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Phosphorylation of Bovine Platelet Myosin by Protein Kinase C†

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ABSTRACT: Bovine platelet myosin is phosphorylated by protein kinase C at multiple sites. Most of the phosphate is incorporated in the 20 000-dalton light chain although some phosphate is incorporated in the heavy chain. Phosphorylation of the 20 000-dalton light chain of platelet myosin is 10 times faster than the phosphorylation of smooth muscle myosin. Platelet myosin light chain is first phosphorylated at a threonine residue followed by a serine residue. Dominant phosphorylation sites of the 20 000-dalton light chain are estimated as serine-1, serine-2, and threonine-9. Prolonged phosphorylation by protein kinase C resulted in an additional phosphorylation site which, on the basis of limited proteolysis, appears to be either serine-19 or threonine-18. Phosphorylation by protein kinase C causes an inhibition of actin-activated ATPase activity of platelet myosin prephosphorylated by myosin light chain kinase. Inhibition of ATPase activity is due to a decreased affinity of myosin for actin, and no change in V_{max} is observed. It is shown that platelet myosin also exhibits the 6S to 10S conformation transition as judged by viscosity and gel filtration methods. Mg^{2+}-ATPase activity of platelet myosin is paralleled with the 10S–6S transition. Phosphorylation by protein kinase C affects neither the 10S–6S transition nor the myosin filament formation. Therefore, the inhibition of actin-activated ATPase activity of platelet myosin is not due to the change in the myosin conformation.

The contractile proteins actin and myosin have been found in many eukaryotic cells and are thought to have important roles in cell function. Blood platelets provide a good model system to study the function of nonmuscle actomyosin, since contractile proteins are the dominant proteins of the platelet cytoplasm (Pollard et al., 1977; Korn, 1978).

In platelets as well as in smooth muscle, skeletal muscle, cardiac muscle, and several nonmuscle cells, it has been shown that the light chain of myosin is phosphorylated (Adelstein et al., 1977; Daniel et al., 1981). The significance of this phosphorylation is best understood in the case of smooth muscle myosin which is phosphorylated by a calcium–calmodulin-dependent protein kinase, myosin light chain (MLC)[1] kinase (Hartshorne, 1987). Phosphorylation of the 20 000-Da light chain of myosin has been shown to be a prerequisite for actin-activated ATPase activity of smooth muscle myosin (Hartshorne, 1987).

Platelets also contain a protein kinase that phosphorylates the 20 000-dalton light chain of platelet myosin (Hathaway et al., 1979), and this activates the actomyosin ATPase activity (Adelstein & Conti, 1975). Thrombin induces several platelet responses including shape change, aggregation, secretion, and clot retraction, and it is thought that phosphorylation of the 20 000 dalton light chain of myosin is associated with the activation of platelets. The protein kinase catalyzing this phosphorylation has been identified as myosin light chain kinase (Adelstein et al., 1977; Daniel et al., 1981).

It is known that tumor-promoting phorbol esters can also cause platelet aggregation and phosphorylation of the 20 000-dalton peptide of intact platelets (Chiang et al., 1981; Caroll et al., 1982). Naka et al. (1983) reported that 12-*O*-tetradecanoylphorbol 13-acetate (TPA) induced the phosphorylation of the 20 000-dalton light chain of myosin during the activation of platelets. They also found that the phosphorylation induced by TPA is mediated mainly by protein kinase C but not by myosin light chain kinase.

Recently, it has been shown that protein kinase C phosphorylates the smooth muscle myosin at a single site on a threonine residue (Nishikawa et al., 1984). Subsequently, it was found that three sites can be phosphorylated, and these sites were determined to be threonine-9, serine-1, and serine-2 of the 20 000-dalton light chain (Ikebe et al., 1987a; Bengur et al., 1987). Phosphorylation alone does not affect the enzymatic properties of HMM, although phosphorylation of

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[1] Abbreviations: MLC, myosin light chain; HMM, heavy meromyosin; HPLC, high-performance liquid chromatography; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMA, phorbol 12-myristate 13-acetate; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

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HMM by both protein kinase C and MLC kinase causes a reduction in actin-activated ATPase activity of HMM compared to the activity obtained following phosphorylation by only MLC kinase (Nishikawa et al., 1984; Ikebe et al., 1987a). The inhibition was due to an increase in the K_m for actin. However, the effects of phosphorylation by protein kinase C on intact myosin function were not studied.

In this paper, we studied the phosphorylation of intact platelet myosin by protein kinase C. It was found that protein kinase C phosphorylates platelet myosin much faster than smooth muscle myosin and that more than two sites of the 20 000-dalton light chain were phosphorylated. The results also suggested that protein kinase C can phosphorylate the same site as myosin light chain kinase as well as the heavy chain of platelet myosin. The effects of phosphorylation of platelet myosin by protein kinase C on myosin function have also been reported.

