition to PP1Mδ. As mentioned, the protein phosphatase 1 and 2A inhibitors, okadaic acid and calyculin-A, cause disassembly of the microtubule coil and platelet shape change.[33] The phosphorylation and inactivation of MAP-regulating phosphatases would promote MAP phosphorylation and subsequent disassembly of the microtubule coil. A number of phosphoproteins have been identified in human platelets that may in the future be identified as the target MAP responsible for stability of the microtubule coil: a 210-kDa MAP,[77] a 51-kDa protein,[3] and a 90-kDa protein.[33] The presence of Y-27632 would prevent p160^{ROCK} (Rho-kinase)-mediated inactivation of phosphatase activity leading to phosphatase-mediated dephosphorylation of MAPs, their association with a growing microtubule, and reformation of the circumferential coil.

In this study, for the first time, we have correlated changes in the aggregometer tracing with specific morphological features of platelet shape change. Either the RhoA/p160^{ROCK} or the calcium-dependent pathway mediates filopodia extension. The calcium-insensitive RhoA/p160^{ROCK} pathway is required for the maintenance of both spherical shape and membrane folding in ADP-induced platelet shape change. The return of the discoid shape in the absence of p160^{ROCK} activity is likely due to the return of the microtubule coil and does not appear to involve the association of actin and myosin in the cytoskeleton. An explanation of the inability of the calcium-sensitive pathway to maintain shape change may be evidenced by the fact the increase in intracellular Ca^{2+} caused by ADP is transient and rapidly returns to baseline. Thus, while Ca^{2+}-regulated events can disrupt the microtubule coil (Figure 6), the p160^{ROCK} pathway is necessary for the

sustained disruption of the microtubule coil and resulting spherical morphology.

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References

1. Holmsen H. Significance of testing platelet functions in vitro. *Eur J Clin Invest* 1994; **24 (Suppl 1)**: 3–8.
2. Wurzinger L J. Histophysiology of the circulating platelet. *Adv Anat Embryol Cell Biol* 1990; **120**: 1–96.
3. Kenney D M, Linck R W. The cytoskeleton of unstimulated blood platelets: structure and composition of the isolated marginal microtubular band. *J Cell Sci* 1985; **78**: 1–22.
4. White J G, Sauk J J. Microtubule coils in spread blood platelets. *Blood* 1984; **64**: 470–8.
5. Allen R D, Zacharski L R, Widirstky S T, Rosenstein R, Zaitlin L M, Burgess D R. Transformation and motility of human platelets: details of the shape change and release reaction observed by optical and electron microscopy. *J Cell Biol* 1979; **83**: 126–42.
6. Barnhart M I, Walsh R T, Robinson J A. A three-dimensional view of platelet responses to chemical stimuli. *Ann NY Acad Sci* 1972; **201**: 360–90.
7. Hantgan R R. A study of the kinetics of ADP-triggered platelet shape change. *Blood* 1984; **64**: 896–906.
8. White J G. An overview of platelet structural physiology. *Scanning Microsc* 1987; **1**: 1677–700.
9. Fox J E. Regulation of platelet function by the cytoskeleton. *Adv Exp Med Biol* 1993; **344**: 175–85.
10. Siess W. Molecular mechanisms of platelet activation. *Physiol Rev* 1989; **69**: 58–178.
11. White J G, Rao G H. Microtubule coils versus the surface membrane cytoskeleton in maintenance and restoration of platelet discoid shape. *Am J Pathol* 1998; **152**: 597–609.
12. Zucker M B, Nachmias V T. Platelet activation. *Arteriosclerosis* 1985; **5**: 2–18.
13. Hartwig J H. Mechanisms of actin rearrangements mediating platelet activation. *J Cell Biol* 1992; **118**: 1421–42.
14. Escolar G, Krumwiede M, White J G. Organization of the actin cytoskeleton of resting and activated platelets in suspension. *Am J Pathol* 1986; **123**: 86–94.
15. Steiner M, Ikeda Y. Quantitative assessment of polymerized and depolymerized platelet microtubules. Changes caused by aggregating agents. *J Clin Invest* 1979; **63**: 443–8.
16. Kenney D M, Chao F C. Ionophore-induced disassembly of blood platelet microtubules: effect of cyclic AMP and indomethacin. *J Cell Physiol* 1980; **103**: 289–98.
