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Thrombin-Induced Activation of RhoA in Platelet Shape Change

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Thrombin-induced activation of RhoA and its involvement in the regulation of myosin II light chain$_{20}$ phosphorylation (MLC-P) in α-toxin permeabilized platelets was investigated. Permeabilized platelets, expressing normal levels of P-selectin, displayed a Ca^{2+}-dependent increase in shape change and MLC-P. Thrombin activated RhoA as measured by a rhotekin-binding assay within 30 s of stimulation under conditions of constant $[Ca^{2+}]_i$. Under the same conditions and timecourse, thrombin or GTPγS induced an increase in MLC-P and platelet shape change which was not dependent on an increase in $[Ca^{2+}]_i$. The thrombin- and GTPγS-induced MLC-P in constant $[Ca^{2+}]_i$ was inhibited by the addition of Y27632, a Rho-kinase inhibitor. This study directly demonstrates that thrombin can activate RhoA in platelets in a timecourse compatible with a role in increasing MLC-P and shape change (not involving an increase in $[Ca^{2+}]_i$). This is also Rho-kinase-dependent. © 2001 Academic Press

Key Words: **rho; rho-kinase; platelet shape change; thrombin; myosin light chain phosphorylation.**

Activation of platelets involves a series of coordinated responses including shape change, aggregation, and secretion (1). These events are associated with an intracellular Ca^{2+} release (2, 3). The increase in $[Ca^{2+}]_i$ has several functions, including activation of MLC-P. It is now clear that phosphorylation of the MLC is necessary for shape change and platelet activation (4). The enzyme predominantly responsible for MLC-P is the Ca^{2+}- and calmodulin-dependent protein kinase, myosin light chain kinase (MLCK). Dephosphorylation occurs via a specific myosin phosphatase (5–7). The shape change induced by platelet-activating agonists, such as thrombin, is therefore the result of the balance between MLCK activity and the myosin phosphatase activity.

Studies have suggested that alternative signaling pathways exist which can contribute to platelet shape change through mechanisms other than direct Ca^{2+}-dependent activation of MLCK (8–10). In particular the shape change induced by platelet agonists, including thrombin, may be partly regulated by activation of $p160^{ROCK}$ (Rho-kinase) which can phosphorylate and inhibit the myosin binding subunit of the myosin phosphatase. The involvement of this pathway is based on the use of a cell permeable Rho-kinase inhibitor and its subsequent effects on MLC-P and platelet shape change (11–15). No study to date has yet demonstrated directly that thrombin can activate RhoA (the upstream effector of Rho-kinase) and that this occurs in a timecourse that is compatible with thrombin-induced shape change. We have used a *Staphylococcus aureus* α-toxin permeabilized platelet model, which minimizes loss of intracellular proteins but allows control of the $[Ca^{2+}]_i$ while maintaining G-protein coupled signaling pathways (16, 17). Using this model we have shown that thrombin induces activation of RhoA (independent of an increase in $[Ca^{2+}]_i$) in a timecourse which is the same as thrombin-induced MLC-P and shape change. This was via a Rho-kinase-dependent pathway.

METHODS

Human blood was collected from informed, healthy volunteers as approved by the local ethics committee. Platelets were isolated by centrifugation and resuspended in washing buffer (0.01 M EDTA, 0.2% bovine serum albumin, 0.9% phosphate-buffered saline). For permeabilization, platelets were suspended in a low Ca^{2+} (pCa < 8.0) mock intracellular buffer (1 mM EGTA, 30 mM Pipes, 10 mM creatine phosphate, 7.3 mM Na_2ATP, 85.8 mM potassium methane sulphonate). *Staphylococcus aureus* α-toxin (50 ng ml^{-1}) was added for 90 min at room temperature. One micrometer thapsigargin was added to disrupt intracellular Ca^{2+} stores and platelets were incubated for a further 30 min. Alternatively, 1 μM Ca^{2+} ionophore A23187 was also used. Results were similar using either thapsigargin or A23187.

In all experiments with permeabilized platelets the $[Ca^{2+}]_i$ was controlled using intracellular solutions with the Ca^{2+} concentration

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buffered by different ratios of Ca- and K_2-EGTA as previously described (18–20). The maximal Ca^{2+} concentrations were pCa 4.5 containing 10 mM Ca-EGTA and minimal Ca^{2+} concentration contained 10 mM K_2-EGTA (pCa $<$ 8.0). Thrombin (0.4 units ml^{-1}) and GTPγS (100 μM) were added to different concentrations of Ca^{2+}-containing buffer as specified in the results. Washed and permeabilized platelets were compared using flow cytometry. The expression of P-Selectin was determined using anti-P-Selectin CD62P PE (Beckman Coulter) in intact and permeabilized platelets in a Becton-Dickinson FACS flow cytometer. Fluorescence channels were set at logarithmic gain, 20,000 particles were acquired for each sample.