Materials and Methods

Platelets were prepared from fresh bovine blood as follows. Whole blood was centrifuged at 500*g* for 10 min. This 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_2PO_4, 0.1% glucose, and 1 mM EDTA) and then centrifuged at 1500*g* for 10 min. The washing step was repeated 3 times. The precipitate was suspended with the washing buffer, loaded on Ficoll Parque (Pharmacia), and centrifuged at 1500*g* for 30 min. The platelets formed a layer on the top of Ficoll Parque. Platelets were washed 2 more times with washing buffer. 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/L ovamucoid trypsin inhibitor) and sonicated for 1–2 min. Three volumes of buffer A were added, and the suspension was gently stirred for 30 min on ice. After the suspension was centrifuged at 50000*g* 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 precipitates were collected by centrifugation at 10000*g* for 15 min and dissolved in buffer C (0.15 M $MgCl_2$, 1 mM EGTA, 5 mM ATP, 2 mM DTT, and 50 mM Tris-HCl, pH 7.5). This suspension was centrifuged at 100000*g* for 3 h, and the supernatant was subjected to Sepharose 4B column chromatography equilibrated with buffer D (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 (Driska et al., 1975). MLC kinase was purified from turkey gizzard as described previously (Ikebe et al., 1987b). Calmodulin was prepared from frozen bull testes as described by Walsh et al. (1983). Protein kinase C was prepared from bovine brain as follows. The brain (200 g) was homogenized with 2 mM EDTA, 10 mM EGTA, 5 mM DTT, 0.25 M sucrose, 0.001% leupeptin, and 20 mM Tris-HCl, pH 7.5, using a Teflon glass homogenizer. The homogenate was centrifuged for 60 min at 100000*g*. Solid $(NH_4)_2SO_4$ was added to the supernatant (480 g/L), and the precipitate was dissolved and dialyzed against 1 mM EDTA, 0.5 mM EGTA, 2 mM DTT, and 20 mM Tris-HCl, pH 7.5. After the dialyzed sample was clarified by centrifugation at 100000*g* for 1 h, 3 mM $CaCl_2$ and 3 mM $MgCl_2$ were added to the sample. The sample was then applied to a phenyl-Sepharose CL 4B column equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 0.5 mM $CaCl_2$, and 2 mM DTT). The column was washed with buffer A, washed with buffer A + 1 M NaCl, and then eluted with buffer B (1 mM EGTA, 2 mM DTT, and 20 mM Tris-HCl, pH 7.5). The kinase activity of the fractions was measured as described in Kikkawa et al. (1986), and the protein kinase C fraction was dialyzed against buffer B and applied to a DEAE-Sephacel column equilibrated with buffer B. Kinase C was eluted with a linear NaCl gradient of 0–0.3 M. The protein kinase C fraction was dialyzed against buffer B and applied to a threonine–Sepharose 4 B column equilibrated with the same buffer. The protein was eluted with a linear NaCl gradient of 0–1.0 M. The protein kinase C fraction was then adjusted to 1.0 M NaCl and applied to a phenyl-Sepharose column equilibrated with buffer B containing 1.0 M NaCl. The kinase was eluted with a linear gradient from 1.0 to 0 M. The final preparation of the enzyme was almost pure as judged by SDS gel electrophoresis.

Trypsin [type XIII, treated with L-1-(tosylamino)-2-phenylethyl chloromethyl ketone], soybean trypsin inhibitor (type II-S), α-chymotrypsin (type VII treated with 1-chloro-3-tosylamido-7-amino-2-heptanone), papain, phosphatidylserine (in chloroform/methanol), and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma. Lysine endopeptidase was obtained from Boehringer Mannheim Biochemicals. [γ-^{32}P]ATP was obtained from Amersham.

To determine the amino acid sequence of the digested fragments of the 20 000-dalton light chain of platelet myosin, the digested platelet myosin was subjected to SDS gel electrophoresis and then transblotted to Immobilon as described by Matsudaira (1987). Immobilon membrane was preactivated with methanol for 10 s followed by wash with the transfer buffer [10 mM CAPS (3-(cyclohexylamino)propane sulfonic acid), pH 10.7, and 0.5 mM DTT]. The SDS–polyacrylamide gel was washed with the transfer buffer for 10 min before electrophoretic transfer. The transfer was conducted for 4 h at 70 V in a cold room with the ice-cold transfer buffer. After the transfer of the proteins, the Immobilon membrane was stained with 0.6% Coomassie Brilliant Blue R-250. The stained protein band was cut out and directly applied to an automated amino acid sequencer (Applied Biosystem 477 A protein sequencer).

Isolation of peptides containing the protein kinase C sites of phosphorylation was as described previously (Ikebe et al., 1987a). Platelet 20 000-dalton light chain was isolated as described previously (Ikebe et al., 1988). The isolated 20 000-dalton light chain (0.2 mg/mL) was phosphorylated with 5 μg/mL protein kinase C in 1 mM $MgCl_2$, 0.2 mM [γ-^{32}P]ATP, 30 mM KCl, 30 mM Tris-HCl (pH 7.5), 1 mM EGTA, 100 ng/mL PMA, and 0.15 mg/mL phosphatidylserine for 60 min at 25 °C. The extent of phosphorylation was 2.1 mol of P/mol of light chain. The phosphorylated light chain was digested by trypsin, and the produced peptides were separated by using a series of gel filtration and C-18 reverse-phase columns. By use of a linear gradient of CH_3CN from 0 to 65%, the major radioactive peak was eluted at approximately 2% CH_3CN. The radioactive fraction was lyophilized and reapplied to a C18 column. The column was developed with a linear gradient, 0.1% trifluoroacetic acid–2% CH_3CN/0.1% trifluoroacetic acid, and three radioactive fractions were obtained (P-1 to P-3). The amino acid sequence of each peptide was determined by using an automated amino acid sequencer. Amino acid analyses were carried out as described by Markham and Satishchandron (1988).

The identification of phosphorylated amino acids was as described previously (Ikebe & Hartshorne, 1985a). For the mapping of the tryptic peptide, platelet myosin was phosphorylated by protein kinase C, and the reaction was stopped by 5% TCA at various times. The precipitate was washed several times with 5% TCA and then dissolved in 8 M urea, and the pH was adjusted to 7.5 with Tris base. After the urea concentration was reduced to 2 M, 1/100 (w/w) trypsin was added to start the proteolysis at 37 °C. After 4 h, another 1/100 (w/w) trypsin was added and the sample hydrolyzed for another 16 h. The reaction was stopped by soybean trypsin inhibitor (1.5 weight excess) and subjected to thin-layer electrophoresis.

Viscosity was measured at 25 °C in Cannon–Ubbelohde viscometer with a water flow time of approximately 54 s. The viscosity data are expressed as η_{rel} (viscosity of protein solution/viscosity of solvent).

