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

(Received for publication, February 18, 1999, and in revised form, June 29, 1999)

Benjamin Z. S. Paul‡, James L. Daniel‡§, and Satya P. Kunapuli‡§

From the Departments of ‡Pharmacology and §Physiology and the §§Sol Sherry Thrombosis Research Center, Temple University Medical School, Philadelphia, Pennsylvania 19140

Platelets undergo shape change upon activation with agonists. During shape change, disc-shaped platelets turn into spiculated spheres with protruding filopodia. When agonist-induced cytosolic Ca2+ increases were prevented using the cytosolic Ca2+ chelator, 5,5'-dimethyl-bis-(o-aminophenoxy)ethane-N,N',N'-N''-tetraacetic acid (5,5'-dimethyl-BAPTA), platelets still underwent shape change, although the onset was delayed and the initial rate was dramatically decreased. In the absence of cytosolic Ca2+, agonist-stimulated myosin light chain phosphorylation was significantly inhibited. The myosin light chain was maximally phosphorylated at 2 s in control platelets compared with 30 s in 5,5'-dimethyl-BAPTA-treated platelets. ADP, thrombin, or U46619-induced Ca2+-independent platelet shape change was significantly reduced by staurosporine, a nonselective kinase inhibitor, by the selective p160 Rho-associated coiled-coil-containing protein kinase inhibitor Y-27632, or by HA 1077. Both Y-27632 and HA 1077 reduced peak levels of ADP-induced platelet shape change and myosin light chain phosphorylation in control platelets. In 5,5'-dimethyl-BAPTA-treated platelets, Y-27632 and HA 1077 completely abolished both ADP-induced platelet shape change and myosin light chain phosphorylation. Our results indicate that Ca2+/calmodulin-stimulated myosin light chain kinase and p160 Rho-associated coiled-coil-containing protein kinase independently contribute to myosin light chain phosphorylation and platelet shape change, through Ca2+-sensitive and Ca2+-insensitive pathways, respectively.

Platelets are anucleate cells that mediate hemostasis through amplifying an initial stimulus and aggregating at a site of injury. Several agents including thrombin, ADP, and thromboxane A2 activate platelets. Activated platelets change shape, secrete α-granules and dense granules, and release positive feedback mediators (1). When platelets are initially stimulated, the first event is a rearrangement of the cytoskeletal proteins (actin and myosin), and the normally disc-shaped cells change into spheres with filopodia (1, 2). Activation of phospholipase A2 releases arachidonic acid from membrane phospholipids, which is converted into thromboxane A2. Serotonin and ADP, released from dense granules, and thromboxane A2 function as positive feedback mediators, which recruit more platelets into a primary hemostatic plug (3).

We have been investigating the intracellular events involved in agonist-induced platelet activation. Recently, we proposed a three-receptor model to explain the signaling events in platelet activation by ADP (for reviews, see Refs. 4 and 5). Through the use of receptor-specific antagonists, we have provided evidence for different functional roles for different P2 receptor subtypes present on platelets (6, 7). Platelets express two G protein-coupled P2 receptor subtypes: a P2TAC receptor subtype, coupled to the inhibition of adenylyl cyclase via the heterotrimeric inhibitory G-protein, Gi, and P2Y1 receptor, coupled to the heterotrimeric protein Gq. Activation of the P2Y1 receptor results in the activation of phospholipase C, production of diacylglycerol, and mobilization of cytosolic Ca2+ in response to IP3 production. ATP acts as an antagonist at both the P2Y1 and P2TAC receptors while acting as an agonist at the ionotropic P2X1 receptor, the third ADP receptor on platelets (7, 8). While ADP-induced platelet aggregation requires coactivation of both the P2Y1 and the P2TAC receptors (9, 10), activation of the P2Y1 receptor is sufficient to cause ADP-induced platelet shape change (6). Similarly, activation of Gq-coupled 5HT2A receptors by serotonin is also sufficient to induce shape change (9, 10).

During platelet shape change, the discoid cells undergo cytoskeletal changes including the disassembly of a microtubule ring that results in an intermediate spherical shape. This is followed by actin polymerization and the slower extension of filopodia (11–13). Previous reports have shown that a strong correlation exists between phosphorylation of the regulatory myosin light chain and the initiation of shape change (14). Agonist-dependent phosphorylation of platelet myosin correlates with its polymerization and association with actin filaments (14–17). The concentration-response curve of ADP-induced myosin light chain phosphorylation closely parallels that of shape change, while both responses have the same half-maximal inhibitory concentration (IC50) toward ATP (14). Myosin light chain kinase is present in platelets (18) and is activated in vitro by Ca2+ and calmodulin (19).