Permeabilized platelets were incubated with equivolume, ice-cold, lysis buffer (50 mM Tris, 500 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.2) followed by centrifugation (21). Supernatant was resuspended in SDS sample buffer and boiled. Rhotekin Rho-binding domain (Upstate Biotechnology) was added and incubated for 45 min. Agarose beads from each sample were resuspended in sample buffer, boiled for 5 min at 100°C, and subjected to SDS–polyacrylamide gel electrophoresis. Separated proteins were transferred to nitrocellulose membrane, incubated in RhoA antibody (Santa Cruz), followed by secondary antibody (goat anti-mouse, Dako). Proteins were detected by enhanced chemiluminescence (Amersham).

Shape change in permeabilized platelets was measured in a platelet aggregation profiler (PAP-4 Bio-data Corporation) by determining the alteration in light transmission through the stirred sample at 37°C. Cell density of samples was 3×10^8 cells ml^{-1}. Platelets were permeabilized as described above and incubated in pCa $<$ 8.0. The Ca^{2+} concentration of the solution was raised to defined $[Ca^{2+}]_i$ by adding the appropriate amount of pCa 4.5 to the pCa $<$ 8.0 solution. Either 0.4 units ml^{-1} thrombin or 100 μM GTPγS was added to the solution and the change in light transmission was recorded.

Following experimental incubations, the phosphorylated state of the MLC was fixed by addition of equivolume 0.6 M perchloric acid. Precipitated proteins were washed with acetone, resuspended in sample buffer, and subjected to 2-dimensional electrophoresis as previously described (19). Resolved proteins were transferred on to nitrocellulose and the membrane stained with colloidal gold solution (Biorad). Phosphorylation was quantitatively compared using a Biorad GS690 densitometer. The total MLC-P in each experiment was expressed as a % of the total MLC protein.

All chemicals and reagents were purchased from Sigma Chemical Company, unless otherwise stated. Results are expressed as mean ± S.E.M. Statistical tests used were paired Student's *t*-tests for single comparisons and 2-way Anova for multiple comparisons.

RESULTS

Characterization and Ca^{2+} Dependency of Permeabilized Platelets

Permeabilized platelets were analyzed for α-granule secretion by assaying for plasma membrane expression of P-selectin by flow cytometry. Platelets permeabilized with α-toxin express similar levels of P-selectin compared to intact platelets (Fig. 1A).

As separated by 2-dimensional electrophoresis, human platelets contained equal amounts of two major isoforms of MLC (Fig. 1B) and confirmed by immunoblotting (not shown) as previously described. This was unchanged by the permeabilization process. Based on their electromobility in the isoelectric focussing gels, these MLC isoforms are the myosin IIa (muscle) and myosin IIb (nonmuscle) forms.

Platelets were permeabilized with α-toxin and incubated in either pCa $<$ 8.0, pCa 6.8 solution or pCa 6.8 following preincubation with a phosphatase inhibitor, 10 nM tautomycin for 1 h. Incubations following the addition of Ca^{2+} were stopped after 15 s. Increasing concentrations of $[Ca^{2+}]_i$ in the permeabilized platelets produced an increase in the MLC-P (Fig. 1). Compared