TSK 4000 SW gel filtration chromatography of myosin was carried out as follows. Platelet and gizzard myosins were dialyzed against 1 mM $MgCl_2$, 0.05 mM ATP, 10 mM HEPES (pH 7.0), and either 0.15 M KCl or 0.5 M KCl. Two hundred micrograms of myosin was applied to the column which had been equilibrated with buffer containing 1 mM $MgCl_2$, 10 μM ATP, 10 mM HEPES (pH 7.0), and 0.15 M KCl or 0.5 M KCl. The flow rate was 0.8 mL/min. The bed volume and the void volume were 16.60 and 7.25 min, respectively. The extent of filament formation of myosin was estimated as follows. Myosin in various concentrations of KCl, 30 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, and 1 mM ATP was centrifuged at 100000g (Beckman airfuge) for 20 min. K^+-EDTA–ATPase activities of supernatant and precentrifuged solutions were measured to estimate the percentage of filament formation. 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 the supernatant followed by densitometric scanning of myosin heavy chain.

SDS gel electrophoresis was carried out in 12.5–25% polyacrylamide gradient slab gels using the discontinuous buffer system of Laemmli (1970). Gels were stained in 0.06% Coomassie Brilliant Blue R-250 (Sigma). Molecular weights were estimated by using the following standards: skeletal muscle myosin heavy chain (205 000), β-galactosidase (116 000), phosphorylase b (97 400), bovine serum albumin (66 000), ovalbumin (45 000), carbonic anhydrase (29 000), soybean trypsin inhibitor (20 100), and lysozyme (14 300).

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).

Results

The phosphorylation of platelet and gizzard myosins is shown in Figure 1. It is suggested from the time course that phosphorylation at one site of platelet myosin occurs relatively rapidly, followed by a slower phosphorylation at a different site. The autoradiogram of SDS gel electrophoresis of platelet myosin revealed that over 95% of phosphate was incorporated into the 20 000-dalton light chain. It was also found that the heavy chain was slightly phosphorylated and the extent of heavy chain phosphorylation was approximately 0.1 mol/mol of heavy chain at 2.5 h of reaction (data not shown).[2] During a prolonged reaction, the phosphorylation levels exceeded 2 mol of P/mol of light chain and reached 2.6 mol of P/mol of light chain at 120 min. It was shown by phosphorylated amino acid analysis that phosphothreonine is first formed, followed by phosphoserine (Figure 1). These results are similar to those

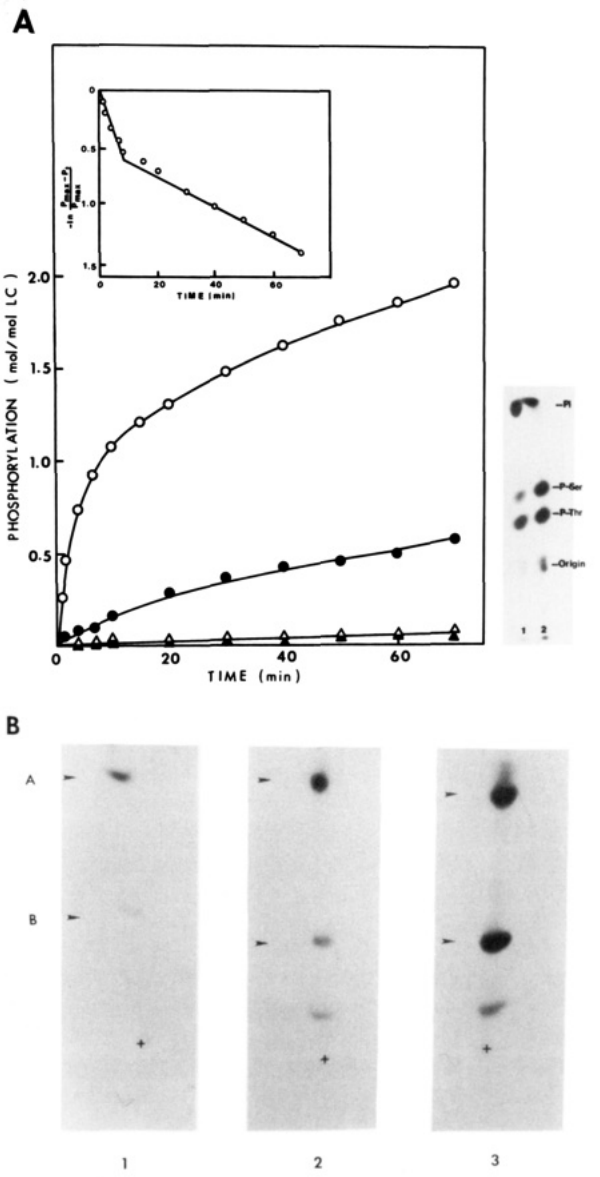

FIGURE 1: Time course of the phosphorylation of platelet and gizzard myosin by protein kinase C. (A) Reaction was started by the addition of 0.5 mM [γ-^{32}P]ATP to 0.24 mg/mL platelet myosin (○, △) or gizzard myosin (●, ▲) in 1 mM EGTA, 1 mM $MgCl_2$, 45 mM KCl, 2 μg/mL protein kinase C, and 30 mM Tris-HCl, pH 7.5, at 25 °C in the presence of 150 μg/mL phosphatidylserine and 100 ng/mL PMA (○, ●) or in their absence (△, ▲). The inset shows a semilogarithmic plot of time course data (○). P_{max} (2.55 mol/mol of LC) was experimentally determined as the extent of phosphorylation at 2 h. The autoradiogram of the phosphorylated amino acids is shown on the right. P-Ser, phosphoserine; P-Thr, phosphothreonine. At different times (6 and 60 min), the reaction was stopped by the addition of 5% trichloroacetic acid; then the mixture was centrifuged at 3000g for 5 min, and the precipitate was washed several times with 6 N HCl. The sample was hydrolyzed in 6 N HCl for 2 h at 110 °C and dried in vacuum. The hydrolysate was suspended in distilled water, and aliquots were applied to a silica gel plate and subjected to electrophoresis at 1000 V for 2 h in acetic acid/formic acid/H_2O (78/25/897). The ^{32}P-labeled amino acids were detected by autoradiography. (B) Electrophoresis of tryptic peptides of platelet myosin. The tryptic peptides of phosphorylated myosin were prepared as described under Materials and Methods. The electrophoresis was carried out according to Elder et al. (1977). Arrows: (A) phosphothreonine-containing peptide; (B) phosphoserine-containing peptide; (+) origin. Time of phosphorylation is 1.5 min (1), 6 min (2), and 60 min (3).