Small GTP-binding proteins have been implicated in rearrangement and activation of cytoskeletal proteins (20). The superfamily of small GTP-binding proteins is divided into subfamilies including Rho, Rac, and Cdc42. There are three forms of Rho proteins (Rho stands for Ras homologous) including RhoA, RhoB, and RhoC (for a review, see Ref. 20) that control the assembly and disassembly of the actin cytoskeleton in...
Role of p160ROCK in Platelet Shape Change

many cell types in response to extracellular signals (21). The activated GTP-bound form of Rho associates specifically with five protein kinases designated as p120 protein kinase N (p120ROCK), RhoA-binding kinase α (p150ROCK).1 Rhon-binding kinase β (p150ROCK). p160 Rho-associated coiled-coil-containing protein kinase (p160ROCK), and p164 Rho kinase (22–26). The pyridine derivative, Y-27632, has been shown to selectively inhibit p160ROCK with an IC50 of 1 μM. This compound has a higher specificity for p160ROCK (200-fold) than PKA or the PKC isoforms present in rat brain; furthermore, its specificity for myosin light chain kinase is 2000-fold lower than p160ROCK (27). Y-27632 has been shown to selectively inhibit both the activity of p160ROCK immunoprecipitated from human platelets and the involvement of this specific kinase in smooth-muscle contraction (24, 27). The homopiperazine derivative, HA 1077, has a slightly lower binding affinity for p160ROCK than Y-27632, but it is also more selective for this kinase than PKC, protein kinase A, and myosin light chain kinase (27, 28).

Following the observation that shape change depends upon stimulation of a Gq-coupled receptor (6, 7, 9), our investigation has focused on the role of intracellular signaling events mediating shape change. Here, we provide evidence that platelet shape change incorporates both Ca2+-dependent and -independent mechanisms for cytoskeletal rearrangement. We have used 5,5′-dimethyl-BAPTA to prevent the increase in cytosolic Ca2+ that occurs following Gα activation. Previous studies (e.g. Jen et al. (29)) have successfully used 5,5′-dimethyl-BAPTA to prevent increases in cytosolic Ca2+ concentration without deleterious effects on either cell viability or morphology. Through the use of p160ROCK-selective inhibitors, Y-27632 and HA 1077, we investigated the role of the RhoA/p160ROCK pathway in platelet response. We show that both Ca2+-calmodulin-dependent myosin light chain kinase and the RhoA/p160ROCK pathways contribute to ADP-induced platelet shape change and regulation of myosin light chain phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—Apase (type V), ADP, fibrinogen, and bovine serum albumin (fraction V) were from Sigma. The acetoxyethyl ester of Fura PE-3 was from Tefabs (Austin, TX). The acetoxyethyl ester of 5,5′-dimethyl-BAPTA, U46619 (a stable analog of thromboxane A2), Stauroporine, HA 1077, GF 109203X (bisindolylmaleimide I), Ro 31–8220 (bisindolymaleimide IX), and phorbol-12-myristyl-13-acetate were from BioMol (Plymouth Meeting, PA). Bovine thrombin was from Parke, Davis and Co. (Detroit, MI). Y-27632 was a gift from Yoshitomi Pharmaceutical Industries, Ltd. (Osaka, Japan). SC-57101 was a gift from Searle Research and Development (Skokie, IL). Ultrapure acrylamide gel reagents were from ICN (Costa Mesa, CA) except for Tris base, dithiothreitol, and glycine, which were purchased from Fisher. Other chemicals were reagent grade, and deionized water was used throughout.

Preparation of Fura PE-3 and 5,5′-Dimethyl-BAPTA-loaded Platelets—Human blood was collected from a pool of informed healthy volunteers, all of whom are students or staff at Temple University School of Medicine. The donated blood was collected into a one-sixth volume of ACD (2.5 g of sodium citrate, 1.5 g of citric acid, and 2.0 g of glucose in 100 ml of deionized H2O). Platelet-rich plasma was isolated by centrifugation of citrated blood at 180 × g for 15 min at room temperature. Platelet-rich plasma was incubated at 37 °C with 5 μM Fura PE-3 acetoxyethyl ester and 1 mM acetylcholine for 15 min followed by the addition of either 50 μM 5,5′-dimethyl-BAPTA acetoxyethyl ester or a corresponding volume of the vehicle, dimethyl sulfoxide, and further incubation for 30 min. After 15 min at room temperature, the platelet-rich plasma was centrifuged at 1000 × g for 10 min at room temperature. The platelet pellet was resuspended in calcium-free HEPES-buffered Tyrode’s solution (7) supplemented with 0.2% bovine serum albumin and 20 μg/ml apyrase. The platelet count was adjusted to 2 × 109 cells/ml. All experiments were repeated at least three times using platelets from different donors.