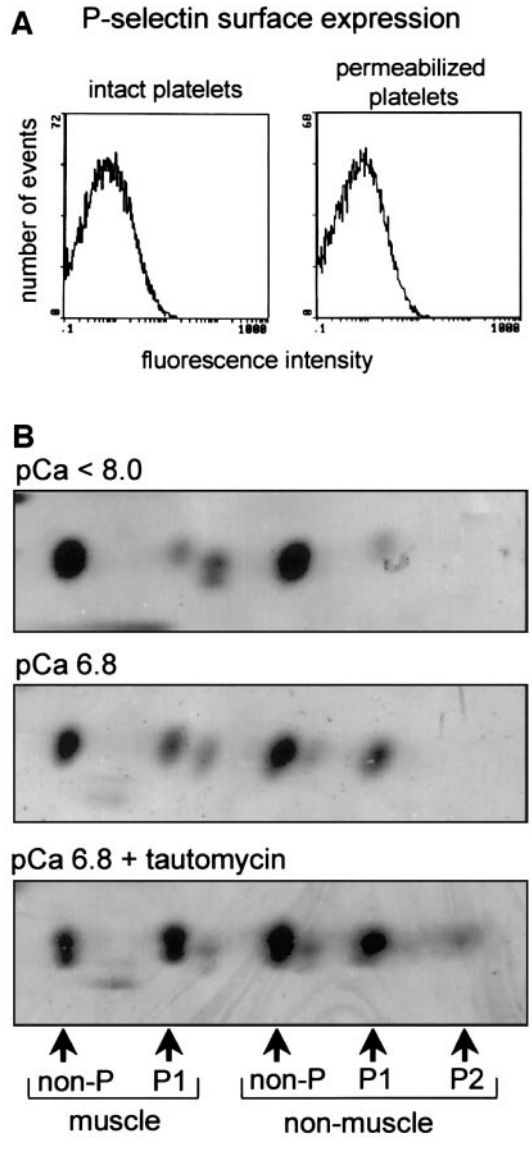

FIG. 1. P-selectin surface expression and Ca^{2+}-dependent myosin light $chain_{20}$ phosphorylation in permeabilized platelets. (A) Histogram of flow cytometric analysis of intact and permeabilized platelets in a resting state incubated with anti-P-selectin antibody. Permeabilization of platelets with α-toxin had no effect on P-selection expression. (B) α-toxin permeabilized platelets in a mock intracellular buffer containing pCa $<$ 8.0 revealed a 12 ± 1% (n = 10) phosphorylation of the total MLC_{20} present. When the $[Ca^{2+}]_i$ was increased to pCa 6.8, the increase in phosphorylation was 18 ± 2% (n = 13) of the total MLC_{20}. The MLC-P of permeabilized platelets in pCa 6.8 following pretreatment with 10 nM tautomycin was 49 ± 5% (n = 4). The MLC-P induced by pCa 6.8 was significantly increased compared to pCa $<$ 8.0. The MLC-P with pCa 6.8 following tautomycin pretreatment was significantly increased compared to pCa 6.8.

to permeabilized platelets incubated in pCa < 8.0, incubations with pCa 6.8 for 15 s significantly increased the MLC-P (Fig. 1). Tautomycin pretreatment followed by a 15 s incubation in pCa 6.8 produced a significant increase in MLC-P compared to pCa < 8.0 or pCa 6.8. Diphosphorylated MLC was also observed with tautomycin pretreatment which accounted for a small percentage of the total phosphorylation.

Activation of RhoA at Constant $[Ca^{2+}]_i$ by Thrombin and GTPγS in Permeabilized Platelets

Using a GST-rhotekin binding assay to assess RhoA activation, permeabilized platelets in pCa 6.8 were compared to platelets in pCa 6.8, and stimulated with thrombin or GTPγS for 30 s. These conditions were the same as those used to measure MLC-P. When normalized to total RhoA extracted for each sample, there was an increase (0.6-fold) in RhoA activation following 30 s stimulation with 0.4 units/ml thrombin in pCa 6.8 compared to platelets incubated in pCa 6.8 alone (Fig. 2). Similarly, stimulation with 100 μM GTPγS in the same constant $[Ca^{2+}]_i$ produce an increase in RhoA activation as measured by rhotekin binding (1.5-fold, Fig. 2).

Thrombin- and GTPγS-Stimulated Myosin Light Chain$_{20}$ Phosphorylation of Permeabilized Platelets in Constant $[Ca^{2+}]_i$

The effects of thrombin and GTPγS stimulation on MLC-P with buffered $[Ca^{2+}]_i$ were investigated. Incubation times were chosen with regard to data obtained from platelet shape change experiments. Permeabilized platelets were incubated in pCa 6.8 alone or simultaneously with 0.4 units ml^{-1} thrombin or 100 μM GTPγS. In permeabilized platelets incubated with pCa 6.8, the simultaneous addition of either thrombin or GTPγS for 15 s produced a significant increase in MLC-P compared to time-matched pCa 6.8 incubations (Fig. 3A). This significant increase was still observed after 30 s of thrombin or GTPγS incubation compared to the pCa 6.8 control. Thrombin or GTPγS added to permeabilized platelets in pCa < 8.0 produced no increase in MLC-P following 15 or 30 s stimulation (data not shown).

