Phosphorylation of a Second Site for Myosin Light Chain Kinase on Platelet Myosin†

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ABSTRACT: The 20 000-dalton light chain of bovine platelet myosin is phosphorylated at two sites by myosin light chain kinase. The first and second phosphorylation sites are at a serine and a threonine residue, respectively. The location of the phosphorylation sites was determined by using limited proteolysis. The N-terminal sequence of the 17 000-dalton tryptic fragment of platelet myosin 20 000-dalton light chain was found to be identical with that of gizzard 20 000-dalton light chain from Ala-17 to Phe-33. On the basis of these results and the distribution of ^{32}P among the proteolytic fragments, it was concluded that serine-19 and threonine-18 were the two phosphorylation sites. Phosphorylation at the threonine residue markedly increases the actin-activated ATPase activity of myosin. It was found that platelet myosin forms 10S and 6S conformations and its Mg^{2+}-ATPase activity parallels the transition from the 6S to the 10S conformation. The conformational transition was influenced by phosphorylation at both sites, and the phosphorylation at the threonine residue further shifted the equilibrium toward the 6S conformation. The phosphorylation at the threonine residue also induced thick filament formation in the presence of ATP. These results suggest that the phosphorylation at the threonine residue as well as at the serine residue may play an important role in the contractility of nonmuscle cells.

It is known that thrombin induces several platelet responses including shape change aggregation, secretion, and clot retraction, and it is thought that the phosphorylation of the 20 000-dalton light chain of myosin is associated with the activation (Zucker et al., 1985). The protein kinase catalyzing this phosphorylation has been identified as the Ca^{2+}–calmodulin-dependent kinase myosin light chain kinase (MLC kinase)[1] (Adelstein et al., 1977; Daniel et al., 1981). It was also shown with platelet myosin (Adelstein & Conti, 1975) as well as with smooth muscle myosin (Hartshorne, 1985) that the phosphorylation activates the actin-activated ATPase activity of myosin. Thus, it is apparent that the phosphorylation of myosin is an essential component of the activation of platelets.

Although the phosphorylation site of the 20 000-dalton light chain of platelet myosin by MLC kinase is not known, it is assumed that a site similar to smooth muscle myosin is phosphorylated. For smooth muscle myosin, the bulk of biochemical evidence indicated that each 20 000-dalton light chain contained one residue that could be phosphorylated by MLC kinase and this was identified as serine-19 (Jakes et al., 1976; Pearson et al., 1984). Recently, it was reported that smooth muscle myosin can be phosphorylated by MLC kinase at two distinct sites (Ikebe & Hartshorne, 1985a; Tanaka et al., 1985) and threonine-18 was identified as a second phosphorylation site for MLC kinase (Ikebe & Hartshorne, 1986).

Nonmuscle cell myosin in the monomeric state can exist in two conformations, i.e., the folded (10 S) and extended (6 S) forms (Citi & Kendrick-Jones, 1986; Higashihara et al., 1989) as well as smooth muscle myosin (Trybus et al., 1982; Onishi & Wakabayashi, 1982; Craig et al., 1983). The properties of the two conformations are studied in detail with smooth muscle myosin. In general, higher ionic strength (Ikebe et al., 1983; Trybus & Lowey, 1984) and phosphorylation (Ikebe et al., 1983; Craig et al., 1983; Trybus & Lowey, 1984; Onishi et al., 1983) favor the 6S conformation. The 10S–6S transition is accompanied by an alteration of the enzymatic activity of myosin (Ikebe et al., 1983), and it was suggested that the effect of phosphorylation is to change the conformation which would be reflected by altered ATPase activity (Ikebe et al., 1983, 1984). Recently, it was found (Ikebe & Hartshorne, 1984) that the S1–S2 junction is altered by the 10S–6S transition, and it is suggested (Ikebe & Hartshorne, 1984, 1985b) that this region of the molecule is involved in the regulation of enzymatic activity. In this paper, we report that platelet myosin is phosphorylated at two distinct sites by MLC kinase. The location of these sites is studied by using limited proteolysis. The effects of dual phosphorylation on the platelet myosin function are also reported.

