Effect of Phosphorylation of Myosin Light Chain by Myosin Light Chain Kinase and Protein Kinase C on Conformational Change and ATPase Activities of Human Platelet Myosin

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Human platelet myosin forms 10S and 6S conformations, and its Ca\(^{2+}\) and Mg\(^{2+}\)-ATPase activities are parallel with the transition between 10S and 6S conformation, as judged by the gel filtration, intrinsic fluorescence, and viscosity methods. The 20,000-dalton myosin light chain (LC20) is phosphorylated by both myosin light chain kinase (MLC kinase) and Ca\(^{2+}\), phospholipid-dependent protein kinase (protein kinase C [PKC]). The phosphorylation (1 mol of phosphate/mol of LC20) by MLC kinase shifts the equilibrium toward the 6S conformation, but that by PKC does not. The prephosphorylation of myosin by PKC prevents the effect of phosphorylation by MLC kinase on actin-activated Mg\(^{2+}\)-ATPase activity, but not the effect on conformational change. Inhibition of actin-activated ATPase activity by PKC is due to a decreased affinity of myosin for actin, and no change in Vmax is observed. These results suggest that sequential phosphorylation of myosin by both kinases plays an important role in the ATPase activities of human platelet myosin.

Thus, the effects of phosphorylation by both kinases should be considered in evaluating the role of myosin phosphorylation in the platelet activation.

Some nonmuscle myosins\(^{15-17}\) as well as smooth muscle myosin\(^ {14,18}\) in the monomeric state are reported to exist in two conformations, ie, the folded (10S) and extended (6S) forms. In smooth muscle myosin, these conformations are characterized by distinct enzymatic properties,\(^ {20}\) ie, 6S is active and 10S is inactive. Among the nonmuscle myosins characterized so far, only myosins from bovine erythrocytes\(^ {17}\) were shown to have correlation of enzymatic properties and conformations.

In this report, we show that human platelet myosin also shows the correlation of enzymatic properties and conformations. The effects of phosphorylation by MLC kinase and/or PKC on the myosin function are also investigated.

MATERIALS AND METHODS

Materials. Ovomucoid trypsin inhibitor, [eihylenbis(oxyethyl)eneneritrol] tetraacetic acid (EGTA), disodium ethylenediaminetetraacetate (EDTA), ATP, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), and TPA were obtained from Sigma Chemical Co (St Louis, MO). Sodium dodecyl (SDS), acrylamide, and urea were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). (γ-32P)ATP was obtained from Amersham Japan Ltd (Tokyo, Japan). Other materials are of the highest grade available.

Proteins. Platelet myosin was prepared as follows. Platelet concentrates (15 U) were washed with phosphate-buffered saline (PBS; pH 7.3) once, and resuspended in HEPES-Tyrode's buffer with 4 mmol/L EGTA, 4 mmol/L EDTA, 0.1 mg/mL trypsin inhibitor, 4 mmol/L DTT, 1 mmol/L PMSF, and 0.04% sodium azide. Platelets were resuspended with the same volume of buffer A (0.5 mol/L KCl, 0.15 mol/L potassium phosphate, pH 6.8, 2 mmol/L EGTA, 2 mmol/L EDTA, 0.2 mg/mL trypsin inhibitor, and 1 mmol/L PMSF) and sonicated for 5 minutes on ice. Two volumes of buffer A were further added, and the suspension was put in ice for 60 minutes. After the suspension was centrifuged at 48,000g for 120 minutes at 4°C, the supernatant was dialyzed against buffer B (10 mmol/L imidazole, pH 7.0, 10 mmol/L MgCl\(_2\), 2 mmol/L EGTA, 1 mmol/L DTT, and 5% sucrose). The pellet by centrifugation at 10,000g for 15 minutes was washed once with buffer B, and centrifuged again as above. The pellet was dissolved in buffer C (0.5 mol/L NaCl, 30 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EGTA, 1 mmol/L PMSF, and 1 mmol/L DTT) and subjected on Sepharose CL-4B column (3 cm × 90 cm) equilibrated with buffer C. The crude myosin fractions judged by SDS gel

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Submitted April 8, 1991; accepted August 26, 1991.

Supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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0006-4971/91/7812-0017$3.00/0

PHOSPHORYLATION OF PLATELET MYOSIN

RESULTS

Phosphorylation of platelet myosin by MLC kinase or PKC.

Purified platelet myosin consists of an approximate 200-Kd heavy chain and two light chains of 20 Kd and 17 Kd in SDS-PAGE, as reported by others. To prevent phosphorylation during preparations, we first applied low ionic strength precipitation followed by Sepharose CL-4B chromatography without Mg²⁺-ATP. Finally, rechromatography with Sepharose CL-4B with Mg²⁺-ATP was used to separate the contaminated actin. The purity was greater than 95% as judged by the densitometry of a Coomassie Brilliant Blue-stained gel (data not shown). Figure 1 shows the data on the time course of phosphorylation of purified myosin by both kinases. In our conditions, 0.9 to 1.0 mol of phosphate per mole of LC20 was maximally incorporated by MLC kinase and 1.8 to 2.1 mol of phosphate per mole of LC20 by PKC in five separate experiments. In addition, prephosphorylated myosin by MLC kinase (1 mol of phosphate/mol of LC20) was further phosphorylated with two more phosphate/mol of LC20 by PKC (Fig 1). Reversing the order of incubation with kinase (ie, phosphorylation with PKC first and then with MLC kinase) also resulted in 3 mol of phosphate/mol of LC20 (data not shown). Although the autoradiogram shows that only LC20 was phosphorylated by both kinases (inset in Fig 1), the heavy chain of myosin treated with PKC was found to be slightly phosphorylated with a prolonged incubation, ic, 2.5 hours (data not shown). Aliquots of myosin from each phosphorylation series were applied to urea gel electrophoresis (inset in Fig 1). Purified myosin was mostly dephosphorylated because the band of LC20 (LC20 · P0) was single and the mobility was the same as that of myosin treated with phosphatase (data not shown), and a single phosphorylated band (LC20 · P1) was seen with MLC kinase with 20 minutes of incubation. In the case of PKC, a more rapidly migrating doubly phosphorylated light chain (LC20 · P2) was also observed with 20 minutes of incubation, and, finally, only LC20 · P2 band was observed with 80 minutes of incubation (data not shown).

Conformational change of dephosphorylated myosin of human platelets. Conformational change was monitored by analytical ultracentrifugation. The sedimentation coefficient values at 0.4 mol/L KCl were 9.5, 9.6, and 10.0 for three myosin samples prepared separately, and 6.2, 6.0, and 6.1, respectively, at 0.1 mol/L KCl.

Viscosity. Viscosity was measured at 23°C in Cannon-Ubbelohde viscometers (Cannon Instrument Co, State College, PA) with a water flow of approximately 54 seconds. The myosin concentration was 0.5 mg/mL. The viscosity data are expressed as ηvisc (viscosity of protein solution/viscosity of solvent).

Sedimentation velocity. Sedimentation velocity measurements were performed in a Beckman Model E analytical ultracentrifuge (Beckman Instruments, Inc, Palo Alto, CA) operated at 56,000 rpm, as described by Umekawa et al. Platelet myosin was dialyzed against 1 mmol/L ATP, 1 mmol/L MgCl₂, 30 mmol/L Tris-HCl, pH 7.4, and 0.1 mol/L KCl or 0.4 mol/L KCl. Another 0.5 mmol/L ATP was added to the sample solution just before analysis. Myosin concentration was adjusted to 0.8 mg/mL. The value of sedimentation coefficient (S₂₀w = 8 x 10⁻⁹ seconds) was the mean of three experiments in each sample.

Gel filtration. The TSK 4000SW column was attached to a TOSO high performance liquid chromatography (HPLC) system (Toyo Soda Industries Ltd, Tokyo, Japan) at a flow rate of 0.7 mL/min. Platelet myosins were dialyzed against 1 mmol/L MgCl₂, 0.1 mmol/L ATP, 30 mmol/L Tris-HCl, pH 7.0, and various concentrations of KCl (0.1 to 0.5 mol/L KCl). The bed volume determined with DTT was 20.8 minutes, and the void volume determined with blue-dextran was 8.80 minutes.

Intrinsic fluorescence measurement. Fluorescence was measured at 25°C with a Shimadzu UV-2100 spectrophotometer equipped with a cell holder temperature-controlled by circulating water. Excitation and emission wavelengths were 295 nm and 335 nm, respectively.