[2] These illustrations were submitted to the scrutiny of the reviewers and will be furnished to the interested reader by writing directly to the authors.

Table I: Amino Acid Sequence of the 20-kDa Light Chain of Platelet Myosin and Phosphopeptides of the 20-kDa Light Chain

	protease used	amino acid sequence
Gizzard LC_{20}	---	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 LC_{20}	lysine endopeptidase	A-K-T-T-K-K-R-P-Q-R-A
	Trypsin	A-T-S-N-V-F-A-M-F-D-Q-S
	α-chymotrypsin	A-M-F-D-Q-S-Q-I-Q-E-F-*-*-A
	papain	K-K-R-P-Q-R-A-T-S-N
P-1		A-K-A-K-T-T-K
P-2		Ac-S-S-K-R-A-K
P-3		Ac-S-S-K-R

obtained with smooth muscle HMM (Ikebe et al., 1987a). To confirm that the first phosphorylation site was the threonine-containing peptide, phosphorylated myosin was digested by trypsin, and the phosphorylated peptides were mapped by electrophoresis (Figure 1B). As shown in Figure 1B, the phosphorylation of the peptide which showed higher mobility appeared much faster than the phosphorylation of the other peptide. It was shown previously (Bengur et al., 1987) that the former peptide was the threonine-containing peptide. It can be estimated from the inset of Figure 1 that the rate of phosphorylation of threonine was approximately 5 times faster than the rate for the second site. This figure also shows that the rate of phosphorylation of platelet myosin was approximately 10 times faster than the rate of phosphorylation of gizzard myosin.

To investigate the location of the phosphorylation sites, platelet and gizzard myosins were phosphorylated by protein kinase C, and then the phosphorylated myosin was proteolyzed by trypsin, papain, and α-chymotrypsin. For both gizzard and platelet myosins, the 20000-dalton light chains were degraded to a 17000-dalton fragment by trypsin proteolysis, an 18000-dalton fragment and then a 17000-dalton fragment by papain proteolysis, and a 16000-dalton fragment by α-chymotrypsin proteolysis (data not shown).[2] The sites for the proteolysis were determined by analyzing the N-terminal sequence of these fragments of platelet myosin light chain, and the results were shown in Table I. The N-termini of the 17000-dalton tryptic fragment, the 18000-dalton papain fragment, and the 16000-dalton α-chymotryptic fragment were estimated as alanine-17, lysine-11, and alanine-23 by comparing with the known sequence of gizzard 20000-dalton light chain. The 20000-dalton light chain of platelet myosin was also proteolyzed by lysine endopeptidase to an 18000-dalton fragment which was subjected to sequence analysis (Table I). The sequences of these fragments of platelet light chain completely coincide with the known sequence of gizzard 20000-dalton light chain (Jakes et al., 1976; Pearson et al., 1984).

The phosphorylation site of gizzard myosin phosphorylated by protein kinase C was removed by tryptic, papain, and α-chymotryptic cleavage (data not shown).[2] This result is consistent with a previous report which showed that the phosphorylation sites of gizzard myosin by protein kinase C were threonine-9, serine-1, and serine-2 (Ikebe et al., 1987a; Bengur et al., 1987). Although some of the phosphorylation sites of platelet myosin phosphorylated by protein kinase C were still retained with the 17000-dalton trypsin fragment and 18000-dalton papain fragment, most phosphorylation sites were removed by trypsin proteolysis of the 20000-dalton light chain. Therefore, these phosphorylation sites are likely to be the N-terminus side of alanine-17. The major phosphorylation sites were identified by isolating the phosphorylated tryptic peptides of the 20-kDa platelet myosin light chain. Amino acid analyses of the phosphorylated peptides P-1, P-2, and P-3 are shown in Table II. Sequence analysis of P-1 gave the sequence Ala–Lys–Ala–Lys–X–Thr–Lys. Thus, it is concluded that threonine-9 is the initial phosphorylation site by protein kinase C. Sequence analysis of P-2 and P-3 gave no sequence which suggests that the N-terminus of P-2 and P-3 is blocked. Since the amino acid sequence of the 20-kDa light chain of platelet myosin was identical with the gizzard 20-kDa light chain between Ala-7 and Phe-33, the N-terminus sequences of P-2 and P-3 were deduced from the amino acid analyses and the N-terminus sequence of gizzard 20-kDa light chain. As a result, it was concluded that serine-1 and serine-2 are the second phosphorylation sites.

Table II: Amino Acid Compositions of Phosphorylated Tryptic Peptides

	P-1		P-2		P-3	
amino acid	obsd	expected	obsd	expected	obsd	expected
Lys	2.8	3	1.8	2	1.1	1
Arg			1.0	1	1.0	1
Thr[a]	1.3	2				
Ser[a]			1.5	2	1.6	2
Ala	2.1	2	1.0	1		

[a] Values for threonine and serine are expected to be low due to phosphorylation.