Materials and Methods

Platelet was prepared from fresh bovine blood as follows. Whole blood was centrifuged at 500*g* for 10 min. The resulting platelet-rich plasma was then centrifuged at 1500*g* for 10 min, and the pellet was resuspended with washing buffer (113 mM NaCl, 4.3 mM K_2HPO_4, 4.3 mM Na_2HPO_4, 24.4 mM NaH_2HPO_4, 0.1% glucose, and 1 mM EDTA) and then centrifuged at 1500*g* for 10 min. The washing step was repeated 3 times, and then the precipitate was suspended with washing buffer loaded on Ficoll Parque (Pharmacia) and centrifuged at 1500*g* for 30 min. Platelets formed the layer on the top of Ficoll Parque. The platelets were further washed with washing buffer twice. About 10–15 mL of packed platelets was suspended with the same volume of buffer A (0.4 M KCl, 0.15 M potassium phosphate, pH 6.8, 2 mM EGTA, 3 mM DTT, 1 mM PMSF, 1 mM EDTA, and 0.1 mg/mL ovomucoid trypsin inhibitor) and sonicated for 1–2 min. Three

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[1] Abbreviations: MLC, myosin light chain; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

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volumes of buffer A were further added, and the suspension was gently stirred for 30 min in ice. After the suspension was centrifuged at 50000g for 40 min, the supernatant was dialyzed against buffer B (10 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 10 mM imidazole, pH 7.0, and 5% sucrose). The precipitate formed during dialysis was collected by centrifugation at 10000g for 15 min and then dissolved in 0.15 M $MgCl_2$, 1 mM EGTA, 5 mM ATP, 2 mM DTT, and 50 mM Tris-HCl, pH 7.5. The protein solution was centrifuged at 100000g for 3 h, and the supernatant was subjected to Sepharose 4B column chromatography (3 cm × 90 cm) equilibrated with 0.5 M NaCl, 1 mM DTT, 1 mM EGTA, 0.1 mM ATP, 1 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.5. The pure myosin fraction as judged by SDS gel electrophoresis was combined and used for the experiments. The purity was more than 97% as judged from the densitometry of a Coomassie Brilliant Blue stained gel. Actin from rabbit skeletal muscle was prepared according to Driska and Hartshorne (1975). MLC kinase was purified from turkey gizzard as described previously (Ikebe et al., 1987). Calmodulin was prepared from frozen bull testes (Walsh et al., 1983). Trypsin (type XIII), α-chymotrypsin (type VII), and soybean trypsin inhibitor (type II-s) were obtained from Sigma.

ATPase assay was carried out at 25 °C as described previously (Ikebe & Hartshorne, 1985b). The extent of protein phosphorylation was measured according to Walsh et al. (1983). The identification of phosphorylated amino acids was as described previously (Ikebe & Hartshorne, 1985c). SDS gel electrophoresis was carried out on 10–23% polyacrylamide gradient slab gels using the discontinuous buffer system of Laemmli (1970). Molecular weights were estimated by using the following standards: skeletal muscle myosin heavy chain (205000), β-galactosidase (116000), phosphorylase b (97000), bovine serum albumin (66000), ovalbumin (45000), carbonic anhydrase (29000), trypsin inhibitor (20100), and lysozyme (14300).

To determine the N-terminal sequence of proteolyzed fragments of the 20000-dalton light chain, the protein was subjected to SDS gel electrophoresis and then transblotted to Immobilon for 4 h at 70 V as described by Matsudaira (1987). The transblotted Immobilon membrane was stained with Coomassie Brilliant Blue R-250 (0.06% dye, 45% ethanol, and 10% acetic acid) and destained with 90% ethanol and 2% acetic acid for a few minutes. The membrane was soaked in the water, and the protein band was cut out and subjected to the protein sequencer. The amino acid sequence was determined by using an Applied Biosystems 477A protein sequencer.

The extent of filament formation of myosin was estimated as follows. Myosin (0.5 mg/mL) in 50 or 100 mM KCl, 1 mM $MgCl_2$, 30 mM Tris-HCl, pH 7.5, and 0.1 mM $CaCl_2$ was incubated with 0.5 mM ATP, or with 0.5 mM ATP, 2 μg/mL MLC kinase, and 1 μg/mL calmodulin, or with 0.5 mM ATP, 40 μg/mL MLC kinase, and 10 μg/mL calmodulin at 25 °C for 20 min. The extent of phosphorylation was approximately 0, 2.0, and 3.8 mol of phosphate/mol of myosin, respectively. The samples were centrifuged by an airfuge (Beckman) at 100000g for 20 min. K^+-EDTA–ATPase activities of the supernatant and precentrifuged solution were measured to estimate the percentage of filament formation. The myosin concentration in the supernatant was estimated by calculating the ATPase activity in the supernatant divided by the activity of the precentrifuged solution. The extent of filament formation was obtained by subtracting the myosin concentration in the supernatant from the myosin concentration in the precentrifuged solution. Assay conditions were 0.5 M KCl, 50 mM Tris-HCl, pH 8.5, and 10 mM EDTA. Filament formation was also confirmed by SDS gels of supernatant followed by densitometric scanning of myosin heavy chain.

