Role of Phosphorylation in Mediating the Association of Myosin with the Cytoskeletal Structures of Human Platelets*

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The effect of myosin light chain phosphorylation on the association of myosin with the cytoskeletal structures of platelets was quantitated. In unstimulated platelets, little myosin light chain was phosphorylated and myosin remained in solution when cytoskeletons from Triton X-100 lysates of platelets were sedimented by centrifugation. In platelets activated by thrombin, the calcium ionophore A23187, or collagen, the rate and extent of myosin light chain phosphorylation paralleled the association of myosin with platelet cytoskeletal structures. Dephosphorylation of myosin light chain and myosin dissociation from the cytoskeleton occurred at comparable rates at longer times after addition of the stimulating agents to platelets. Quantitation of radioactive phosphate in the cytoskeleton-associated myosin and in the soluble myosin showed that the phosphorylated myosin light chain was selectively isolated with the Triton-insoluble cytoskeletons, whereas nonphosphorylated myosin was not associated. Inhibition of the light chain kinase with the calmodulin antagonist trifluoperazine inhibited myosin light chain phosphorylation and incorporation of myosin into the platelet cytoskeletons. Inhibition of light chain phosphorylation by prostaglandin E\textsubscript{1} and prostacyclin produced similar effects. Thus, phosphorylation of the myosin light chain stabilizes the association of myosin with the contractile structures within platelets.

When platelets are stimulated with thrombin or other stimuli such as collagen or the ionophore A23187, they change shape, release the contents of their granules, and, in the presence of Ca\textsuperscript{2+} ions, aggregate. Platelets contain high concentrations of the contractile proteins actin and myosin, and it has long been thought that platelet responses to stimulation involve contractile mechanisms (1, 2). Since contractile processes within platelets presumably depend on force-generating interactions of myosin with actin filaments, two fundamental questions can be asked. What changes occur in these two proteins during platelet activation? What regulates their interaction?

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Recent studies have shown that actin undergoes pronounced changes during platelet activation. In unstimulated platelets, about 40% of the platelet actin is filamentous. In platelets activated with thrombin for only 30 to 60 s, this value increases to about 60% (3-6). Actin filaments are insoluble in Triton X-100 and can be sedimented from Triton X-100 extracts of either control or activated platelets by low speed centrifugation (8,700 \times g for 4 min), permitting ready evaluation of their structure and composition (5). Filaments from unstimulated platelets are dispersed but those from thrombin-activated platelets are more structured, often retaining the shape of the platelet that existed before it was extracted with Triton X-100. The protein composition of the filamentous structures from unstimulated platelets consists primarily of actin (41% of the total actin in platelets), actin-binding protein (6% of the total), myosin (14% of the total), and an undetermined amount of a 31,000 M\textsubscript{r} polypeptide. The filamentous structures from platelets activated with thrombin for 30 to 60 s contain more actin (60% of the total), actin-binding protein (20% of the total), and essentially all of the platelet myosin (>90% of the total). While the amounts of actin and actin-binding protein in cytoskeletons remain at these levels at various times after thrombin addition, the amount of myosin decreases (to 60% of the total within 30 min). Thus, thrombin activation of platelets causes actin to polymerize into filaments. These filamentous structures, or cytoskeletons, can be readily isolated and contain several proteins found in the cytoskeletons of a variety of cell types (4, 5).

Since actin-binding protein binds to actin filaments (7-11), the small increase in concentration of the actin-binding protein in the cytoskeletons obtained from activated platelets is not surprising. However, the almost total conversion of myosin from a Triton-soluble form to a form isolated by low speed centrifugation and the subsequent reversal of this conversion is unexpected. Clearly, platelet activation has transiently altered the physical state of myosin within these cells.