Although most phosphorylation was found to be on the N-terminus side of alanine-17, some phosphorylation sites remained in the tryptic 17000-dalton fragment with prolonged phosphorylation. When the extent of phosphorylation was 1.2 mol of P/mol of light chain, all of the phosphorylation sites were removed during the proteolysis of the 20000-dalton light chain to the 17000-dalton fragment while on more prolonged incubation, some phosphorylation sites were found in the 17000-dalton light chain fragment (data not shown).[2] This phosphorylation was dependent on phospholipid/phorbol ester; therefore, it is likely that this phosphorylation is catalyzed by protein kinase C. Since this site was removed by prolonged digestion by α-chymotrypsin, which cleaves between phenylalanine-22 and alanine-23 (Table I), it is reasonable to assume that this phosphorylation site by protein kinase C is either serine-19 or threonine-18. This assumption is supported by previous studies which demonstrated the sites phosphorylated by myosin light chain kinase (serine-19 and threonine-18) were also removed by a prolonged digestion by α-chymotrypsin

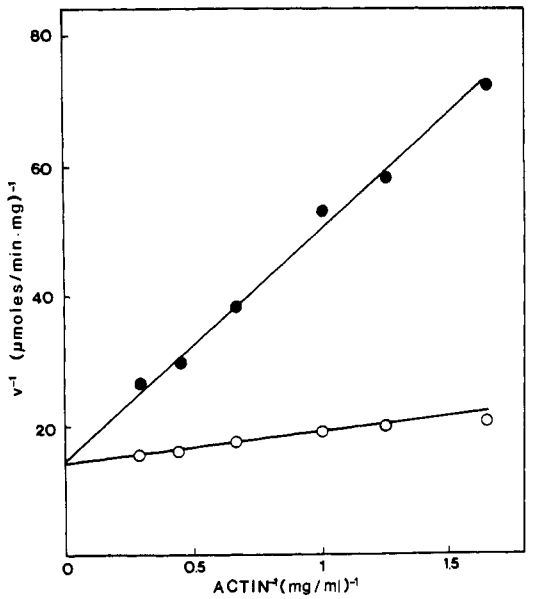

FIGURE 2: Effect of the phosphorylation by protein kinase C on the actin-activated ATPase activity of platelet myosin. Platelet myosin (1.0 mg/mL) was phosphorylated by MLC kinase (2 μg/mL) in 30 mM Tris-HCl (pH 7.5), 0.3 M KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mM ATP, and 2 μg/mL calmodulin for 15 min at 25 °C. Reaction was stopped by addition of EGTA to a final concentration of 1 mM, and the solution was applied to a TSK-SW 4000 column attached to a Perkin-Elmer HPLC system. Myosin was collected, concentrated, and dialyzed against 50 mM KCl, 1 mM DTT, and 30 mM Tris-HCl (pH 7.5). The level of phosphorylation was checked by urea gel electrophoresis, and the level was approximately 2 mol of P/mol of myosin. The phosphorylated myosin was incubated with protein kinase C with conditions as in Figure 1 with PMA, [γ-^{32}P]ATP, and phosphatidylserine. Myosin was then dialyzed against 10 mM $MgCl_2$, 1 mM DTT, and 30 mM Tris-HCl (pH 7.5). The precipitate was collected by centrifugation (10000g for 5 min), washed, and dissolved in 0.3 M KCl, 2 mM DTT, and 30 mM Tris-HCl (pH 7.5). The extent of phosphorylation by protein kinase C was 1.9 mol of P/mol of myosin. ATPase activity was assayed with 0.24 mg/mL myosin, 4 mM $MgCl_2$, 55 mM KCl, 30 mM Tris-HCl (pH 7.5), and 0.5 mM [γ-^{32}P]ATP at 25 °C. (○) Myosin phosphorylated by MLC kinase alone; (●) myosin phosphorylated by MLC kinase and protein kinase C.

(Ikebe & Hartshorne, 1985a).

The influence of phosphorylation by protein kinase C on the actin-activated ATPase activity of platelet myosin was examined. Figure 2 shows the actin concentration dependence of ATPase activity of platelet myosin. Platelet myosin was prephosphorylated by MLC kinase (to approximately 2 mol of P/mol of myosin). For myosin phosphorylated only by MLC kinase, the K_a (the apparent dissociation constant for actin as determined by ATPase measurements) and V_{max} are 8.8 μM and 71 nmol/(min·mg), respectively. Additional phosphorylation of platelet myosin by protein kinase C (to an additional 2.3 mol of P/mol of light chain) did not alter V_{max} but markedly increased K_a to 63 μM.

It has been suggested that phosphorylation of the 20000-Da light chain by MLC kinase does not alter the ATPase activity of smooth muscle myosin directly but via a change in myosin conformation (Ikebe et al., 1983; Ikebe & Hartshorne, 1985b). Therefore, we studied whether or not the inhibition of the actin-activated ATPase activity of platelet myosin by the phosphorylation by protein kinase C involved a change in myosin conformation. Figure 3 shows the KCl concentration dependence of Mg^{2+}-ATPase activity of platelet myosin. For dephosphorylated myosin, activity decreased at KCl concentrations below 0.3 M. On the other hand, a decrease in the activity was observed at lower KCl concentrations for the myosin phosphorylated by MLC kinase. This [KCl] dependence of Mg^{2+}-ATPase activity is similar to that of smooth muscle myosin (Ikebe et al., 1983). It was found previously (Ikebe et al., 1983) that the decrease in the ATPase activity of smooth muscle myosin is paralleled with the change in the myosin conformation from 6 S to 10 S. Therefore, we monitored the change in the conformation of platelet myosin by viscosity measurements and gel filtration. It was shown previously that the decrease in the viscosity of myosin is due to the change in myosin conformation from an extended (6S myosin) to a folded (10S myosin) form (Ikebe et al., 1983). As shown in Figure 3, the viscosity of dephosphorylated platelet myosin decreased at KCl concentrations below 0.3 M, where ATPase activity also decreased. The effects of phosphorylation by protein kinase C on platelet myosin conformation were also examined. The [KCl] dependence of Mg^{2+}-ATPase activity of both dephosphorylated and phosphorylated platelet myosin by MLC kinase was not altered by protein kinase C phosphorylation (Figure 3). This suggests that the conformational transition of platelet myosin is not affected by protein kinase C phosphorylation. This was confirmed by viscosity measurements. As shown in Figure 3, the viscosity of neither dephosphorylated myosin nor phosphorylated myosin by MLC kinase was affected by protein kinase C phosphorylation. The change in the ATPase activity and the viscosity of platelet myosin was more gradual to the change in the KCl concentration as compared with smooth muscle myosin (Ikebe et al., 1983). For example, the ATPase activity and viscosity of dephosphorylated platelet myosin at 0.2 M KCl were considerably higher than those at 0.1 M KCl, while those of dephosphorylated smooth muscle myosin at 0.2 M KCl were almost the same as those at 0.1 M KCl. Similar results were obtained with erythrocyte myosin (Higashihara et al., 1989).