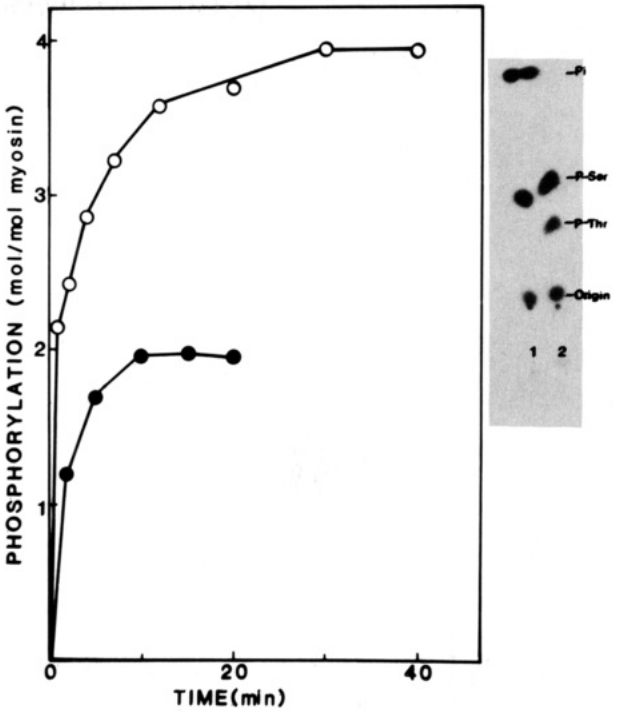

FIGURE 1: Time course of the phosphorylation of platelet myosin by MLC kinase. Platelet myosin (0.5 mg/mL) was phosphorylated either with 2 μg/mL MLC kinase and 1 μg/mL calmodulin (●) or with 40 μg/mL MLC kinase and 10 μg/mL calmodulin (○) at 25 °C in a solution containing 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 mM [γ-^{32}P]ATP, 30 mM Tris-HCl (pH 7.5), and 85 mM KCl. Reactions were stopped by addition of 5% trichloroacetic acid. The right side shows autoradiograms of electrophoresis patterns of acid-hydrolyzed platelet myosin. Lane 1, myosin phosphorylated for 20 min in 2 μg/mL MLC kinase; lane 2, myosin phosphorylated for 20 min in 40 μg/mL MLC kinase.

Viscosity was measured at 23 °C in Cannon–Ubbelohde viscometers with a water flow time of approximately 54 s. The relative viscosity (η_{rel}) of platelet myosin (0.5 mg/mL) was measured in the solution containing 0.5 mM ATP, 1 mM $MgCl_2$, and 30 mM Tris-HCl (pH 7.5). To obtain the singly and doubly phosphorylated platelet myosin, the myosin was phosphorylated as described for the experimental procedure for the assay of myosin filament formation. The viscosity was expressed as η_{rel} (flow time of protein solution/flow time of solvent). The assays are repeated for 10 times, and the mean value was calculated.

The protein concentration of platelet myosin was determined by the biuret method (Itzhaki & Gill, 1964) using gizzard myosin as a standard. Concentrations of gizzard MLC kinase and calmodulin were determined by absorbance measurements using the following molar extinction coefficients: $\epsilon_{278}^{1\%} = 11.4$ for MLC kinase (Adelstein & Klee, 1981) and $\epsilon_{276}^{1\%} = 1.8$ for calmodulin (Watterson et al., 1976). The molar concentration of myosin was calculated by using a molecular weight for myosin of 480000.