17. Nachmias V T. Cytoskeleton of human platelets at rest and after spreading. *J Cell Biol* 1980; **86**: 795–802.
18. Nachmias V T, Sullender J, Fallon J, Asch A. Observations on the 'cytoskeleton' of human platelets. *Thromb Haemost* 1980; **42**: 1661–6.
19. Mattson J C, Zuiches C A. Elucidation of the platelet cytoskeleton. *Ann NY Acad Sci* 1981; **370**: 11–21.
20. Lewis J C, White M S, Prater T, Taylor R G, Davis K S. Ultrastructural analysis of platelets in nonhuman primates. III. Stereo microscopy of microtubules during platelet adhesion and the release reaction. *Exp Mol Pathol* 1982; **37**: 370–81.
21. White J G, Rao G H. Influence of a microtubule stabilizing agent on platelet structural physiology. *Am J Pathol* 1983; **112**: 207–17.
22. Hartwig J H, DeSisto M. The cytoskeleton of the resting human blood platelet: structure of the membrane skeleton and its attachment to actin filaments. *J Cell Biol* 1991; **112**: 407–25.
23. Nishimura Y, Itoh K, Yoshioka K, Uehata M, Himeno M. Small guanosine triphosphatase Rho/Rho-associated kinase as a novel regulator of intracellular redistribution of lysosomes in invasive tumor cells. *Cell Tissue Res* 2000; **301**: 341–51.
24. Takaishi K, Kikuchi A, Kuroda S, Kotani K, Sasaki T, Takai Y. Involvement of rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI) in cell motility. *Mol Cell Biol* 1993; **13**: 72–9.
25. Prendergast G C, Khosravi-Far R, Solski P A, Kurzawa H, Lebowitz P F, Der C J. Critical role of Rho in cell transformation by oncogenic Ras. *Oncogene* 1995; **10**: 2289–96.
26. Perona R, Esteve P, Jimenez B, Ballestero R P, Ramon y Cajal S, Lacal J C. Tumorigenic activity of rho genes from *Aplysia californica*. *Oncogene* 1993; **8**: 1285–92.
27. Paterson H F, Self A J, Garrett M D, Just I, Aktories K, Hall A. Microinjection of recombinant p21rho induces rapid changes in cell morphology. *J Cell Biol* 1990; **111**: 1001–7.
28. Kishi K, Sasaki T, Kuroda S, Itoh T, Takai Y. Regulation of cytoplasmic division of *Xenopus* embryo by rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI). *J Cell Biol* 1993; **120**: 1187–95.
29. Hall A. Small GTP-binding proteins and the regulation of the actin cytoskeleton. *Annu Rev Cell Biol* 1994; **10**: 31–54.
30. Palazzo A F, Cook T A, Alberts A S, Gundersen G G. mDia mediates Rho-regulated formation and orientation of stable microtubules. *Nat Cell Biol* 2001; **3**: 723–9.
31. Pletjushkina O J, Rajfur Z, Pomorski P, Oliver T N, Vasiliev J M, Jacobson K A. Induction of cortical oscillations in spreading cells by depolymerization of microtubules. *Cell Motil Cytoskeleton* 2001; **48**: 235–44.
32. Roychowdhury S, Panda D, Wilson L, Rasenick M M. G protein alpha subunits activate tubulin GTPase and modulate microtubule polymerization dynamics. *J Biol Chem* 1999; **274**: 13 485–90.
33. Yano Y, Sakon M, Kambayashi J, Kawasaki T, Senda T, Tanaka K, Yamada F, Shibata N. Cytoskeletal reorganization of human platelets induced by the protein phosphatase 1/2 A inhibitors okadaic acid and calyculin A. *Biochem J* 1995; **307**: 439–49.
34. Drewes G, Ebneth A, Mandelkow E M. MAPs, MARKs and microtubule dynamics. *Trends Biochem Sci* 1998; **23**: 307–11.
35. Desai A, Mitchison T J. Microtubule polymerization dynamics. *Annu Rev Cell Dev Biol* 1997; **13**: 83–117.
36. Nogales E. Structural insights into microtubule function. *Annu Rev Biochem* 2000; **69**: 277–302.
37. Verin A D, Birukova A, Wang P, Liu F, Becker P, Birukov K, Garcia J G. Microtubule disassembly increases endothelial cell barrier dysfunction: role of MLC phosphorylation. *Am J Physiol Lung Cell Mol Physiol* 2001; **281**: L565–74.