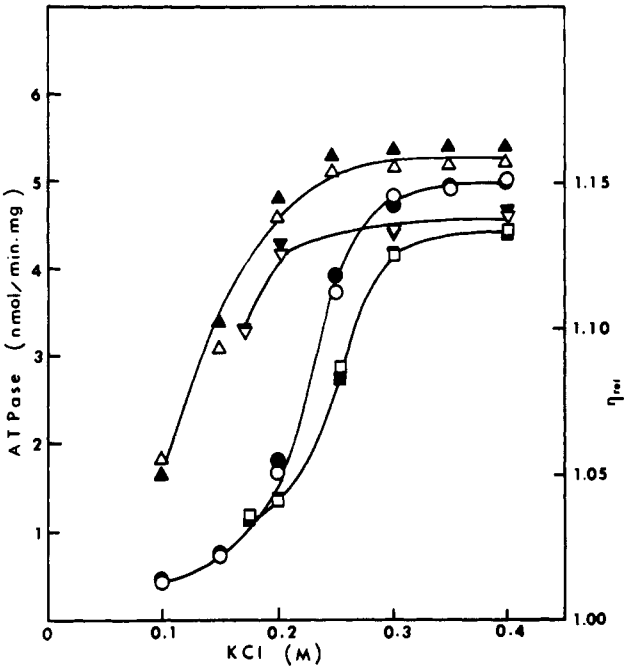

FIGURE 3: [KCl] dependence of Mg^{2+}-ATPase activity and viscosity of platelet myosin. Myosin was phosphorylated by MLC kinase and protein kinase C as described in Figure 2. ATPase activity (0.24 mg/mL myosin) and viscosity (0.5 mg/mL myosin) were assayed with 1 mM $MgCl_2$, 30 mM Tris-HCl, pH 7.5, and 0.5 mM [γ-^{32}P]ATP at 25 °C. ATPase: (○) dephosphorylated myosin; (●) myosin phosphorylated by protein kinase C; (△) myosin phosphorylated by MLC kinase; (▲) myosin phosphorylated by MLC kinase and protein kinase C. Viscosity: (□) dephosphorylated myosin; (■) myosin phosphorylated by protein kinase C; (▽) myosin phosphorylated by MLC kinase; (▼) myosin phosphorylated by MLC kinase and protein kinase C.

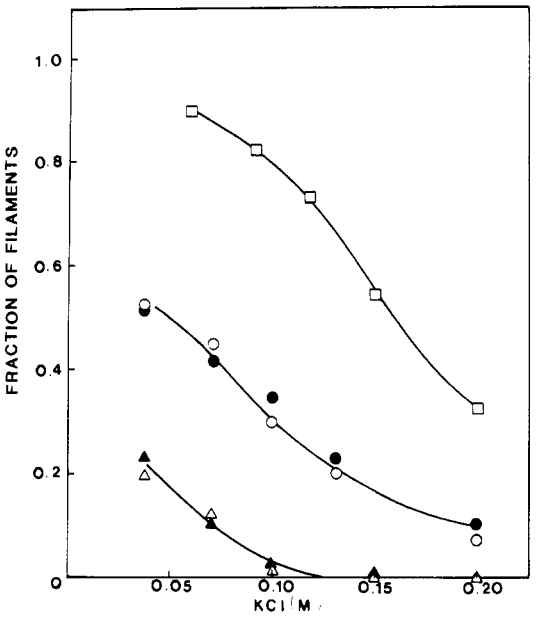

FIGURE 4: [KCl] dependence of myosin filament formation. The amount of myosin filaments was determined as described under Materials and Methods. Conditions are the same as in Figure 3 except 6 mM $MgCl_2$ and 0.24 mg/mL myosin were used. Myosin was phosphorylated by MLC kinase and protein kinase C as described in Figure 4. (○) Myosin phosphorylated by MLC kinase; (●) myosin phosphorylated by MLC kinase and protein kinase C; (△) dephosphorylated myosin; (▲) phosphorylated myosin by protein kinase C; (□) dephosphorylated myosin minus ATP.

A change in the conformation of platelet myosin was confirmed by using TSK 4000 SW gel filtration. The elution positions of smooth muscle 6S myosin (0.5 M KCl) and 10S myosin (0.15 M KCl) were 7.56 and 9.30 min, respectively. Dephosphorylated platelet myosin eluted at 7.60 min at 0.5 M KCl and at 9.24 min at 0.15 M KCl. Therefore, it was concluded that dephosphorylated platelet myosin forms an extended (6S) conformation at 0.5 M KCl and a folded (10S) conformation at 0.15 M KCl.