Others. Protein concentrations were determined by the method of Bradford. SDS gel electrophoresis was performed in 12.5% polyacrylamide slab gels (SDS-PAGE). Gels were stained in 0.06% Coomassie Brilliant Blue R-250 (Sigma). Silver staining was performed as described previously.
Time (min)
Flg 1. Time course of phosphorylation of platelet myosin by MLC kinase and/or PKC. Platelet myosin (0.8 mg/ml) was phosphorylated at 25°C with either MLC kinase solution (C) or PKC solution (D), as described in Materials and Methods. Reactions were stopped either by the addition of 5% trichloroacetic acid (for phosphorylation assay) or by the addition of urea to 8 mol/L (for urea gel electrophoresis). Myosin prephosphorylated by MLC kinase for a 20-minute reaction period was further phosphorylated by adding PKC solution (final reaction solution was the same as mentioned above). The inset shows the autoradiogram of 12.5% SDS-PAGE (A) and silver staining of urea-gel electrophoresis (B). Myosin phosphorylated by MLC kinase for 20 minutes (lanes 1 and 5) or PKC for 20 minutes (lanes 2 and 4); dephosphorylated myosin (lanes 3 and 6). Four micrograms of myosin was applied to each gel lane. LC20, 20-Kd MLC; LC20 - P0, dephosphorylated LC20; LC20 - P1, single-phosphorylated LC20; LC20 - P2, double-phosphorylated LC20; LC17, 17-Kd MLC.

Correlation of enzymatic activity and conformation. Figure 2 shows the data of KCl-dependence of Ca²⁺-ATPase activities of platelet myosin. Between 0.1 and 0.2 mol/L KCl, the ATPase activity was very low. The activity showed a sharp increase between 0.2 and 0.3 mol/L KCl and reached a plateau over 0.3 mol/L KCl.

To investigate the correlation of enzymatic activity and myosin conformation, gel filtration technique was applied. According to previous reports, 6S myosin eluted faster than 10S myosin. Figure 3 shows the elution time as the function of KCl concentration. The elution time changed rapidly between 0.2 and 0.3 mol/L KCl. Taken together with the result of KCl-dependence of Ca²⁺-ATPase activity (Fig 2), this finding led us to conclude that the elution time, which represents the conformational change, is paralleled by the Ca²⁺-ATPase activity.

The effect of phosphorylation on KCl-dependence of Mg²⁺-ATPase activity. The correlation of KCl-dependence of Ca²⁺- and Mg²⁺-ATPase activities and conformation was previously shown for smooth muscle myosin. 39 Thus, KCl-dependence of Mg²⁺-ATPase activity was also investigated for platelet myosin. Furthermore, the effect of phosphorylation by MLC kinase or PKC on the Mg²⁺-ATPase activities was monitored (Fig 4). For the dephosphorylated platelet myosin, the change of Mg²⁺-ATPase activity as the function of KCl concentration showed almost the same dependency as that of Ca²⁺-ATPase activity (Fig 2). The phosphorylation by MLC kinase (1 mol of phosphate/mol of LC20) shifts the equilibrium toward the low concentration of KCl, i.e., at the concentration of 0.2 mol/L KCl, Mg²⁺-ATPase activity of phosphorylated myosin was as high as that at 0.3 mol/L KCl. At 0.1 mol/L KCl, the activity was as high as that of dephosphorylated myosin at 0.25 mol/L KCl. In contrast, neither Mg²⁺-ATPase activity of dephosphorylated myosin nor that of prephosphorylated myosin by MLC kinase was affected by PKC phosphorylation.

The effect of phosphorylation on the KCl-dependence of viscosity. The viscosity is another probe for the measurement of conformational change of myosin. 39 The decrease in the viscosity of gizzard myosin was shown to be due to the change in myosin conformation from an extended (6S myosin) to a folded (10S myosin) form. 36 Like the gizzard myosin, the viscosity of dephosphorylated platelet myosin at 0.1 mol/L KCl was low (Fig 5). On the other hand, at 0.4 mol/L KCl, viscosity was high. The change of viscosity of dephosphorylated platelet myosin as the function of KCl concentration showed a similar pattern as KCl-dependence of Ca²⁺- and Mg²⁺-ATPase activities. These results provide
Fig 2. KCl-dependence of Ca$^{2+}$-ATPase activity of platelet myosin. ATPase activity was assayed with myosin (0.24 mg/mL), 10 mmol/L CaCl$_2$, 30 mmol/L Tris-HCl, pH 7.5, 0.5 mmol/L $[^{32}P]$ATP, and various concentrations of KCl at 25°C.