38. Kolodney M S, Wysolmerski R B. Isometric contraction by fibroblasts and endothelial cells in tissue culture: a quantitative study. *J Cell Biol* 1992; **117**: 73–82.
39. Danowski B A. Microtubule dynamics in serum-starved and serum-stimulated Swiss 3T3 mouse fibroblasts: implications for the relationship between serum- induced contractility and microtubules. *Cell Motil Cytoskeleton* 1998; **40**: 1–12.
40. Jin J, Kunapuli S P. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci USA* 1998; **95**: 8070–4.
41. Savi P, Beauverger P, Labouret C, Delfaud M, Salel V, Kaghad M, Herbert J M. Role of P2Y1 purinoceptor in ADP-induced platelet activation. *FEBS Lett* 1998; **422**: 291–5.
42. Jantzen H M, Gousset L, Bhaskar V, Vincent D, Tai A, Reynolds E E, Conley P B. Evidence for two distinct G-protein-coupled ADP receptors mediating platelet activation. *Thromb Haemost* 1999; **81**: 111–7.
43. Kunapuli S P. Multiple P2 receptor subtypes on platelets: a new interpretation of their function. *Trends Pharmacol Sci* 1998; **19**: 391–4.
44. Kunapuli S P. Functional characterization of platelet ADP receptors [Review]. *Platelets* 1998; **9**: 343–51.
45. Kunapuli S P, Daniel J L. P2 receptor subtypes in the cardiovascular system. *Biochem J* 1998; **336**: 513–23.
46. Paul B Z, Daniel J L, Kunapuli S P. Platelet shape change is mediated by both calcium-dependent and -independent signaling pathways. Role of p160 rho-associated coiled-coil-containing protein kinase in platelet shape change. *J Biol Chem* 1999; **274**: 28293–300.
47. Bauer M, Retzer M, Wilde J I, Maschberger P, Essler M, Aepfelbacher M, Watson S P, Siess W. Dichotomous

regulation of myosin phosphorylation and shape change by Rho-kinase and calcium in intact human platelets. *Blood* 1999; **94**: 1665–72.
48. Klages B, Brandt U, Simon M I, Schultz G, Offermanns S. Activation of G12/G13 results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets. *J Cell Biol* 1999; **144**: 745–54.
49. Nakai K, Suzuki Y, Kihira H, Wada H, Fujioka M, Ito M, Nakano T, Kaibuchi K, Shiku H, Nishikawa M. Regulation of myosin phosphatase through phosphorylation of the myosin-binding subunit in platelet activation. *Blood* 1997; **90**: 3936–42.
50. Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase) [see comments]. *Science* 1996; **273**: 245–8.
51. Noda M, Yasuda-Fukazawa C, Moriishi K, Kato T, Okuda T, Kurokawa K, Takuwa Y. Involvement of rho in GTP gamma S-induced enhancement of phosphorylation of 20 kDa myosin light chain in vascular smooth muscle cells: inhibition of phosphatase activity. *FEBS Lett* 1995; **367**: 246–50.
52. Kozasa T, Jiang X, Hart M J, Sternweis P M, Singer W D, Gilman A G, Bollag G, Sternweis P C. p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13 [see comments]. *Science* 1998; **280**: 2109–11.
53. Amano M, Chihara K, Kimura K, Fukata Y, Nakamura N, Matsuura Y, Kaibuchi K. Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. *Science* 1997; **275**: 1308–11.
54. Kureishi Y, Kobayashi S, Amano M, Kimura K, Kanaide H, Nakano T, Kaibuchi K, Ito M. Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. *J Biol Chem* 1997; **272**: 12 257–60.
55. Chihara K, Amano M, Nakamura N, Yano T, Shibata M, Tokui T, Ichikawa H, Ikebe R, Ikebe M, Kaibuchi K. Cytoskeletal rearrangements and transcriptional activation of c-fos serum response element by Rho-kinase. *J Biol Chem* 1997; **272**: 25 121–7.
56. Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension [see comments]. *Nature* 1997; **389**: 990–4.
57. Amano M, Chihara K, Nakamura N, Fukata Y, Yano T, Shibata M, Ikebe M, Kaibuchi K. Myosin II activation promotes neurite retraction during the action of Rho and Rho-kinase. *Genes Cells* 1998; **3**: 177–88.
58. Essler M, Amano M, Kruse H J, Kaibuchi K, Weber P C, Aepfelbacher M. Thrombin inactivates myosin light chain phosphatase via Rho and its target Rho kinase in human endothelial cells. *J Biol Chem* 1998; **273**: 21867–74.