The effect of phosphorylation of platelet myosin by protein kinase C on myosin filament formation is shown in Figure 4. Higher concentrations of $MgCl_2$ (6 mM) were used to promote myosin filaments. Platelet myosin formed filaments at KCl concentrations below 0.2 M, but most filaments of dephosphorylated myosin were dissolved by the addition of ATP. This is confirmation of the results by Takeuchi and Ishimura (1985) and is in agreement with studies using thymus, brush border (Kendrick-Jones et al., 1987), and erythrocyte myosins (Higashihara et al., 1989). Phosphorylation by MLC kinase induced platelet myosin filament formation, although considerable amounts of platelet myosin were still not in the filamentous form (Figure 4). This stimulation of filament formation by MLC kinase phosphorylation is also similar to other nonmuscle cell myosins such as thymus, brush border (Kendrick-Jones et al., 1987), and erythrocyte (Higashihara et al., 1989) myosins. On the other hand, phosphorylation of platelet myosin by protein kinase C did not affect filament formation (Figure 4). Myosin phosphorylated by MLC kinase was further phosphorylated by protein kinase C, and the extent of filament formation was compared with filament formation of myosin phosphorylated by MLC kinase alone. No significant change in filament formation was observed (Figure 4).

Discussion

This study shows that multiple sites on platelet myosin are phosphorylated by protein kinase C. Naka et al. reported recently (Naka et al., 1988) that doubly phosphorylated MLC was detected in thrombin-stimulated human platelets. It was suggested that two different sites were phosphorylated in doubly phosphorylated MLC and one of these sites was phosphorylated by MLC kinase and the other site was phosphorylated by protein kinase C by the analysis of two-dimensional peptide mapping. Although most of the phosphate is incorporated in the 20 000-dalton light chain, some phosphorylation of heavy chain is also observed with a prolonged incubation with protein kinase C. Phosphorylation of myosin heavy chain in intact platelets was recently reported, and it was suggested that the phosphorylation of the heavy chain was catalyzed by protein kinase C (Kawamoto et al., 1988). Phosphorylation of myosin heavy chain was also demonstrated in some vertebrate and invertebrate nonmuscle cell myosins (Kuznicki, 1986). In *Acanthamoeba* and *Dictyostelium* cells, it was shown that phosphorylation of myosin heavy chain influences the self-assembly of myosin and the actin–myosin interaction (Collins et al., 1982; Kuczmarski & Spudich, 1980; Rovner et al., 1986). In this study, the extent of heavy-chain phosphorylation is small, so the affect of platelet heavy-chain phosphorylation on the self-assembly of myosin and the actin–myosin interaction was not studied.

Several sites on the 20 000-dalton light chain of platelet myosin are phosphorylated by protein kinase C. Threonine is phosphorylated first followed by serine phosphorylation (Figure 1). This is similar to the phosphorylation of smooth muscle HMM by protein kinase C (Ikebe et al., 1987a). Most of the phosphate incorporated in the 20 000-dalton light chain of platelet myosin could be removed by trypsin, papain, and α-chymotrypsin proteolysis. Isolation of the phosphopeptides suggested the major phosphorylation sites by protein kinase C are threonine-9, serine-1, and serine-2. These sites are identical with the phosphorylation sites of smooth muscle HMM (Ikebe et al., 1987a; Bengur et al., 1987).

Although most phosphate is removed from the 20 000-dalton light chain by limited tryptic proteolysis, some phosphate remains with the proteolyzed 17 000-dalton light-chain fragment of platelet myosin. Since this phosphorylation site is removed by prolonged α-chymotryptic proteolysis, it can be concluded that protein kinase C also phosphorylates either serine-19 or threonine-18. As the rate of phosphorylation at this site is much slower than the phosphorylation of the first threonine site and also is much slower than the phosphorylation by MLC kinase at serine-19, phosphorylation at this site by protein kinase C may not have physiological relevance in the platelet cell. However, it cannot be ruled out that phosphorylation at this site by protein kinase C may play a role in other cell systems where MLC kinase is not expressed.

The rate of phosphorylation is about 10 times faster for platelet myosin than for gizzard myosin (Figure 1). Since the amino acid sequence of the 20 000-dalton light chain of platelet myosin is identical from alanine-7 to phenylalanine-33, the marked difference in the phosphorylation rate may be due to the difference in the heavy-chain sequence of the heavy–light chain interface. The fast rate of phosphorylation of platelet myosin by protein kinase C suggests that this phosphorylation is relevant in physiological conditions. This is consistent with a previous report which showed that platelet myosin was readily phosphorylated in vivo after the addition of phorbol ester (Naka et al., 1983).

Platelet myosin can form 6S and 10S conformations (Figure 3) similar to smooth muscle myosin. Since other nonmuscle cell myosins from thymus, brush border (Kendrick-Jones et al., 1987), and erythrocytes (Higashihara et al., 1989) are reported to form 6S and 10S conformations, this is not

unexpected. A more interesting observation is the correlation of the enzymatic activity with the conformation of platelet myosin. This has been demonstrated before with smooth muscle myosin (Ikebe et al., 1983) and recently with erythrocyte myosin (Higashihara et al., 1989). We have developed the hypothesis that conformational changes within the 10S–6S transition form part of the in vivo regulatory mechanism of smooth muscle, and it is important to determine if this hypothesis can be extended to nonmuscle systems. Our present results as well as the results of erythrocyte myosin suggest that the "shape–activity" hypothesis might be a general mechanism applicable to those systems which are regulated by the phosphorylation of myosin light chain.

Phosphorylation of platelet myosin by protein kinase C inhibits the actin-activated ATPase activity of platelet myosin prephosphorylated by MLC kinase. Previously it was reported that actin-activated ATPase activity of smooth muscle HMM was inhibited by protein kinase C phosphorylation (Nishikawa et al., 1984; Ikebe et al., 1987a). However, the effect of this phosphorylation on the function of intact myosin was not reported. The present study is the first to show the inhibition of actin-activated ATPase of intact myosin by protein kinase C phosphorylation.

An obvious concern is whether or not the inhibition of actin-activated ATPase activity by protein kinase C phosphorylation is related to the change in myosin conformation in terms of 10S and 6S conformation. We investigated this problem by measuring the viscosity of platelet myosin, but observed no effect of protein kinase C mediated phosphorylation on myosin conformation. It was reported previously (Ikebe et al., 1983) that a decrease in the Mg^{2+}-ATPase activity of smooth muscle myosin is paralleled with the change in myosin conformation from 6 S to 10 S. Mg^{2+}-ATPase activity of platelet myosin also parallels the change in viscosity, but the Mg^{2+}-ATPase activity is not influenced by protein kinase C phosphorylation. These results indicate that the conformational change of myosin is not involved in the inhibition of actin-activated myosin ATPase activity by protein kinase C phosphorylation.