Results

The time course of the phosphorylation of platelet myosin at two MLC kinase concentrations is shown in Figure 1. At lower (2 μg/mL) MLC kinase concentration, platelet myosin was phosphorylated up to 2 mol of phosphate/mol of myosin. On the other hand, 4 mol of phosphate/mol of myosin was

Table I: Amino Acid Sequence of the 17 000-Dalton Tryptic Fragment and the 16 000-Dalton α-Chymotryptic Fragment of the 20 000-Dalton Light Chain of Platelet Myosin

	amino acid sequence
gizzard 20 000-dalton light chain	Ac-S-S-K-R-A-K-A-K-T-T-K-K-R-P-Q-R-A-T-S-N-V-F-A-M-F-D-Q-S-Q-I-Q-E-F-K-E-A-F
platelet 17 000-dalton tryptic fragment	A-T-S-N-V-F-A-M-F-D-Q-S
platelet 16 000-dalton α-chymotryptic fragment	A-M-F-D-Q-S-Q-I-Q-E-F-*-*-A

incorporated at higher (40 μg/mL) concentrations of MLC kinase. Platelet myosin phosphorylated 2 mol/mol of myosin, 3.8 mol/mol of myosin was subjected to partial acid hydrolysis, and the hydrolysate was electrophoresed (Figure 1). At a phosphorylation level of 2 mol of phosphate/mol of myosin, only phosphoserine was detected. On the other hand, both phosphothreonine and phosphoserine were found at a phosphorylation level of 3.8 mol of phosphate/mol of myosin. This shows that the serine residue is the preferred site.

To identify the phosphorylation sites, limited proteolysis of platelet myosin was carried out. Figure 2 shows the SDS–polyacrylamide electrophoresis profiles and autoradiogram of tryptic and α-chymotryptic digests of platelet myosin (phosphorylated 3.8 mol of phosphate/mol of myosin). The ^{32}P was initially confined to the 20 000-dalton light chain; no labeling of the heavy chain or the 17 000-dalton light chain was found. Following proteolysis with trypsin, the 20 000-dalton light chain was degraded to a 17 000-dalton fragment that retained both phosphorylation sites. When the light chain was proteolyzed with α-chymotrypsin, the light chain was initially degraded to an 18 000-dalton fragment that retained the phosphorylation sites. Further digestion produced a 16 000-dalton fragment that had no phosphate on the basis of autoradiography (Figure 2).

The SDS–polyacrylamide gel of dephosphorylated platelet myosin digests was transblotted to an Immobilon membrane, and the N-terminal sequences of the 17 000-dalton tryptic fragments and the 16 000-dalton α-chymotryptic fragments were determined (Table I). The amino acid sequences of the 17 000-dalton tryptic fragment and the 16 000-dalton α-chymotryptic fragment were identical with the known sequence of the 20 000-dalton gizzard myosin light chain. It was also found that both phosphorylation sites were present in the 17 000-dalton tryptic fragment but absent in the 16 000-dalton α-chymotryptic fragment as judged by the autoradiogram (Figure 2). When platelet myosin was phosphorylated at only the serine site, the phosphorylation site was located only in the 17 000-dalton tryptic fragment but not in the 16 000-dalton α-chymotryptic fragment (data not shown). Previously, it was reported for smooth muscle myosin that serine-19 and threonine-18 are the sites phosphorylated by MLC kinase and that these sites are present in 17 000-dalton tryptic fragment but not the 16 000-dalton α-chymotryptic fragment (Ikebe & Hartshorne, 1985a; Ikebe et al., 1986). Therefore, it was concluded that the first phosphorylation site is serine-19 and the second phosphorylation site is threonine-18.

We examined the effect of the second phosphorylation on myosin function. The actin-activated ATPase activity of platelet myosin as a function of the level of phosphorylation is shown in Figure 3. The actin-activated ATPase activity increased parabolically with increase in the phosphorylation level up to 2 mol of phosphate/mol of myosin and then increased almost linearly with increasing of the phosphorylation level. This indicates that the phosphorylation of the threonine residue activates the actin-activated ATPase activity of platelet myosin.

The effect of the phosphorylation on platelet myosin conformation was studied. It has been shown (Citi & Kendrick-Jones, 1986; Higashihara et al., 1989) that nonmuscle