59. Hirose M, Ishizaki T, Watanabe N, Uehata M, Kranenburg O, Moolenaar W H, Matsumura F, Maekawa M, Bito H, Narumiya S. Molecular dissection of the Rho-associated protein kinase (p160ROCK)-regulated neurite remodeling in neuroblastoma N1E-115 cells. *J Cell Biol* 1998; **141**: 1625–36.
60. Majumdar M, Seasholtz T M, Goldstein D, de Lanerolle P, Brown J H. Requirement for Rho-mediated myosin light chain phosphorylation in thrombin-stimulated cell rounding and its dissociation from mitogenesis. *J Biol Chem* 1998; **273**: 10 099–106.
61. Nemoto Y, Namba T, Teru-uchi T, Ushikubi F, Morii N, Narumiya S. A rho gene product in human blood platelets. I. Identification of the platelet substrate for botulinum C3 ADP-ribosyltransferase as rhoA protein. *J Biol Chem* 1992; **267**: 20 916–20.
62. Paul B Z, Jin J, Kunapuli S P. Molecular mechanism of thromboxane A(2)-induced platelet aggregation. Essential role for p2t(ac) and alpha(2a) receptors. *J Biol Chem* 1999; **274**: 29 108–14.
63. Jin J, Daniel J L, Kunapuli S P. Molecular basis for ADP-induced platelet activation II: the P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem* 1998; **273**: 2030–4.
64. Zablocki J A, Miyano M, Garland R B, Pireh D, Schretzman L, Rao S N, Lindmark R J, Panzer-Knodle S G, Nicholson N S, Taite B B, Salyers A K, King L W, Campion J G, Feigen L P. Potent in vitro and invivo inhibitors of platelet aggregation based upon the Arg-Gly-Asp-Phe sequence of fibrinogen. *J Med Chem* 1993; **36**: 1811–9.
65. Weisel J W, Nagaswami C. Computer modeling of fibrin polymerization kinetics correlated with electron microscope and turbidity observations: clot structure and assembly are kinetically controlled. *Biophys J* 1992; **63**: 111–28.
66. Wang D, Villasante A, Lewis S A, Cowan N J. The mammalian beta-tubulin repertoire: hematopoietic expression of a novel, heterologous beta-tubulin isotype. *J Cell Biol* 1986; **103**: 1903–10.
67. Cox A C, Carroll R C, White J G, Rao G H. Recycling of platelet phosphorylation and cytoskeletal assembly. *J Cell Biol* 1984; **98**: 8–15.
68. Daniel J L, Adelstein R S. Isolation and properties of platelet myosin light chain kinase. *Biochemistry* 1976; **15**: 2370–7.
69. Hathaway D R, Adelstein R S. Human platelet myosin light chain kinase requires the calcium-binding protein calmodulin for activity. *Proc Natl Acad Sci USA* 1979; **76**: 1653–7.
70. Suzuki Y, Yamamoto M, Wada H, Ito M, Nakano T, Sasaki Y, Narumiya S, Shiku H, Nishikawa M. Agonist-induced regulation of myosin phosphatase activity in human platelets through activation of Rho-kinase. *Blood* 1999; **93**: 3408–17.
71. Daniel J L, Molish I R, Rigmaiden M, Stewart G. Evidence for a role of myosin phosphorylation in the initiation of the platelet shape change response. *J Biol Chem* 1984; **259**: 9826–31.
72. Offermanns S, Toombs C F, Hu Y H, Simon M I. Defective platelet activation in G alpha(q)-deficient mice. *Nature* 1997; **389**: 183–6.
73. Behnke O. Incomplete microtubules observed in mammalian blood platelets during microtubule polymerization. *J Cell Biol*. 1967; **34**: 697–701.
74. Behnke O. Microtubules in disk-shaped blood cells. *Int Rev Exp Pathol* 1970; **9**: 1–92.
75. White J G, Krivit W. An ultrastructural basis for the shape changes induced in platelets by chilling. *Blood* 1967; **30**: 625–35.
76. White J G. Platelet morphology. In: Johnson S A, ed. *The Circulating Platelet*. New York: Academic Press; 1971: 45–121.
77. Tablin F, Reeber M J, Nachmias V T. Platelets contain a 210K microtubule-associated protein related to a similar protein in HeLa cells. *J Cell Sci* 1988; **90**: 317–24.