Measurement of Ca2+ Using Fura PE-3 and Platelet Activation—Aliquots (1.0 ml) of the platelet suspension were stirred in a water-jacketed cuvette maintained at 37 °C during activation. Fluorescence was constantly measured using a Perkin-Elmer LS-5 spectrofluorimeter with settings of 340 nm (excitation) and 510 nm (emission). Fura PE-3 fluorescence signals were calibrated as described previously (30). Fmax was determined by the addition of 2 mM EGTA and 20 mM Tris base. Fmin was determined by lysing the cells with 40 μg digitonin in the presence of saturating CCl4.

Platelet Aggregation and Analysis of Shape Change—Agonist-induced platelet aggregation was determined by measuring the transmission of light through a 0.5-ml sample of aspirinated washed platelets (2 × 109 cells/ml) with stirring in a lumiaggregometer at 37 °C (Chrono-Log, Haverton, PA). The base line was set using 0.5 ml of Tyrode’s solution as a blank. Aggregation of washed platelets required the addition of fibrinogen (1 mg/ml) prior to the addition of an agonist, with the recorder output speed set to 0.2 mm/s. Platelet shape change was observed by the addition of 10 μM SC-57101 before agonist stimulation as described earlier (6, 14). SC-57101 is a known inhibitor of platelet aggregation through blocking fibrinogen binding to its receptor (31). Three shape change curve characteristics were measured. First, the time from initiation of shape change to 50% of the final time point (t1/2); second, the time from initiation of shape change to the point where shape change is half complete (t1/4); third, the initial rate constant of the shape change curve. In order to better resolve these three shape change curve characteristics, results were printed out using a Kipp and Zonen BD-41 ( Fisher) analog recorder with the output speed set to 1 mm/s. The initial rate constant was determined using the program Kaleidagraph (Synergy Software, Reading, PA) by graphing the fraction of shape change complete ( fractional light absorbance) versus time, and the resulting points were fit with the exponential equation, y = 1 − e−kt, where k represents the initial rate constant of platelet shape change and t is the time.

Measurement of Myosin Light Chain Phosphorylation—The percent- age of myosin light chain in the phosphorylated form was determined using a protocol (32) that was adapted from a modification (33) of the method described by Perrie and Perry (34). In brief, aspirinated platelets were resuspended in Tyrode’s solution at a concentra- tion of 2 × 109 cells/ml. Aliquots (0.5 ml) were stirred at 37 °C during stimulation in the lumiaggregometer. At specific time points, 25 μl of 6.6 μl HClO4, was added, and the resulting acid precipitate was collected for 30 min at 4 °C. The precipitate was washed three times and resuspended with 0.5 ml of 0.5 M NH4OH. The sample was then centrifuged at 10,000 × g for 2 min. Protein pellets were dissolved in 50 μl of sample buffer containing 8 M urea, 20 mM Tris, 122 mM glycine, 5 mM dithiothreitol, pH 8.6, with approximately 0.1% bromphenol blue dye. The suspended pellets were further dissolved by sonication in a Branson (Shelton, CT) sonication bath. Gel electrophoresis was performed using 10% polyacrylamide slab gels containing 40% (v/v) glycerol with a 3.6% polyacrylamide stacking gel containing 2% of 2× Tris. Gels were stained for 0.05% (w/v) Coomassie Brilliant Blue R-250, destained, and scanned using a Hoeffer (San Francisco, CA) scanning densitometer hooked up to a Macintosh II computer via a National Instruments Corporation (Austin, TX) DAQ conversion board. The densitometry peaks correlating to the phosphorylated and nonphosphorylated myosin light chains as well as the 16-kDa band were analyzed using the program Kaleidagraph (Synergy Software, Reading, PA). The data points were fit using a three-peak gaussian equation. The amount of total myosin light chain in the phosphorylated form was determined by dividing the area of the phosphorylated peak by the combined areas of the phosphorylated and the nonphosphorylated myosin light chain peaks. Results were expressed as the percentage of total myosin light chain in the phosphorylated form.

RESULTS

Effects of 5,5′-Dimethyl-BAPTA on Agonist-induced Increase in Cytosolic Ca2+, Platelet Aggregation, and Platelet Shape

1 The abbreviations used are: ROCK, Rhon-binding kinase; ROCK, Rhon-associated coiled-coil-forming kinase; P2Y5, platelet ADP receptor coupled to inhibition of adenylate cyclase; MBS, myosin-binding subunit; 5,5′-dimethyl-BAPTA, 5,5′-dimethyl-bis(o-aminophenoxy)ethane-N,N,N,N′-tetracetic acid; PKC, protein kinase C.
increased in cytosolic Ca\(^{2+}\), indicating that an increase in cytosolic Ca\(^{2+}\) does not aggregate in response to ADP, thrombin, or U46619 (Fig. 1A).