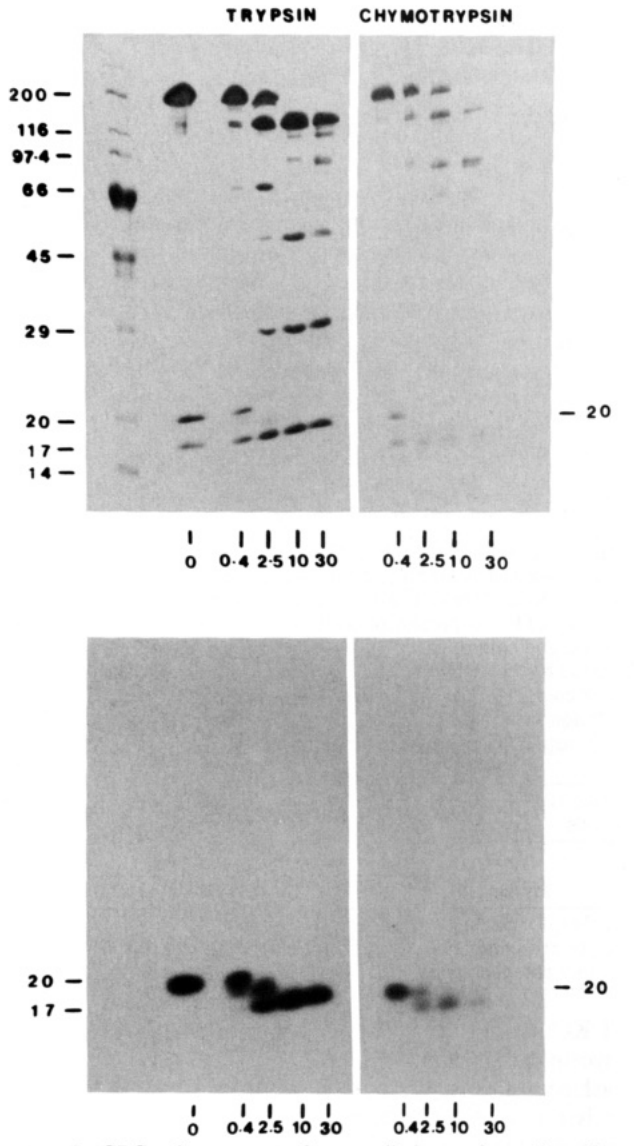

FIGURE 2: SDS gel patterns and autoradiogram of tryptic and α-chymotryptic digests of platelet myosin. Platelet myosin (1 mg/mL) was phosphorylated at 25 °C for 20 min with 40 μg/mL MLC kinase plus 6 μg/mL calmodulin. Phosphorylated myosin was digested by 2 μg/mL trypsin or 25 μg/mL α-chymotrypsin at 25 °C. Times of digestion (minutes) are as indicated in the figure. Twenty micrograms of protein was applied on the gel. The lower panel shows an autoradiogram of the SDS gel. Lane 1 shows the molecular weight standards. Molecular weights ($\times 10^{-3}$) are indicated on the left of the SDS gel.

cell myosins can form a folded (10 S) and an extended (6 S) structure as has been shown previously for smooth muscle myosin (Trybus et al., 1982; Onishi & Wakabayashi, 1982; Craig et al., 1983; Ikebe et al., 1983). It has also been shown that the two conformations of nonmuscle cell myosin are characterized by distinct enzymatic activities using erythrocyte myosin (Higashihara et al., 1989). Figure 4 shows the [KCl] dependence of Mg^{2+}-ATPase activities of dephosphorylated, singly phosphorylated, and doubly phosphorylated myosin. The Mg^{2+}-ATPase activities of dephosphorylated and singly phosphorylated myosin decreased sharply below 0.3 and 0.2