Shape Change in Permeabilized Platelets

In aggregometry experiments, permeabilized platelets did not produce any aggregation (as manifested by an increase in light transmission) at lower $[Ca^{2+}]_i$ of pCa 6.8 but did aggregate when the $[Ca^{2+}]_i$ was raised up to pCa 4.5 (not shown). The shape change induced in permeabilized platelets with a defined $[Ca^{2+}]_i$ and following stimulation with thrombin or GTPγS was investigated. Permeabilized platelets in pCa < 8.0 at

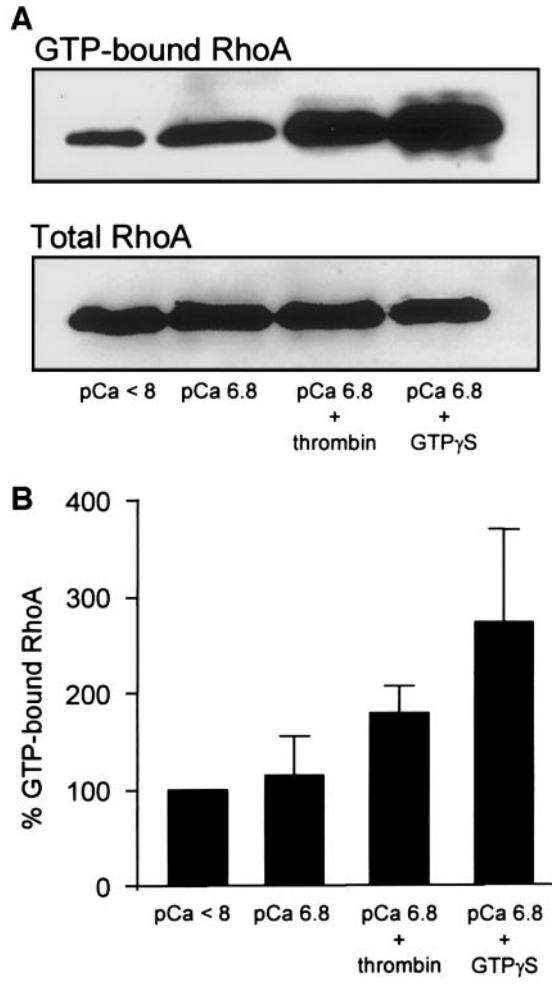

FIG. 2. Thrombin- and GTPγS-stimulated RhoA activation as measured by Rhotekin-binding assay. (A) Typical immunoblots of GTP-bound (active) RhoA immunoprecipitated with anti-rhotekin antibody and total RhoA protein in each sample. (B) Mean data for % of RhoA bound to Rhotekin (normalized to Rho activation levels in pCa < 8). Permeabilized platelets in pCa 6.8 in the presence of 0.4 units ml^{-1} thrombin for 30 s showed an increase in RhoA activation (n = 4). Similarly, 100 μM GTPγS also produced an increase in RhoA activation following incubation for 30 s in permeabilized platelets in pCa 6.8 solution (n = 4).

37°C showed no change in light transmission. When the $[Ca^{2+}]_i$ was increased to pCa 6.8, a decrease in light transmission was observed (Fig. 3B). When the $[Ca^{2+}]_i$ was increased to pCa 6.8, the simultaneous addition of thrombin produced a decrease in light transmission (Fig. 3B). The magnitude of this decrease was significantly different compared to the shape change induced by pCa 6.8 alone. Similarly, the addition of GTPγS following incubation with pCa 6.8 also produced a change in light transmission which was significantly increased in magnitude compared to pCa 6.8 alone. In platelets maintained in pCa < 8.0, the addition of either thrombin or GTPγS produced no change in light transmission (data not shown).