To investigate the role of the increase in cytosolic Ca\(^{2+}\) in agonist-induced platelet shape change, we loaded the platelets with Fura PE-3, either alone or in combination with the cytosolic Ca\(^{2+}\) chelator 5,5'-dimethyl-BAPTA, and measured the fluorescence response. The normal increase in the cytosolic Ca\(^{2+}\) concentration, which occurs in response to ADP, thrombin, and U46619 (Fig. 1A), did not occur in the platelets loaded with 5,5'-dimethyl-BAPTA (Fig. 1B). These traces are representative of experiments performed to establish (and reconfirm) the absence of an increase in cytosolic Ca\(^{2+}\) due to Ca\(^{2+}\) chelation by 5,5'-dimethyl-BAPTA.

In the absence of an increase in cytosolic Ca\(^{2+}\), platelets did not aggregate in response to ADP, thrombin, or U46619 (Fig. 2), indicating that an increase in cytosolic Ca\(^{2+}\) is essential for fibrinogen receptor activation. However, agonist-induced platelet shape change still occurred. Close examination revealed that shape change in platelets treated with 5,5'-dimethyl-BAPTA possess different characteristics from shape change occurring in control platelets. It is first apparent that the rate of increase in light absorbance (indicating the change in platelet shape from disc to spiny sphere) was significantly slower. Moreover, a delay in the initiation of shape change following the addition of an agonist can be observed. After repeating each of these conditions three or more times in three different donors, we quantitated and compared these three features of platelet shape changes in normal platelets and 5,5'-dimethyl-BAPTA-treated platelets. In order to do so, the recorder’s printout speed was increased to give greater resolution. In the absence of an increase in cytosolic Ca\(^{2+}\) concentration, the time to initiate shape change increased substantially for all agonists examined (Fig. 3A). The time for half-completion of shape change was also dramatically increased in 5,5'-dimethyl-BAPTA-treated platelets (Fig. 3B). A significant decrease in the initial rate of shape change was observed for U46619 (~40% of control) and both ADP and thrombin (~60% of control) (Fig. 3C).

Effect of 5,5'-Dimethyl-BAPTA on Agonist-induced Myosin Light Chain Phosphorylation—In normal platelets following stimulation by ADP, thrombin, and U46619, there was a large increase in the percentage of phosphorylated myosin light chain (Fig. 4). When the increase in cytosolic Ca\(^{2+}\) concentration was prevented with 5,5'-dimethyl-BAPTA, the levels of phosphorylated myosin light chain were dramatically reduced in platelets stimulated with ADP, thrombin, and U46619 compared with vehicle-loaded platelets. The difference in levels of phosphorylated myosin light chain in 5,5'-dimethyl-BAPTA-treated platelets following agonist stimulation was significant (p < 0.05; n = 3). In the absence of agonist stimulation, there was a very small increase in the level of phosphorylated myosin light chain in 5,5'-dimethyl-BAPTA-treated platelets; however, the difference between the level of phosphorylation in unstimulated treated platelets and unstimulated control platelets was not significant (Fig. 4B).

Measuring the amount of phosphorylated myosin light chain at the above time points (2, 20, and 45 s) gave only a partial indication of the signaling events following agonist stimulation. Shape change begins 2 s after the addition of ADP to control platelets. This is in contrast to 5,5'-dimethyl-BAPTA-treated platelets, in which ADP-induced shape change did not begin until after 7.5 s following the addition of agonist. Therefore, we analyzed the changes in myosin light chain phosphorylation over time in vehicle-treated control platelets and compared our findings to changes in myosin light chain phosphorylation over time in 5,5'-dimethyl-BAPTA-treated platelets (Fig. 5). Both the extent and rate of myosin light chain phosphorylation were dramatically inhibited in the absence of an increase in cytosolic Ca\(^{2+}\). A peak in myosin light chain phosphorylation occurred at 2 s in control platelets in contrast to a lesser peak in myosin light chain phosphorylation occurring at 30 s in 5,5'-dimethyl-BAPTA-treated platelets.

Effect of Protein Kinase C Inhibitors on Ca\(^{2+}\)-independent Shape Change—ADP-induced shape change is mediated through stimulation of the G\(_{q}\)-coupled P2Y1 receptor (6). G\(_{q}\)-coupled receptors mediate the activation of serine/threonine PKC isoforms (35). Using two cell-permeable inhibitors of PKC, Ro 31–8220 and GF 109203X, we investigated whether PKC stimulated by ADP, thrombin, or U46619 mediates Ca\(^{2+}\)-independent platelet shape change. The \(\alpha\)-, \(\beta\)-, and \(\gamma\)-isoforms of PKC are potently inhibited by Ro 31–8220 and GF 109203X with an in vitro IC\(_{50}\) values of 5–27 nM and 16–20 nM, respectively (36). The compound GF 109203X inhibits the \(\delta\)-isoform of PKC with an in vitro IC\(_{50}\) value of 210 nM and inhibits the \(\epsilon\)-isoform with an in vitro IC\(_{50}\) value of 132 nM (37). The compound Ro 31–8220 inhibits the \(\epsilon\)-isoform of PKC with an in vitro IC\(_{50}\) of 24 nM (38). The activity of these compounds was determined by...
Staurosporine to establish the potential role of a Ca\(^{2+}\) both tyrosine and serine/threonine protein kinases. We used 

\[ \text{Change} \]