Myosin filament formation also is not influenced by protein kinase C phosphorylation (Figure 4). Since 6S myosin tends to form filaments while 10S myosin does not, the lack of an effect on thick filament formation by protein kinase C phosphorylation is consistent with the results of the viscosity and Mg^{2+}-ATPase activity of myosin. In the case of phosphorylation by MLC kinase, the V_{max} of actomyosin ATPase is markedly increased. On the other hand, additional phosphorylation by protein kinase C alters the K_a but not the V_{max} (Figure 2). A change in the 10S–6S transition might be accompanied with a change in the V_{max} of actomyosin ATPase activity. Umekawa et al. (1985) reported that the protein kinase C phosphorylation of smooth muscle myosin phosphorylated by MLC kinase shifts the myosin conformation from 6 S to 10 S. However, we could not detect any change in the conformation of platelet myosin by additional phosphorylation by protein kinase C to the MLC kinase phosphorylated myosin. This discrepancy could be due to the difference between smooth muscle myosin and platelet myosin, but it is still obscure. During the preparation of this paper, Kawamoto et al. (1989) suggested that serine-1, serine-2, and threonine-9 of human platelet myosin light chain can be phosphorylated by protein kinase C by comparing two-dimensional mapping of tryptic peptides of platelet myosin light chain with gizzard myosin light chain. According to them, the serine phosphorylation site was more preferable in vivo, and the myosin heavy chain was readily phosphorylated in phorbol ester treated platelets. We changed several parameters ($[MgCl_2]$, ionic strength, $[CaCl_2]$) for the phosphorylation of platelet myosin by protein kinase C; however, the relative rate of phosphorylation of different sites was not changed. We also checked the effect of actin on phosphorylation; however, no significant effect was observed. The putative myosin binding protein may alter the environment of the phosphorylation site in vivo; however, the apparent discrepancy between in vitro and in vivo phosphorylation is still obscure.

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Registry No. Ser, 56-45-1; Thr, 72-19-5; ATPase, 9000-83-3; protein kinase C, 9026-43-1.

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Reexamination of the Role of Nonhydrolyzable Guanosine 5′-Triphosphate Analogues in Tubulin Polymerization: Reaction Conditions Are a Critical Factor for Effective Interactions at the Exchangeable Nucleotide Site

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ABSTRACT: Recently it was proposed [O'Brien, E. T., & Erickson, H. P. (1989) *Biochemistry 28*, 1413-1422] that tubulin polymerization supported by guanosine 5′-(β,γ-imidotriphosphate) [p(NH)ppG], guanosine 5′-(β,γ-methylenetriphosphate) [p(CH_2)ppG], and ATP might be due to residual GTP in reaction mixtures and that these nucleotides would probably support only one cycle of assembly. Since we had observed polymerization with these three compounds, we decided to study these reactions in greater detail in two systems. The first contained purified tubulin and a high concentration of glycerol, the second tubulin and microtubule-associated proteins (MAPs). In both systems, reactions supported by nucleotides other than GTP were most vigorous at lower pH values. In the glycerol system, repeated cycles of polymerization *were* observed with ATP and p(CH_2)ppG, but not with p(NH)ppG. With p(NH)ppG, a single cycle of polymerization was observed, and this was caused by contaminating GTP. In the MAPs system, repeated cycles of polymerization were observed with both nonhydrolyzable GTP analogues, even without contaminating GTP, but ATP was not active at all in this system. Binding to tubulin of p(NH)ppG, p(CH_2)ppG, and, to a lesser extent, ATP was demonstrated indirectly, since high concentrations of the three nucleotides displaced radiolabeled GDP originally bound in the exchangeable site, with p(NH)ppG the most active of the three compounds in this displacement assay. The failure of GTP-free p(NH)ppG to support tubulin polymerization in our glycerol system even though it displaced GDP from the exchangeable site was further investigated by examining the effects of p(NH)ppG on polymerization and polymer-bound nucleotide with low concentrations of GTP. The two nucleotides appeared to act synergistically in supporting polymerization, so that a reaction occurred with a subthreshold GTP concentration if p(NH)ppG was also in the reaction mixture. Analysis of radiolabeled exchangeable-site nucleotide in polymers formed in reaction mixtures containing both GTP and p(NH)ppG demonstrated that p(NH)ppG which entered polymer did so primarily at the expense of GDP originally bound in the exchangeable site rather than at the expense of GTP. It appears that in the glycerol reaction condition, tubulin–p(NH)ppG cannot initiate tubulin polymerization but that it can participate in polymer elongation. ATP and p(CH_2)ppG also entered the exchangeable site during polymerization without GTP in glycerol, as demonstrated by displacement of radiolabeled GDP from polymer when these alternate nucleotides were used. Moreover, at pH 6.1 in the glycerol system, binding of alternate nucleotides to unpolymerized tubulin was demonstrated with radiolabeled p(NH)ppG and ATP and to polymer by high-performance liquid chromatographic analysis of acid extracts of pellets harvested by centrifugation.

Microtubule assembly normally is accompanied by the hydrolysis of stoichiometric amounts of GTP bound at the exchangeable nucleotide site of tubulin (Kobayashi, 1975; MacNeal & Purich, 1978; Hamel et al., 1986a; O'Brien et al., 1987). Nonetheless, many reports of polymerization not dependent on GTP (or GTP analogue) hydrolysis have appeared over the years. These have included reactions induced by non guanosine triphosphates (Penningroth & Kirschner, 1978; Zabrecky & Cole, 1980; Duanmu et al., 1986), by GDP

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