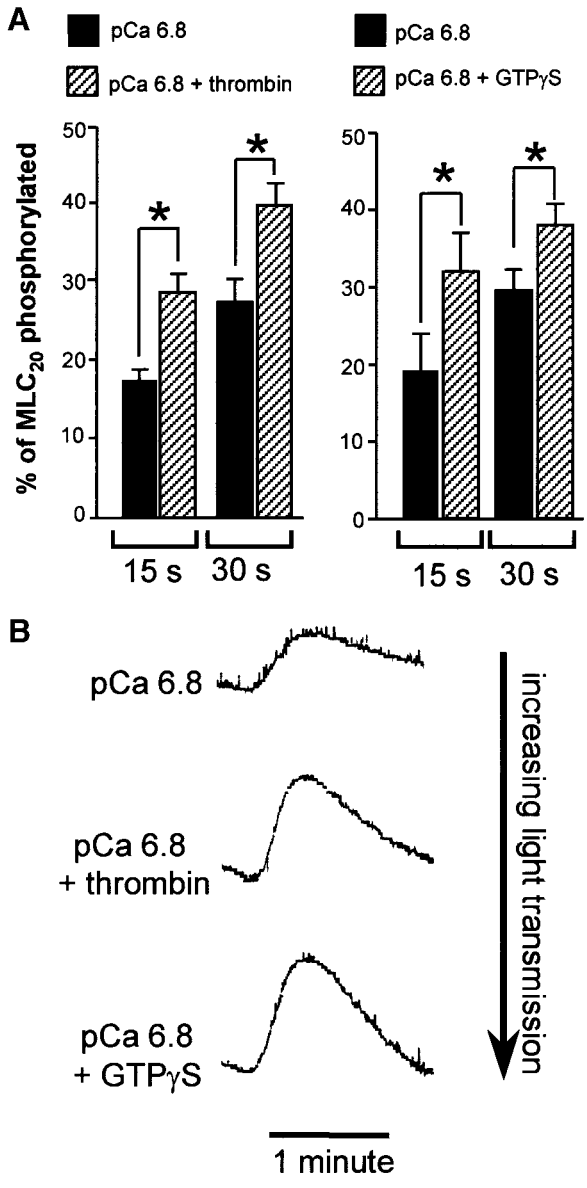

FIG. 3. Myosin light chain$_{20}$ phosphorylation and shape change of thrombin- and GTPγS-stimulated permeabilized platelets in constant $[Ca^{2+}]_i$. (A) In pCa 6.8, thrombin significantly increased the MLC-P of permeabilized platelets in comparison to pCa 6.8 alone after 15 s stimulation and this was maintained after 30 s ($n = 6$). Asterisks denote statistical significance $P < 0.05$ between values indicated. Similarly, GTPγS significantly increased the MLC-P of permeabilized platelets in pCa 6.8 when compared to pCa 6.8 alone after 15 s stimulation ($n = 6$). This was also maintained after 30 s. (B) Aggregometry measurements in permeabilized platelets. When the intracellular Ca^{2+} concentration of permeabilized platelets was rapidly raised from pCa < 8.0 to pCa 6.8, a decrease in light transmission, indicative of shape change, was observed. When the intracellular Ca^{2+} concentration was raised to pCa 6.8 in the presence of either thrombin or GTPγS (B), a decrease in light transmission was also observed. This change in light transmission was significantly greater in magnitude in the presence of thrombin or GTPγS in pCa 6.8 compared to pCa 6.8 alone (pCa 6.8, 8 ± 3 mm deflection; pCa 6.8 + thrombin, 13 ± 3 mm; pCa 6.8 + GTPγS, 15 ± 3 mm).

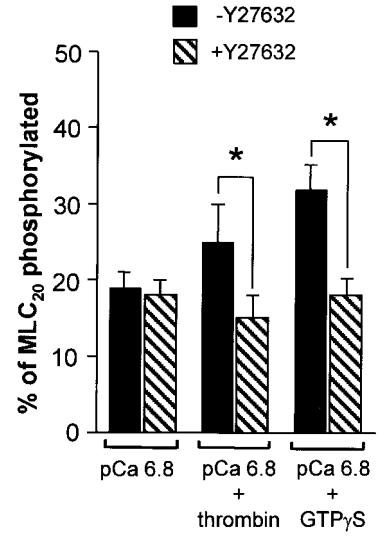

FIG. 4. Effect of rho-kinase inhibition on MLC-P of thrombin- and GTPγS-stimulated permeabilized platelets in fixed $[Ca^{2+}]_i$. Following incubation with 10 μM Y27632, permeabilized platelets were incubated with either pCa 6.8, pCa 6.8 + 0.4 units ml^{-1} thrombin, or pCa 6.8 + 100 μM GTPγS for 15 s. Y27632 had no effect on MLC-P stimulated by pCa 6.8. However, both the thrombin- and GTPγS-stimulated MLC-P in pCa 6.8 was significantly inhibited to phosphorylation levels similar to pCa 6.8 alone. Asterisks denote statistical significance $P < 0.05$ between values indicated ($n = 4$ for each sample).