Staurosporine is a potent and nonspecific inhibitor of change was induced by 10 \( \mu M \) ADP, 0.1 unit/ml thrombin, or 1 \( \mu M \) U46619 in aspirinated platelets that were previously treated with either vehicle (dimethyl sulfoxide, labeled control) or with 50 \( \mu M \) 5,5'-dimethyl-BAPTA acetoxyethyl ester as indicated. Agonist-induced platelet shape change characteristics were measured as described under “Experimental Procedures.” The time from the addition of agonist until the initiation of platelet shape change (A), the time to half-complete shape change (point at which half-maximal light absorbance was reached) (B), and the initial rate of light absorbance during platelet shape change (change in absorbance/s) (C) as compared with control values are presented. Results are presented as mean values ± S.E. (\( n = 6 \)). The effect of 5,5'-dimethyl-BAPTA as compared with control is significant in each case (\( p < 0.01 \), Student’s unpaired \( t \) test).

Blocking aggregation induced by 100 \( \mu M \) phorbol-12-myristyl-13-acetate (not shown). Neither of these compounds had an effect on agonist-induced platelet shape change in either the presence or absence of an increase in cytosolic Ca\(^{2+}\) (data not shown).

**Effect of Stauroporine on Ca\(^{2+}\)-independent Shape Change**—Staurosporine is a potent and nonspecific inhibitor of both tyrosine and serine/threonine protein kinases. We used staurosporine to establish the potential role of a Ca\(^{2+}\)-independent kinase pathway in platelet shape change. We observed that ADP-induced shape change was inhibited by ~56% in vehicle-treated platelets pretreated with 0.3 \( \mu M \) staurosporine. Inhibition of shape change by staurosporine also occurred in control platelets stimulated by thrombin or U46619 (data not shown). However, 0.3 \( \mu M \) staurosporine completely inhibited

**Effect of p160ROCK-selective Inhibitors on Ca\(^{2+}\)-independent Shape Change**—The RhoA/p160ROCK pathway has been shown to play a role in smooth muscle contraction (27, 39, 40) as well as a role in the contractile responses of fibroblasts (41), endothelial cells (42), and neuronal cell lines (43–45). Staurosporine has recently been reported to dramatically inhibit ROKα at a concentration of 1 \( \mu M \) (46). Hence, we investigated the role of the RhoA/p160ROCK pathway in Ca\(^{2+}\)-independent platelet shape change. Both HA 1077 and Y-27632 show selective inhibition of p160ROCK purified from human platelets with IC\(_{50}\) values of ~2 \( \mu M \) and 1–1.5 \( \mu M \), respectively (27). In vehicle-treated platelets, 10 \( \mu M \) HA 1077 (Fig. 6A) and 10 \( \mu M \) Y-27632 (Fig. 6B) inhibited the extent of platelet shape change by ~30% and ~35% each. In the absence of an increase in cytosolic Ca\(^{2+}\) concentration caused by 5,5'-dimethyl-BAPTA, both 10 \( \mu M \) HA 1077 and 10 \( \mu M \) Y-27632 completely abolished ADP-induced platelet shape change. Moreover, the IC\(_{50}\) for the inhibition of

![Image](http://www.jbc.org/)

**Fig. 3.** The effect of 5,5'-dimethyl-BAPTA on three characteristics of agonist-induced platelet shape change. Platelet shape change was induced by 10 \( \mu M \) ADP, 0.1 unit/ml thrombin, or 1 \( \mu M \) U46619 in aspirinated platelets that were previously treated with either vehicle (dimethyl sulfoxide, labeled control) or with 50 \( \mu M \) 5,5'-dimethyl-BAPTA acetoxyethyl ester as indicated. Agonist-induced platelet shape change characteristics were measured as described under “Experimental Procedures.” The time from the addition of agonist until the initiation of platelet shape change (A), the time to half-complete shape change (point at which half-maximal light absorbance was reached) (B), and the initial rate of light absorbance during platelet shape change (change in absorbance/s) (C) as compared with control values are presented. Results are presented as mean values ± S.E. (\( n = 6 \)). The effect of 5,5'-dimethyl-BAPTA as compared with control is significant in each case (\( p < 0.01 \), Student’s unpaired \( t \) test).