Platelet myosin has been purified to homogeneity and has been shown to contain six polypeptides, two 200,000 M\textsubscript{r}, heavy chains, two 20,000 M\textsubscript{r}, light chains, and two 16,000 M\textsubscript{r}, light chains (12-14). While purified myosin can form bipolar filaments under physiological ionic conditions in vitro, such filaments have not yet been detected in intact platelets, possibly because filaments are structured, retain the shape of activated platelets, and are found associated with other proteins, they have been termed the platelet cytoskeletons when isolated from either control or activated platelets. This term will be used throughout this manuscript. Since Triton X-100 is used for their preparation, use of this term is not meant to imply that the structural elements within cells consist of only those proteins isolated, i.e. actin, myosin, actin-binding protein, and a 31,000 M\textsubscript{r}, protein. Other proteins could have been actin filament-associated and solubilized by the extraction procedure.
because relatively few would be present even if all myosin molecules were polymerized into filaments (15). Several groups have reported that the 20,000 M, light chain is rapidly phosphorylated and then slowly dephosphorylated during activation of platelets (16-20). Phosphorylation is catalyzed by a light chain kinase that contains calmodulin as part of its structure and is accordingly activated by Ca++ (21, 22). Recently, Daniel et al. (20) showed that approximately 10% of the 20,000 M, myosin light chain in unstimulated platelets was phosphorylated. This value approached 100% within 30 s of platelet activation. Thus, during platelet activation, there is the transient conversion of myosin to a phosphorylated form.

The extent of phosphorylation and the time course for the occurrence of the phosphorylation of the 20,000 M, light chain appear to correlate with the association of myosin with the cytoskeleton. The present studies were designed to determine whether association of myosin with cytoskeletons during platelet stimulation is regulated by the extent of phosphorylation of the myosin light chain within platelets.

MATERIALS AND METHODS

Preparation of Suspensions of Labelled Platelets—Venous blood from healthy adult donors was collected into ¼ volume of a solution containing 85 mM sodium citrate, 111 mM dextrose, and 71 mM citric acid (23). The blood was centrifuged at 160 X g for 20 min, and the platelet-rich plasma removed and centrifuged at 730 X g for 10 min to sediment the platelets. The platelet pellet was washed two times by resuspension in the original plasma volume of a buffer containing 120 mM sodium chloride, 13 mM trisodium citrate, and 30 mM dextrose, pH 7.4, and isolated by centrifugation at 730 X g for 10 min. They were then resuspended at about 2 X 10^8 platelets/ml in a buffer, pH 7.4, containing 150 mM sodium chloride, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 mM EDTA, and about 0.8 mCi of carrier-free (^32)P phosphate/ml (New England Nuclear). The suspension was incubated for 30 min, then the platelets were isolated by centrifugation, washed once in the same buffer, but without (^32)P phosphate, and finally resuspended at 2 X 10^8 platelets/ml in a buffer containing 138 mM sodium chloride, 2.9 mM potassium chloride, 12 mM sodium bicarbonate, 0.36 mM sodium phosphate, 5.5 mM glucose, and 1 mM EDTA, pH 7.4. All steps in the washing procedure were performed in polystyrene tubes at 22 °C. Platelet counts were determined with an Electrozone/Celloscope Counter (Particle Data, Inc., Elmhurst, IL). Platelet lysis was monitored by the release of lactate dehydrogenase assayed by the method of Bergmeyer et al. (24).

Incubations—Samples of platelet suspension (0.4 ml) were incubated in microfuge tubes (1.5 ml) with either 0.1 NIH unit of thrombin (a generous gift from Dr. J. W. Fenton, II, of the New York Department of Health, Albany, NY), 20 μg of collagen/ml (Horm, Munich, West Germany), 50 μM ADP, or 0.4 μM ionophore A23187 (Calbiochem). Thrombin, collagen, and ADP were each added in 8 μl of buffer. Ionophore A23187 was added to the platelet suspension in 0.8 μl of dimethyl sulfoxide. This volume of solvent alone had no effect on any of the parameters studied. Incubations with collagen were performed with constant agitation of the platelet suspension. In some experiments, platelet suspension was preincubated for 2 min with 5 to 50 μM trifluoperazine (a gift from Dr. Robert Wallace, St. Jude Children's Research Hospital, Memphis, TN), 2 μM PGE_2, or 50 μM PGI_2 (both supplied by Dr. J. Fiks of the Upjohn Co., Kalamazoo, MI).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—For the study of platelet cytoskeletons, incubations were terminated by the addition of an equal volume of Triton extraction buffer (15% Triton X-100 (Sigma) 10 mM EGTA, and 100 mM Tris, pH 7.4) containing 0.2 NIH unit of hirudin/ml (Sigma). Insoluble cytoskeletons were immediately isolated by centrifugation as previously described (4). For electrophoretic analysis, cytoskeletons were solubilized in a buffer containing 2% SDS (w/v), 5% 2-mercaptoethanol (v/v), 10% glycerol (v/v), 0.002% bromphenol blue (w/v), 225 mM Tris- HCl, pH 6.8. Triton-soluble fractions were dissolved in SDS by addition of ½ volume of a 4 times concentrated solubilization buffer. In experiments in which protein phosphorylation was studied in intact platelets, incubations were terminated by the addition of an equal volume of a 2 times concentrated solubilization buffer. All samples for electrophoresis were incubated at 100 °C for 5 min. Triton (10 to 50 μg) was electrophoresed through slab gels according to the method of Laemmli (25), using a 5 to 20% exponential gradient of acrylamide in the resolving gel and 3% acrylamide in the stacking gel. Protein was stained with Coomassie brilliant blue. The relative amounts of myosin heavy chain present were determined from scans of stained wet gels, using an Ortec densitometer linked to a Hewlett-Packard 9845A computer, through which areas under peaks were integrated. To determine the distribution of radioactivity in phosphopolypeptides, gels were dried under vacuum on Whatman No. 3MM chromatography paper and exposed to Trilum x-ray film for 1 to 5 days. Autoradiographs were developed in a Kodak RP X-Omat processor. The relative amounts of radioactivity in phosphopolypeptides were determined either by integration of densitometric scans of autoradiograms, as described above for stained protein bands, or by cutting out the areas of dried gels that superimposed with the bands of interest on the processed autoradiographs. These gel slices were counted at 37 °C for 18 h with 0.4 ml of Protosol (New England Nuclear) and 50 μl of water. Samples were neutralized with 40 μl of glacial acetic acid and radioactivity determined by scintillation counting in 3 ml of scintillation fluid (ACS, New England Nuclear).