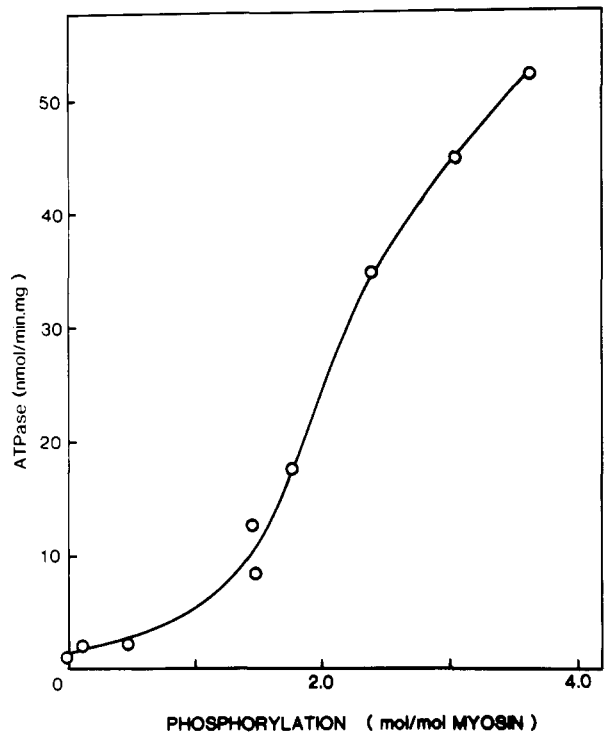

FIGURE 3: Actin-activated ATPase activity of platelet myosin as a function of the extent of phosphorylation. Myosin in 0.1 mM $CaCl_2$, 85 mM KCl, 30 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, and 0.5 mM [γ-^{32}P]ATP was prephosphorylated with various amounts of MLC kinase and calmodulin and times at 25 °C. The ATPase assay was started by the addition of 1 mg/mL actin and 1 mM EGTA, and the time course of P_i liberation was measured. The extent of phosphorylation was also monitored. During the ATPase assay, the extent of phosphorylation was stable.

Table II: Relative Viscosity of Myosin at Different Phosphorylation Levels

myosin	η_{rel} 0.3 M KCl	0.2 M KCl	0.16 M KCl
dephosphorylated	1.14 ± 0.01	1.05 ± 0.01	1.03 ± 0.01
singly phosphorylated	1.14 ± 0.01	1.12 ± 0.01	1.08 ± 0.01
doubly phosphorylated	1.14 ± 0.01	1.14 ± 0.01	1.13 ± 0.01

M KCl, respectively. It is known for both smooth muscle myosin (Ikebe et al., 1983) and nonmuscle cell myosin (Higashihara et al., 1989) that this decrease in the Mg^{2+}-ATPase activity is due to the transition of the myosin conformation from 6 S (an extended conformation) to 10 S (a folded conformation). Therefore, these results suggest that dephosphorylated myosin and singly phosphorylated platelet myosin form the 10S conformation below 0.3 and 0.2 M KCl, respectively. This was confirmed by the viscosity measurement (Table II). It was shown previously (Ikebe et al., 1983) that the reduction in viscosity corresponds to the transition from 6S to 10S myosin. The viscosity of dephosphorylated platelet myosin was significantly reduced in 0.2 and 0.16 M KCl. Although the reduction of viscosity of singly phosphorylated myosin was not as large as dephoshorylated myosin, the viscosity also decreased in 0.16 M KCl. On the other hand, the viscosity of doubly phosphorylated myosin was not significantly decreased at 0.16 M KCl. Consistent with the viscosity data, the Mg^{2+}-ATPase activity of doubly phosphorylated myosin did not significantly decrease at low ionic strength, although the ATPase activity was slightly decreased below 0.2 M KCl (Figure 4).

It has been known that the phosphorylation of nonmuscle cell myosin favors thick filament formation. We, therefore,

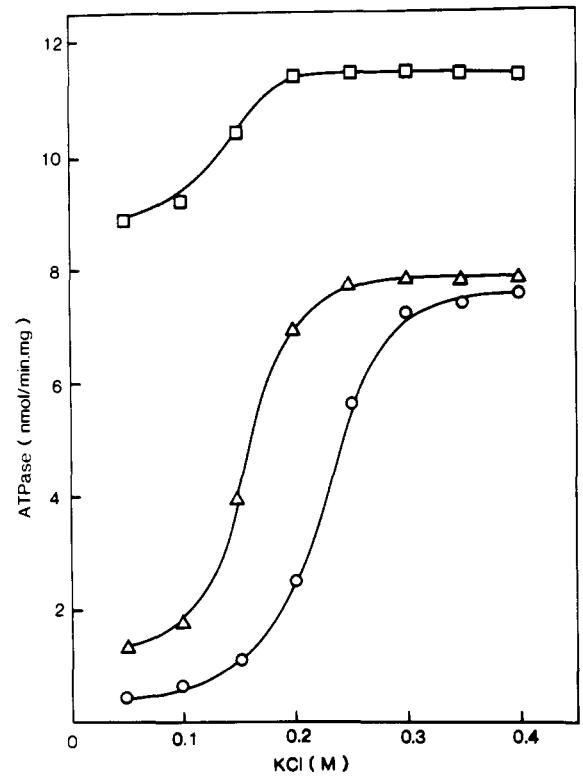

FIGURE 4: KCl concentration dependence of Mg^{2+}-ATPase activity of platelet myosin at different phosphorylation levels. Doubly and singly phosphorylated myosins were produced as described under Materials and Methods. The extent of phosphorylation of singly (△) and doubly (□) phosphorylated myosins was approximately 2.0 and 3.8 mol/mol of myosin, respectively. Conditions: 0.5 mg/mL dephosphorylated (○), singly phosphorylated (△), and doubly phosphorylated (□) platelet myosin, 1 mM $MgCl_2$, and 30 mM Tris-HCl (pH 7.5), 25 °C.