![Image](http://www.jbc.org/)

**Fig. 4.** Effect of 5,5'-dimethyl-BAPTA on levels of agonist-stimulated myosin light chain phosphorylation. A, alkaline-urea-PAGE of HClO\(_4\) pellets from platelets treated with either dimethyl sulfoxide (labeled Control) or 50 \( \mu M \) 5,5'-dimethyl-BAPTA acetoxyethyl ester (labeled 5,5'-dimethyl BAPTA). A 0.5-ml volume of washed and aspirinated platelets (2 × 10\(^6\) cells/ml) was stimulated with one of the following: 10 \( \mu M \) ADP, 0.5 unit/ml thrombin, or 1 \( \mu M \) U46619 before the addition of HClO\(_4\) at the indicated times. Each sample was treated at 37 °C with stirring (900 rpm). The top band is the nonphosphorylated 20-kDa myosin light chain (indicated by MLC), and the band in the middle position is the phosphorylated 20-kDa myosin light chain (indicated by MLC-P). The bottom band is the 16-kDa myosin light chain. The above results are representative of three experiments each performed using platelets from different donors. B, densitometric analysis of the above experiment. These results are representative of the results from three experiments.

![Image](http://www.jbc.org/)
shape change by HA 1077 was −1.2 μM (Fig. 6A), and that for Y-27632 was −1.1 μM (Fig. 6B), in excellent agreement with that for inhibition of purified platelet p160<sup>ROCK</sup> (27). In control platelets, Y-27632 (10 μM) did not inhibit ADP-induced platelet aggregation (Fig. 7A); however, it inhibited the extent but not the rate of ADP-induced platelet shape change (Fig. 7B). We investigated whether the effects of Y-27632 on platelet shape change in both control and 5,5'-dimethyl-BAPTA-treated platelets are reversible. PRP was incubated with Y-27632 (10 μM) for 30 min at 37 °C. Upon resuspension in HEPES-buffered Tyrode’s solution, these platelets behaved no differently than control platelets (Fig. 7). Thus, the effects of Y-27632 appear to be reversible.

Effect of HA 1077 and Y-27632 on ADP-stimulated Myosin Light Chain Phosphorylation—We investigated the contribution of the p160<sup>ROCK</sup> to myosin light chain phosphorylation in Ca<sup>2+</sup>-dependent and -independent pathways. The effect of Y-27632 (10 μM) on the extent of ADP-induced myosin light chain phosphorylation in platelets at different time points was measured. This phosphorylation was significantly decreased at all time points in comparison with control platelets (Fig. 8). The difference in the levels of phosphorylated myosin light chain at 2 and 5 s were the most significant (p < 0.01; n = 3). The increase in myosin light chain phosphorylation that occurred in 5,5'-dimethyl-BAPTA-treated platelets in response to 10 μM ADP was completely abolished by 10 μM Y-27632 (Fig. 8). The effects of HA 1077 on ADP-induced myosin light chain phosphorylation were very similar to those of Y-27632. At 2 s following the addition of ADP, the peak level of phosphorylated myosin light chain in control platelets was reduced to similar levels by both 10 μM Y-27632 and 10 μM HA 1077 in 5,5'-dimethyl-BAPTA-treated platelets (Fig. 9). Furthermore, in 5,5'-dimethyl-BAPTA-treated platelets, both Y-27632 and HA 1077 abolished the peak level of myosin light chain phosphorylation observed at 30 s to levels observed in unstimulated platelets.

**DISCUSSION**

Shape change is considered to be the first measurable physiological response produced by platelets following exposure to an agonist. We and others have previously shown that ADP or serotonin-induced shape change is solely mediated by G<sub>q</sub>-coupled receptors on platelets (6, 9, 10). Since activation of G<sub>q</sub> leads to mobilization of calcium from cytosolic stores and activation of PKC, it is expected that these signaling events play an important role in agonist-induced platelet shape change. Offermans et al. (47) determined that G<sub>q</sub> is essential for platelet aggregation by producing G<sub>q</sub>-deficient mice. Interestingly, platelets from these mice still undergo agonist-stimulated aggregation.
shape change even in the absence of an increase in the cytosolic Ca\(^{2+}\) concentration. Previous observations indicated that substantially lower cytosolic Ca\(^{2+}\) concentrations were sufficient for agonist-stimulated shape change and myosin light chain phosphorylation than the Ca\(^{2+}\) concentrations required for the Ca\(^{2+}\) ionophore, ionomycin, to produce the same responses (48, 49). Ionomycin induces a maximum of 70% phosphorylated myosin light chain in 5,5'-dimethyl-BAPTA-treated platelets (Fig. 5). We have confirmed that shape change is preceded by myosin light chain phosphorylation, as has been previously reported (14). Our results indicate that the maximum myosin light chain phosphorylation is occurring ~2 s following the addition of ADP and that the extent of phosphorylation is substantially diminished by 15 s. In the absence of an increase in cytosolic Ca\(^{2+}\), the onset of platelet shape change was delayed. Changes in the kinetics of shape change were mirrored by a dramatic decline in the time and peak level of myosin light chain phosphorylation. In control platelets, we observed shape change beginning soon after the peak in myosin light chain phosphorylation, which is in agreement with the findings reported earlier (14). In the absence of increase in cytosolic Ca\(^{2+}\), the level of myosin light chain phosphorylation peaked well after shape change had begun. It appears that an increase of ~50% above the basal level of phosphorylated myosin light chain is sufficient to initiate shape change. In contrast, ADP-treated platelets, where the level of phosphorylated myosin light chain decreased after 2 s, the phosphorylation of myosin light chain in 5,5'-dimethyl-BAPTA-treated platelets continued to increase throughout shape change. Thus, both calcium-dependent and -independent pathways contribute to agonist-induced platelet shape change.