RESULTS

Time Course of Thrombin-induced Phosphorylation of Myosin Light Chain and Incorporation of Myosin into Cytoskeletons—We have previously identified the 200,000 M, polyepitide in the Triton-insoluble cytoskeletons from human platelets as the heavy chain of myosin (5). Fig. 1 shows the time course of phosphorylation of myosin by addition of thrombin to intact platelets. This was quantitated by densitometry of Coomassie blue-stained SDS-polycrylamide gels. The amount of myosin associated with the cytoskeletons rose from 13.2 ± 1.4% (mean ± S.E. for platelet preparations from 3 donors) of the total platelet myosin in unstimulated platelets to 92.6 ± 1.4% within 60 s of the addition of thrombin.

FIG. 1. Time-dependent changes in myosin phosphorylation and its association with cytoskeletons during thrombin activation of platelets. Platelets that had been labeled with (^32)P phosphate were treated at 22 ± 2 °C with 0.1 unit of thrombin/ml for the times indicated. The extent of phosphorylation of the myosin light chain was determined after electrophoresis of platelet samples solubilized directly into SDS. The amount of myosin associated with cytoskeletons was determined by measuring the myosin sedimenting after lysis of platelets with Triton X-100, as described in the text. These results were obtained from 3 different platelet preparations (mean ± S.E.).
FIG. 2. Thrombin-induced phosphorylation reactions in intact platelets and the recovery of phosphopolypeptides from Triton lysates. Platelets that had been labeled with \(^{32}P\)-labeled intact platelets with thrombin results in a rapid increase in the labeling of two major polypeptides of 47,000 M, and one of 20,000 M (16, 17). Fig. 2A shows the \(^{32}P\)-labeling of polypeptides in control and thrombin-treated platelets. The 20,000 M, polypeptide that was phosphorylated during thrombin activation appeared to be a homogeneous peak. Based on the work of Daniel et al. (17, 20), who showed that myosin light chain is the only polypeptide of this molecular weight that incorporates increased phosphate during thrombin activation of platelets, we identified this 20,000 M, polypeptide as the myosin light chain. Fig. 1 shows the incorporation of radioactivity into this polypeptide at various times after thrombin was added to intact platelets. The extent of labeling of all platelet polypeptides varied with the three different platelet suspensions used for these experiments. Thus, the amount of radioactivity incorporated into myosin light chain at each time point was expressed as a percentage of the maximum incorporated. The extent and time course of myosin light chain phosphorylation closely paralleled the amount of myosin association with the cytoskeletons. Maximum phosphorylation was observed approximately 30 s after thrombin addition. After this time, the amount of radioactivity declined in parallel with the amount of sedimentable myosin in the Triton extracts. Unlike that in myosin light chain from thrombin-activated platelets (see later), the radioactivity present in the 20,000 M, region of SDS gels from unstimulated platelets was not recovered in cytoskeletons nor was it a substrate for phosphatase in platelet lysates. Thus, this radioactivity is probably in a polypeptide other than myosin light chain, and to compensate for this background we have adjusted the axes of Figs. 1, 3, and 4 to maximize the overlap of curves.