Table III: Effects of Phosphorylation on Myosin Filament Formation

	ATPase act. of supernatant (nmol/min)[a]		fraction of filamentous myosin	
	0.1 M KCl	0.05 M KCl	0.1 M KCl	0.05 M KCl
dephosphorylated +ATP	272	269	0	0
dephosphorylated −ATP	13.5	5.4	0.95	0.98
singly phosphorylated +ATP	287	242	0	0.15
doubly phosphorylated +ATP	86.5	20.2	0.70	0.93

[a] ATPase activities of the precentrifuged solutions are 270, 285, and 288 nmol/min for dephosphorylated, singly phosphorylated, and doubly phosphorylated myosin, respectively.

examined the effects of two sites of phosphorylation on the thick filament formation of platelet myosin. As shown in Table III, the thick filaments of dephosphorylated myosin were disassembled by the addition of ATP. This was consistent with earlier reports (Takeuchi & Ishimura, 1985). Although thick filament formation is only very slightly favored by phosphorylation at the first site, it is markedly stabilized when both sites are phosphorylated.

Discussion

Platelet myosin was phosphorylated by MLC kinase at two distinct sites. The amino acid residues for the first and the

second site were serine and threonine, respectively. Although the complete amino acid sequence of 20000-dalton light chains of platelet myosin is not known, the N-terminal amino acid sequence of the proteolyzed 20000-dalton light chains of platelet myosin was found to be identical with the corresponding region of the gizzard 20000-dalton light chain. By use of limited proteolysis, the two phosphorylation sites were shown to be confined between alanine-17 and phenylalanine-22. Therefore, it is reasonable to assume that the two phosphorylation sites are serine-19 and threonine-18. The present work and previous studies with erythrocyte myosin (Higashihara et al., 1989) suggest that phosphorylation at two distinct sites by MLC kinase is common to myosins whose activity is regulated by the phosphorylation at 20000-dalton light chains. It is of interest to note that protein kinase C results in phosphorylation of the 20000-dalton light chain of platelet myosin but at different residues than found in the present work using MLC kinase (Kawamoto et al., 1989).

The interesting aspect of the present study is the effects of dual phosphorylation of the biological properties of myosin. The actin-activated ATPase activity increased almost linearly with increasing phosphorylation of above 2 mol of phosphate/mol of myosin. Since no threonine residue was phosphorylated at 2 mol of phosphate/mol of myosin, this further increase in the activity is due solely to the phosphorylation at the threonine residue.

Several different results have been reported concerning the relationship between ATPase activity and the level of serine phosphorylation. For gizzard smooth muscle, it was shown that the actin-activated ATPase activity did not increase linearly with phosphorylation, and it is thought that the phosphorylation of both myosin heads is required for the activation of ATPase activity (Persechini & Hartshorne, 1981; Ikebe et al., 1982; Sellers et al., 1983). On the other hand, it was reported that the actin-activated ATPase of calf thymus myosin increased linearly with light-chain phosphorylation, and it was proposed that the phosphorylation of one head activates the ATPase activity of the same head independent of the other head (Wagner et al., 1985). The phosphorylation dependence of the actin-activated ATPase of platelet myosin up to 2 mol of phosphate/mol of myosin was similar to that of gizzard myosin; therefore, the mechanism of activation due to serine phosphorylation may be similar to that of gizzard myosin. On the other hand, the phosphorylation above 2 mol of phosphate/mol of myosin increased the ATPase activity almost linearly, and this suggests that the threonine phosphorylation activates the ATPase of the myosin head independently of the other head.

There are a number of observations that the 10S–6S transition of smooth muscle myosin is influenced by phosphorylation. A similar relationship was also made using nonmuscle cell myosin (Citi & Kendrick-Jones, 1986; Higashihara et al., 1989). The present study showed that platelet myosin also forms 10S and 6S conformations and that this transition is influenced by phosphorylation. However, a particularly interesting aspect of this study is the effect of threonine phosphorylation on the 10S–6S transition. It has been previously suggested (Ikebe et al., 1983) that the [KCl] dependence of Mg^{2+}-ATPase activity is a useful monitor of myosin conformation. The [KCl] dependence of Mg^{2+}-ATPase activity suggests that the formation of the 10S conformation is inhibited by the threonine phosphorylation, and this is also confirmed by the viscosity data. These results are essentially the same as those obtained with gizzard myosin (Ikebe et al., 1988) and suggest that the increase in the actin-activated ATPase activity by the threonine phosphorylation is due to the conformational transition of myosin to 6 S.