We examined the possible contribution of calcium-independent protein kinase activity by using protein kinase-selective inhibitors. Since ADP and serotonin induce shape change...
Role of p160ROCK in Platelet Shape Change

through G \text{q}-coupled receptors, leading to activation of PKC, we used selective PKC inhibitors, GF 109203X (36) and Ro 31–8220 (50), to examine the possible role of PKC in calcium-independent shape change. These agents did not have any effect on platelet shape change, either alone or in combination with 5,5′-dimethyl-BAPTA. In agreement with our finding, it has been reported that PKC did not have any effect on agonist-induced Ca\textsuperscript{2+} sensitization of smooth muscle from guinea pig vas deferens (51). Since these compounds are known to inhibit the classical PKC isomers α, β, and γ as well as the novel isomers δ and ε (36–38), these isomers probably do not contribute to either Ca\textsuperscript{2+}-dependent or Ca\textsuperscript{2+}-independent mechanisms of platelet shape change. However, the role of other PKC isoforms in these processes cannot be ruled out.

Myosin light chain phosphorylation plays a central role in agonist-stimulated smooth muscle contraction (52, 53). A receptor-mediated increase in cytosolic Ca\textsuperscript{2+} binds calmodulin and activates the Ca\textsuperscript{2+}/calmodulin-dependent myosin light chain kinase. Myosin light chain kinase primarily phosphorylates myosin light chain at Ser-19, which induces the interaction of actin and myosin, resulting in increased actin-stimulated myosin ATPase activity and smooth muscle contraction (53, 54). Analogous to the situation in platelets, it has been reported that levels of smooth muscle cell contraction are not always proportional to cytosolic Ca\textsuperscript{2+} concentration (55). In smooth muscle cells, the Rho family of Ras-like small GTPases has been identified as a mediator in the enhancement of smooth muscle cell sensitivity to Ca\textsuperscript{2+}-induced contraction. RhoA activates RhoA-binding kinase, which phosphorylates the myosin-binding subunit (MBS) of myosin phosphatase and inhibits its activity (56, 57). RhoA-binding kinase has also been shown to directly phosphorylate myosin light chain and activate myosin in vitro as well as inducing smooth muscle contraction in the absence of Ca\textsuperscript{2+} (40).

Human platelets contain myosin phosphatase consisting of a 38-kDa catalytic subunit of protein phosphatase type 1 δ, a

FIG. 9. Comparison of the effect of Y-27632 with HA 1077 on peak levels of myosin light chain phosphorylation induced by 10 μM ADP in both normal and 5,5′-dimethyl-BAPTA-treated platelets. Denensitometrical analysis of alkaline-ures-PAGE. The values represent the percentage of total myosin light chain in the phosphorylated form. A 0.5-ml volume of washed and aspirinated platelets (2 × 10\textsuperscript{9} cells/ml) was stimulated either with 10 μM ADP for 2 s in vehicle-treated, control platelets or for 30 s in 5,5′-dimethyl-BAPTA-treated platelets before the addition of HClO\textsubscript{4}. Samples were incubated with 10 μM Y-27632 or HA 1077 for 3 min with stirring at 37 °C before agonist stimulation. Data are expressed as the means ± S.E. from three experiments. The effects of HA 1077 and Y-27632 in 5,5′-dimethyl-BAPTA-treated platelets as compared with each matched control are significant (p < 0.01, Student’s unpaired t test).

FIG. 10. Model depicting the intracellular events mediating platelet shape change. The solid arrows indicate a stimulatory effect, and dashed arrows indicate an inhibitory effect. Double bars indicate the inhibitory action of Y-27632 and HA 1077.