Selective Recovery of Phosphorylated Myosin with Cytoskeletons—We next wanted to determine whether the phosphorylated myosin light chain was associated with the cytoskeletons of Triton-lysed platelets. However, we were aware of the fact that a phosphatase active against myosin light chain has been observed in platelet extracts (26, 27). Therefore, in preliminary experiments, we treated thrombin-activated platelets with the Triton-lysis buffer and determined the level of phosphate in platelet polypeptides by SDS gel electrophoresis at various times after lysis. It was observed that within 10 s after addition of Triton buffer at 22 ± 2°C, essentially all of the light chain was dephosphorylated, while the 47,000 M, polypeptide was unaffected (data not shown). To prevent dephosphorylation of myosin during isolation of cytoskeletons, we used the EGTA, pyrophosphate, and sodium molybdate as inhibitors. However, these inhibitors not only inhibited the phosphatase activity, but also prevented the sedimentation of actin-binding protein and partially inhibited the sedimentation of myosin with the Triton-insoluble cytoskeletons. This may have resulted from the high ionic strength of the lysates (approximately 0.4), or from the known inhibitory effect of pyrophosphate on the actin-myosin interaction (29). Table I shows results in which we lowered the EGTA concentration, eliminated Trit from the Triton extraction buffer, and attempted to find a combination of phosphatase inhibitors that prevented dephosphorylation without inhibiting the sedimentation of myosin with cytoskeletons. Lower concentrations of the inhibitors did not affect the sedimentation properties of myosin but they did not inhibit the phosphatase either. Most of the myosin was recovered in cytoskeletons even though it was extensively dephosphorylated during the isolation procedure. The lowest concentrations of inhibitors that completely inhibited dephosphorylation (20 mM potassium phosphate, 40 mM sodium pyrophosphate, and 10 mM sodium molybdate) still partially inhibited myosin sedimentation (only 62% of the total platelet myosin sedimented with cytoskeletons compared with the usual 90 to 100%). These concentrations also prevented the sedimentation of actin-binding protein with cytoskeletons (data not shown). Electron microscopy of negatively stained preparations showed that cytoskeletons of individual platelets were present in preparations containing the phosphatase inhibitors (data not shown). However, these structures were not as tightly condensed as those isolated by our previous procedures (5). We decided to use the modified lysis buffer in which dephosphorylation was completely inhibited to determine whether phosphorylated myosin was preferentially associated...
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TABLE I
Inhibition of dephosphorylation of myosin light chain in Triton lysates and the effect of inhibitors on the recovery of myosin in cytoskeletons

| Inhibitor concentration | Triton-insoluble myosin | Triton-soluble myosin | Total recovered |
|-------------------------|-------------------------|-----------------------|----------------|
| Potassium phosphate     | Sodium pyrophosphate    | Sodium molybdate      | % of total     |
|                         | (mM)                    | (mM)                  | %              |
| 20                      | 5                       | 10                    | 106.36 ± 7.83  |
| 10                      | 10                      | 5                     | 104.68 ± 1.28  |
| 10                      | 20                      | 5                     | 75.61 ± 2.34   |
| 20                      | 40                      | 10                    | 62.09 ± 2.04   |

$^{32}$P-labeled platelets were incubated at 25 °C either alone or with 0.1 unit of thrombin/ml for 60 s. Platelets were solubilized in SDS for electrophoresis or lysed by the addition of an equal volume of an ice-cold buffer, pH 7.4, containing 2% Triton X-100. Platelets that had been labeled with $(^{32}$P)phosphate were treated at 25 °C with 0.4 μM A23187, 20 μg of collagen/ml, or 50 μM ADP in ice-cold buffer containing 2% Triton X-100, 2 mM EGTA, 2 mM N-ethylmaleimide, and inhibitors to give the final concentrations shown. Lysates were immediately centrifuged as described in the text except that all steps were performed at 4 °C. Recovery of $^{32}$P-labeled myosin light chain in the Triton-soluble and -insoluble fractions were determined as described in the text. Values given are mean ± S.E. from 3 different platelet donors.