Another interesting finding is that the dual phosphorylation favors thick filament formation. This is consistent with the observation that dual phosphorylation favors the 6S conformation since 6S myosin forms thick filaments easily at low ionic strength. The formation of thick filaments was not significant when myosin was only phosphorylated at the serine residue, and an additional phosphorylation was required to form considerable amounts of thick filaments. This result appears to contradict previous observations with gizzard and thymus myosin that phosphorylation at a single site induced considerable thick filament formation (Craig et al., 1983). However, thick filament formation of smooth muscle and nonmuscle cell myosin is known to be sensitive to Mg^{2+} concentration (Hartshorne, 1987), and the apparent discrepancy with the present work is likely to be due to differences in the Mg^{2+} concentration used [10 mM Mg^{2+} was used in a previous study (Craig et al., 1983), and 1 mM $MgCl_2$ was used in this study]. Moreover, the Mg^{2+} concentration used in this study is close to the physiological condition.

Since it is thought that 6S myosin forms thick filaments readily whereas 10S myosin cannot, it is reasonable that the dual phosphorylation induces not only the transition of the myosin conformation toward 6 S but also the thick filament formation. Because these two events are closely related, it is difficult to distinguish whether the activation of the actin-activated ATPase activity by the dual phosphorylation is due to the change in the myosin conformation or the thick filament formation. Previously, it was observed for smooth muscle myosin that the dual phosphorylation affected the actin-activated ATPase activity of HMM (Ikebe et al., 1988). Therefore, it is likely that the activation of ATPase activity is due to the change in the myosin conformation which in turn affects the thick filament formation. However, the direct effect of thick filament formation by dual phosphorylation on the actin-activated ATPase activity cannot be ruled out. Enhancement of the thick filament formation by dual phosphorylation was also found previously with smooth muscle myosin (Ikebe et al., 1988); however, this mechanism is probably more important in the platelet system, since the thick filaments of platelet cells are not as stable as smooth muscle cells. Whether or not the dual phosphorylation regulates the thick filament formation in vivo requires further study since other factors such as putative myosin binding proteins and the in vivo myosin concentration could also affect thick filament formation.

Although the present study shows that platelet myosin can be phosphorylated by MLC kinase at two distinct sites in vitro, doubly phosphorylated 20000-dalton light chain has also been detected during the contraction of the intact smooth muscle strip (Barany et al., 1985). However, whether or not the phosphorylation at the second site occurs in intact platelets is not known, and the physiological significance of this phosphorylation remains to be clarified. Daniel et al. (1984) have reported that the ADP-induced platelet shape change is paralleled with the extent of phosphorylation of the myosin light chain. It was estimated by using an alkali urea gel that the singly phosphorylated light chain was produced during the platelet shape change. The production of the doubly phosphorylated band was not mentioned; however, the gel did not show enough resolution of the putative doubly phosphorylated band and the 17000-dalton light chain. Therefore, it is unclear whether doubly phosphorylated myosin contributes to the platelet shape change or whether the single-site phosphorylation is sufficient to bring this shape change about.

The possibility of second-site phosphorylation in vivo would also be closely related to the concentration of MLC kinase in platelets. The concentration of MLC kinase in smooth muscle has been estimated as 1–4 μM (Adelstein & Klee, 1981; Ngai et al., 1984; Walsh et al., 1983), and although MLC kinase concentration in platelets is thought to be lower than that of smooth muscle cells, the MLC kinase concentration used in the present study (0.3 μM) might be available in vivo. Furthermore, one should consider the possibility that the local MLC concentration which is available for 20000-dalton myosin light chain is probably higher than the overall concentration since it has been reported that MLC kinase binds both F-actin and myosin (Sellers & Pato, 1984).

Recently, Haeberle et al. (1988) reported that the phosphorylation at threonine-18 of the myosin light chain did not affect either shortening velocity or force development of glycerinated vascular smooth muscle. The discrepancy between the results obtained in solution and in the fiber remains to be clarified.

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