Effects of Rho-Kinase Inhibitor (Y27632) on Thrombin- and GTPγS-Stimulated Myosin Light Chain$_{20}$ Phosphorylation of Permeabilized Platelets in Constant $[Ca^{2+}]_i$

The effects of preincubation with a selective Rho-kinase inhibitor, Y27632, on the thrombin and GTPγS-stimulated increases in MLC-P were investigated. Following a preincubation for 30 min in 10 μM Y27632, permeabilized platelets were stimulated either with pCa 6.8, pCa 6.8 + 0.4 units ml^{-1} thrombin, or pCa 6.8 + 100 μM GTPγS for 30 s. Y27632 had no effect on the MLC-P following pCa 6.8 alone. However, preincubation with Y27632 significantly decreased the MLC-P produced by thrombin or GTPγS (Fig. 4).

DISCUSSION

Several studies have suggested that a rise in the $[Ca^{2+}]_i$ is not essential for platelet shape change (8–10) and that this Ca^{2+}-independent component can be abolished by inhibition of Rho-kinase (11–15). This study has used a *Staphylococcus aureus* α-toxin permeabilized platelet model (16, 17) to examine directly the thrombin-induced activation of RhoA. The advantage of this permeabilized platelet model is that it allows the ability to separate the Ca^{2+}-independent signalling pathways of thrombin-induced shape change from the Ca^{2+}-dependent mechanisms of shape change whilst

maintaining Ca^{2+} levels at resting or above $[Ca^{2+}]_i$. This is the first study to use such an approach to study shape change in platelets. Previous studies have relied on buffering the $[Ca^{2+}]_i$ to artificially low levels (11, 12), or used concentrations of platelet agonists which do not increase $[Ca^{2+}]_i$ as assessed by global Ca^{2+} measurements (11, 14). In addition, α-toxin permeabilized platelets have been utilised in other studies which, in agreement with our study, has shown that these platelets express P-selectin levels within the range expressed in nonpermeabilized platelets (16, 17). The kinetics of the shape change following increased $[Ca^{2+}]_i$ in permeabilized platelets was approximately 20–30 s from the increase in $[Ca^{2+}]_i$ to peak of shape change. This correlates with the timecourse of the Ca^{2+}-induced MLC-P and is comparable to that observed in nonpermeabilized platelets.

This is the first study to show that the platelet agonist, thrombin, can activate RhoA in platelets without an increase in $[Ca^{2+}]_i$. Other recent studies using selective inhibitors have previously implied the involvement of rho (11–15). We have also demonstrated that this increase in RhoA activation occurs in a timecourse that is compatible with a role in platelet shape change using a method which allows immunoprecipitation of only activated (rhotekin-bound) RhoA (21). Measurements using RhoA translocation does not allow such timecourse information to be assessed.

It has been established that a downstream target of the RhoA/Rho-kinase pathway is the myosin phosphatase (22–24). Phosphorylation of the myosin-binding subunit of myosin phosphatase by Rho-kinase leads to a decrease in phosphatase activity. This results in an increase in MLC-P. This mechanism does not therefore increase phosphorylation directly but decreases the dephosphorylation of MLC_{20}. In platelets, Rho-kinase can phosphorylate the myosin binding subunit, decreasing the myosin phosphatase activity (6). In addition, an inhibition of the myosin phosphatase in platelets, using selective phosphatase inhibitors such as tautomycin as shown in this study, can induce a significant increase in MLC_{20} phosphorylation as well as platelet shape change (25). In permeabilized platelets, thrombin (as well as GTPγS) induced RhoA activation should therefore result in Ca^{2+}-independent effects on MLC-P and shape change. Thrombin- and GTPγS induced significant increases in MLC-P under constant $[Ca^{2+}]_i$ of pCa 6.8 (154 nM). This was correlated with a potentiation of the shape change response. This is not Ca^{2+} independent *per se*, but is a Ca^{2+} sensitization of the MLC-P. These changes occurred within the timecourse compatible for RhoA-mediated mechanism. Further evidence for the involvement of the Rho pathway is demonstrated by the effects of Y-27362, a selective Rho-kinase inhibitor. Inhibition of Rho-kinase significantly decreased the thrombin-induced MLC-P in constant $[Ca^{2+}]_i$.

In conclusion, this study has used a permeabilized platelet model to elucidating the mechanisms that control platelet shape change. We have directly demonstrated that thrombin can activate RhoA under conditions of constant $[Ca^{2+}]_i$ in permeabilized platelets. The timecourse of this activation correlates with the thrombin and GTPγS-induced Ca^{2+} sensitization of MLC-P and shape change. This occurs at least partly via a Rho-kinase mechanism.

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