Fig. 3. Selective incorporation of phosphorylated myosin into cytoskeletons. Platelets that had been labeled with $(^{32}$P)phosphate were treated at 25 °C with 0.1 unit of thrombin/ml for increasing times then lysed by the addition of an equal volume of ice-cold buffer containing 2% Triton X-100, 2 mM EGTA, 2 mM N-ethylmaleimide, 40 mM potassium phosphate, 80 mM sodium pyrophosphate, and 20 mM sodium molybdate, pH 7.4. Cytoskeletons were isolated at 4 °C by centrifugation and solubilized immediately with SDS. The amount of myosin and the amount of radioactivity associated with myosin light chain in the platelet cytoskeletons was determined after electrophoresis of the samples.

Myosin in Cytoskeletons-The phosphorylation of myosin in intact platelets increased the stability of myosin with cytoskeletons. We argued that if phosphorylation of myosin in intact platelets increased the stability of myosin with cytoskeletons, then the ratio of $(^{32}$P)phosphate/unit of myosin in the cytoskeletons would be constant at all levels of phosphorylation of myosin in intact platelets (i.e. at all time points after thrombin addition), even under lysis conditions in which sedimentation of some of the myosin with cytoskeletons was inhibited.

Fig. 2A shows the phosphopolymerptides present in unstimulated platelets and the time-dependent increases in phosphorylation of myosin light chain and the 47,000 M₀ polyptide during thrombin activation. When Triton-soluble (Fig. 2C) and -insoluble (Fig. 2B) fractions were isolated in the presence of phosphatase inhibitors, most of the major phosphopolymerptides, including the one of 47,000 M₀ and the one that comigrated with actin-binding protein, were recovered in the Triton-soluble fraction. Myosin light chain was the only major phosphopolymerptide recovered in cytoskeletons. In the experiment shown in Fig. 2, approximately 80% of the thrombin-induced increase in radioactivity in myosin light chain was in cytoskeletons at all time points after addition of thrombin. The remainder of the thrombin-induced increase and all of the radioactivity in the 20,000 M₀ region of gels from unstimulated platelets was recovered in the Triton-soluble fractions. At all time points the amount of myosin heavy chain that sedimented with cytoskeletons was about 80% of that sedimented in the absence of phosphatase inhibitors.

FIG. 4. Correlation between phosphorylation of myosin in intact platelets and the recovery of myosin in cytoskeletons-The effects of various platelet-stimulating agents on myosin phosphorylation and on the recovery of myosin in cytoskeletons. Platelets that had been labeled with $(^{32}$P)phosphate were treated at 22 ± 2 °C with 0.4 μM A23187, 20 μg of collagen/ml, or 50 μM ADP. The extent of phosphorylation of myosin light chain was determined after solubilization of intact platelets with SDS. The amount of myosin associated with the cytoskeletons was determined by electrophoresis of the Triton-insoluble material.
protein composition of cytoskeletons were examined. Collagen, the divalent cation ionophore A23187, and ADP caused an increase in the amount of actin, actin-binding protein, and the 31,000 M₄ polypeptide in cytoskeletons (data not shown). Although the rate of the responses varied, collagen and ionophore A23187, like thrombin, also stimulated phosphorylation of myosin light chain and the association of myosin with cytoskeletons. Fig. 4 shows the correlation between phosphorylation of myosin and myosin sedimenting with cytoskeletons after addition of A23187, collagen, or ADP. Addition of 0.4 μM A23187 to intact platelets caused both a rapid incorporation of radioactivity into the myosin light chain and a rapid association of myosin with cytoskeletons. The rate and extent of phosphorylation closely correlated with the rate and extent of association of myosin with platelet cytoskeletons. Collagen (20 ng/ml) caused a slower phosphorylation of myosin light chain than did the ionophore but this rate closely paralleled that for the association of myosin with cytoskeletons. In contrast, ADP had little effect on the incorporation of phosphate into myosin light chain or on the association of myosin with cytoskeletons.

**Effect of Inhibitors of Platelet Function on the Phosphorylation of Myosin Light Chain and the Association of Myosin with Cytoskeletons—** Preincubation of platelet suspension with the calmodulin inhibitor trifluoperazine caused a dose-dependent inhibition of thrombin-induced phosphorylation of myosin light chain (Fig. 5). This drug also inhibited the association of myosin with cytoskeletons. The extent of inhibition of phosphorylation resembled that of association of myosin with cytoskeletons at all concentrations of trifluoperazine used. When platelets were centrifuged from suspension, the supernatants from control and trifluoperazine-treated platelets contained 7.7 and 8.5%, respectively, of the total platelet lactate dehydrogenase activity (data not shown), indicating that trifluoperazine did not cause extensive platelet lysis.