130-kDa MBS, and a 20-kDa subunit (46, 58). High levels of RhoA protein are also found in platelets, and both RhoA and p160ROCK co-immunoprecipitate with anti-MBS antibodies (46). Hence, initially we used staurosporine to test for the possibility that p160ROCK plays a role in shape change (46, 58). Staurosporine had been shown to prevent the phosphorylation of the MBS of platelet myosin phosphatase by recombinant p160ROCK (46). In our study, staurosporine (0.3 μM) partially inhibited ADP, thrombin, or U46619-induced shape change in control platelets and completely blocked shape change in 5,5′-dimethyl-BAPTA-treated platelets.

Since staurosporine is a potent inhibitor of many tyrosine and serine/threonine protein kinases including myosin light chain kinase, we decided to use the more selective p160ROCK inhibitors, Y-27632 and HA 1077. Both of these compounds had a similar inhibitory effect on the extent of shape change (~30%) in control platelets (Fig. 6). In the presence of 5,5′-dimethyl-BAPTA, both Y-27632 and HA 1077 completely abolished agonist-induced platelet shape change. The IC\textsubscript{50} values of Y-27632 and HA 1077 for inhibiting shape change in 5,5′-dimethyl-BAPTA-treated platelets (Fig. 6) are similar to those for inhibiting purified human platelet p160ROCK (27). Furthermore, both Y-27632 and HA 1077 reduced myosin light chain phosphorylation during shape change (Fig. 9). The abrogation of both shape change (Fig. 6) and myosin light chain phosphorylation in 5,5′-dimethyl-BAPTA-treated platelets (Figs. 8 and 9) by both Y-27632 and HA 1077 provides strong evidence that RhoA-activated p160ROCK is mediating the Ca\textsuperscript{2+}-independent shape change.

An outline of the intracellular signaling events leading to platelet shape change and their regulation is shown in Fig. 10. One target of p160ROCK has been shown to be myosin phosphatase, which is inactivated by phosphorylation (24, 46, 56–58). While myosin phosphatase dephosphorylates the myosin light chain and counteracts Ca\textsuperscript{2+}/calmodulin-dependent myosin light chain kinase, inactivation of myosin phosphatase would lead to an increase in myosin light chain phosphorylation. It is also possible that p160ROCK directly phosphorylates myosin (39, 40). The presence of two systems regulating myosin phosphorylation and shape change may at first appear unnecessarily redundant, but there are countless examples in biology of redundancy. In addition, dual activation of myosin phosphorylation by kinase activation and phosphatase inhibition may allow for a more rapid and robust response to an external
Role of p160ROCK in Platelet Shape Change

signal. In human endothelial cells, thrombin has been shown to activate PLC phosphatase through a Rho/Rho-operated kinase pathway as part of a signaling network that controls myosin phosphorylation and endothelial cell contractility (42).

We previously have shown that the Gα2-coupled receptor activation, by ADP or epinephrine, does not induce shape change (6, 7, 9). Offermanns et al. (47) have shown that shape change occurs in Goq-deficient platelets. Go11 is not expressed in human platelets (59). Thus, the calcium-independent shape change observed in our study and in the Goq-deficient mice (47) is mediated by G proteins other than Gα, Gq, or Go11. It is possible that Gα2 or Gα11 mediate Ca2+-independent shape change, since it has already been shown that Rho is regulated by Gαq and Gα11 (60).

Since the submission of this paper, Klages et al. (61) have demonstrated a Ca2+-independent mechanism for thromboxane A2-mediated shape change in Goq-deficient mouse platelets. Agonist stimulation of Goq-deficient mouse platelets occurs without mobilization of Ca2+ into the cytoplasm (47). In complete agreement with our results, it was observed that 10 μM Y27632 totally inhibited shape change in Goq-deficient mouse platelets (61). Klages et al. (61) have proposed that Ca2+-independent shape change is mediated by either Gαq or Gα11 (Fig. 10). Since ADP failed to elicit a shape change response in Goq-deficient mouse platelets (47), Offermanns and co-workers (61) did not establish whether the calcium-independent pathway is a general mechanism of platelet shape change or specific for U46619 alone. We have demonstrated with three platelet agonists that both calcium-sensitive and -insensitive pathways independently contribute to platelet shape change and myosin light chain phosphorylation, suggesting that this is the general mechanism of platelet shape change.

In conclusion, we have demonstrated that agonist-induced platelet shape change occurs through both calcium-dependent and -independent mechanisms. RhoA/160ROCK appears to play an important role in the calcium-independent pathway leading to shape change.

Acknowledgments—We thank Drs. J. Bryan Smith and Barrie Ashby (Department of Pharmacology) for critically reviewing the manuscript.

REFERENCES
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

Benjamin Z. S. Paul, James L. Daniel and Satya P. Kunapuli

doi: 10.1074/jbc.274.40.28293

Access the most updated version of this article at http://www.jbc.org/content/274/40/28293

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 60 references, 37 of which can be accessed free at http://www.jbc.org/content/274/40/28293.full.html#ref-list-1