Table II shows that thrombin-induced phosphorylation of myosin was also inhibited by preincubation of platelets with PGE₂ or PGL₂. These drugs also inhibited the thrombin-induced association of myosin with cytoskeletons and again the extent of inhibition of these two processes was similar.

**DISCUSSION**

Several groups have demonstrated that adding thrombin to platelets incubated with (³²P)phosphate increases incorporation of radioactivity into myosin light chain (16-20). Four observations in the present study show that phosphorylation of the light chain of myosin stabilizes the association of myosin with the contractile structures of platelets.

(a) The time course of phosphorylation of myosin was similar to the time course of association of myosin with platelet cytoskeletons isolated by low speed centrifugation after Triton-lysis. This temporal relationship was observed even when we used a variety of stimulating agents that mediate phosphorylation of myosin at very different rates. Thus, the Ca²⁺ ionophore A23187 caused maximum phosphorylation of myosin and maximum association of this protein with cytoskeletons within 10 s. Thrombin induced maximum phosphorylation after 30 to 60 s, and myosin incorporation into cytoskeletons reached a maximum at a similar time and followed a similar time course. Collagen activates platelets more slowly than either A23187 or thrombin and induces phosphorylation of myosin at a much slower rate (18). We found that the collagen-induced association of myosin with cytoskeletons also occurred at this slower rate. Unlike the other stimuli, ADP causes platelets to change shape, but will not induce secretion unless fibrinogen and calcium are added and the suspension is agitated to induce aggregation (see Ref. 30 for review). We found, as have others (18), that ADP did not induce phosphorylation of myosin in unstirred suspensions. Although the amount of actin, actin-binding protein, and the 31,000 M₄ polypeptide increased in the cytoskeletons following ADP stimulation, the amount of myosin remained constant.

(b) The dephosphorylation of the myosin light chain that occurs with time after stimulus addition also caused a dissociation of myosin from cytoskeletal structures. Platelets contain a light chain phosphatase that appears to be active within platelets since the amount of phosphate bound to myosin in activated platelets decreases with time (16-20). We have previously shown that the amounts of actin, actin-binding protein, and a 31,000 M₄ polypeptide in cytoskeletons increase to stable levels after the addition of thrombin to platelets, while the amount of cytoskeleton-associated myosin peaks and then declines (4, 5). The similarity of the rate and extent of myosin dephosphorylation within platelets to the loss of myosin from cytoskeletal structures is consistent with the idea that myosin light chain phosphorylation stabilizes the association of myosin with platelet cytoskeletons. The failure of myosin to dissociate from cytoskeletons when dephosphorylated in Triton lysates may be due to loss of a control mechanism during platelet lysis.

**TABLE II**

| Inhibitor present | Thrombin-induced increase in [³²P]cpm in myosin light chain | Thrombin-induced increase of the percentage of total myosin in cytoskeletons |
|-------------------|--------------------------------------------------|--------------------------------------------------|
| None              | 26,825 (66.9)                                     | 66.9                                             |
| 2 μM PGE₁         | 12,154 (54.7%)                                    | 25.2 (62.3%)                                     |
| 50 nM PGL₂        | 3,850 (85.3%)                                     | 8.2 (87.8%)                                     |

* Values given in parentheses are the percentage of inhibition.
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(c) Phosphorylated myosin light chain was selectively isolated with the Triton-insoluble cytoskeletons. Previously, it has been shown that essentially all myosin light chain is phosphorylated during thrombin-induced activation of platelets (20) at a time when essentially all myosin is isolated with the cytoskeletons (5). The constant specific activity of myosin light chain in cytoskeletons at time points before and after the maximum phosphorylation indicates that only phosphorylated myosin is associated with cytoskeletons during thrombin activation of platelets.

(d) Inhibitors of myosin light chain phosphorylation also inhibited myosin association with platelet cytoskeletons. Trifluoperazine inhibits calmodulin-dependent reactions (31). We found that this compound, when added to intact platelets, caused an identical dose-dependent inhibition of the phosphorylation of myosin light chain and the amount of myosin associated with the cytoskeletons. PGE1 and PGI2 inhibit platelet responses, possibly by lowering the calcium ion concentrations in the cytosol (27). They both inhibited thrombin-induced myosin phosphorylation and association of myosin with the cytoskeletons to similar degrees.

Experiments with purified myosin suggest two mechanisms by which phosphorylation of platelet myosin could cause it to be isolated with the Triton-insoluble cytoskeletons. Adelstein and Conti (32) showed that the actin-activatable ATPase activity of platelet myosin is stimulated by phosphorylation of the 20,000 Mr kinase. This increased activity indicates that phosphorylated myosin interacts with actin filaments differently than unphosphorylated myosin. This property of myosin could account for its Triton-insolubility. Another possibility is suggested by the report of Scholey et al. (39), who showed that phosphorylation of platelet myosin light chain causes myosin to assemble into filaments at physiological ionic strength and Mg-ATP concentrations. This ability to polymerize into filaments. The present results are consistent with a mechanism involving increased affinity of myosin to actin filaments, since the low speed centrifugation used for isolating cytoskeletons would not be expected to sediment myosin filaments that were not bound to other cytoskeletal structures (15). Furthermore, we have found that depolymerization of actin in Triton lysates by DNase I or Ca2+, also prevented the sedimentation of cytoskeletal-associated myosin by low speed centrifugation. Thus, while our data do not permit us to exclude the possibility that phosphorylation of myosin causes it to polymerize in activated platelets, they do suggest that Triton-insolubility of myosin arises from its increased binding to actin filaments, irrespective of the extent of myosin polymerization.

Although the present data show that the phosphorylated form of myosin has increased association with the cytoskeletal structures, it remains to be determined whether (a) phosphorylation is a prerequisite for myosin association or whether (b) myosin association with cytoskeletons is independent of phosphorylation and that the bound myosin serves as a preferred substrate for the light chain kinase. However, the observations that phosphorylated myosin forms filaments (33) and has a higher actin-activatable ATPase activity (32) suggest that phosphorylation may be a prerequisite. Distinguishing between these mechanisms is clearly relevant to understanding the involvement of myosin in the contractile processes of nonmuscle cells.

It has been reported that there is a direct relationship between the extent of phosphorylation of smooth muscle myosin and its actin-activated ATPase activity (34). Similarly, the tension generated by platelet actomyosin threads in vitro was proportional to the level of phosphorylation of myosin (35). In contrast, Percichini and Hartshorne suggest a mechanism in which there is ordered phosphorylation of smooth muscle myosin light chains with phosphorylation of both light chains being required for stimulation of the ATPase activity (36). Our results showed that the extent of myosin light chain phosphorylation closely paralleled the association of myosin with the cytoskeleton, although rate measurements showed that the association of myosin slightly preceded phosphorylation while dephosphorylation preceded dissociation (see Figs. 1 and 4). Thus, our data are most consistent with a mechanism in which phosphorylation and dephosphorylation of the two light chains on a myosin molecule occur randomly and phosphorylation of only one light chain is sufficient for association of a myosin molecule with the cytoskeletal structure.

Experiments by others show that the phosphorylation of myosin correlates with a force-generating system. Lebowitz and Cooke (35) prepared actomyosin threads from human blood platelets and found that the maximum isometric tension of these threads was proportional to the level of phosphorylation of myosin light chain. Likewise, phosphorylation of myosin light chain within cells is associated with contraction. In intact arterial smooth muscle, the contraction-relaxation cycle is coincident with cyclic phosphorylation-dephosphorylation of the myosin light chain (37). Also, in tracheal smooth muscle, the extent of myosin phosphorylation occurred temporarily with the increase in isometric tension (28).

With human platelets, agents that mediate the release reaction as well as shape change and aggregation, such as thrombin, collagen, and ionophore A23187, stimulate myosin phosphorylation. ADP, which causes shape change and aggregation but not the release reaction, does not cause phosphorylation (18). With rat platelets, phosphorylation can occur without the release reaction (38) but the converse has not been observed. Thus, phosphorylation of myosin light chain plays a role early in the secretory process. Our finding that phosphorylation regulates the association of myosin with cytoskeletons suggests that the release reaction is a contractile process involving a force-generating interaction of actin with myosin. Shape change and aggregation, however, can presumably occur without the involvement of myosin, but may require newly formed actin filaments and their interaction with actin-binding protein.

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