Further support for the correlation of enzymatic properties and conformation of platelet myosin. The phosphorylation by MLC kinase shifted the equilibrium toward the low concentration of KCl, i.e., at 0.2 mol/L KCl, viscosity of myosin phosphorylated by MLC kinase was as high as that of dephosphorylated myosin at 0.3 to 0.4 mol/L KCl (6S myosin). The viscosity of neither dephosphorylated myosin nor myosin prephosphorylated by MLC kinase was affected by PKC phosphorylation.

The effect of phosphorylation on intrinsic fluorescence of myosin. In the inset of Fig 6A are shown the fluorescence emission spectra of human platelet myosin before and after addition of 0.1 mmol/L ATP in the presence of MgCl$_2$. The fluorescence intensity at 335 nm was enhanced by about 15% on addition of ATP. At 0.2 mol/L KCl, the fluorescence level of myosin (10S myosin) was higher than that at 0.3 mol/L KCl (6S myosin) with addition of ATP (Fig 6A and B). At 0.2 mol/L KCl in the presence of MLC kinase and calmodulin, the addition of 0.1 mmol/L CaCl$_2$ resulted in a decrease of fluorescence (Fig 6A), whereas the addition of EGTA did not change the fluorescence intensity (data not shown). In contrast, at 0.3 mol/L KCl in the presence of MLC kinase and calmodulin, the fluorescence level did not change after the addition of CaCl$_2$. A similar experiment performed in the presence of PKC, phosphatidyserine, and TPA, with the addition of CaCl$_2$, showed a constant level of fluorescence (data not shown).

The effect of phosphorylation on actin-activated Mg$^{2+}$-ATPase activity and myosin conformation. As shown in Table 1, phosphorylation of platelet myosin by PKC alone had no effect on actin-activated Mg$^{2+}$-ATPase activity,
whereas phosphorylation by MLC kinase resulted in a 15-fold increase in actin-activated Mg\(^2\+)ATPase activity over that of dephosphorylated myosin. The myosin maximally phosphorylated by both kinases (3 mol of phosphate/mol of LC20) was accompanied by a decrease of about 40% in the actin-activated Mg\(^2\+)ATPase activity, compared with that of myosin phosphorylated by MLC kinase alone. The sedimentation coefficients in solutions containing 0.2 mol/L KCl were measured for four species (dephosphorylated myosin, myosin phosphorylated by MLC kinase, myosin phosphorylated by PKC, and myosin phosphorylated by both kinases). Sedimentation coefficients were 9.8 for dephosphorylated myosin, 6.1 for MLC kinase, 9.9 for PKC, and 6.2 for both kinases (Table 1).

Figure 7 shows the actin dependence of Mg\(^2\+)ATPase activity of platelet myosin. For myosin phosphorylated by MLC kinase alone (to approximately 1 mol of phosphate/mol of LC20), the Ka (apparent dissociation constant for actin) and V\(_{\text{max}}\) are 10.2 \(\mu\)mol/L and 56 nmol/(min \cdot mg), respectively. Additional phosphorylation of platelet myosin by PKC (an additional 2.1 mol of phosphate/mol of LC20) did not alter V\(_{\text{max}}\), but increased Ka to 45 \(\mu\)mol/L.

### DISCUSSION

The study described in this report showed that LC20 of purified human platelet myosin was phosphorylated by both MLC kinase and PKC. The analysis of two-dimensional peptide mapping previously showed that the phosphorylation of LC20 by both kinases actually occurred in thrombin-stimulated human platelets. Thus, the effects of phosphorylation of LC20 by both kinases on myosin function should be considered in evaluating the role of myosin in platelet activation. In some vertebrate and invertebrate nonmuscle...
myosin, phosphorylation of myosin heavy chain was also detected. Although phosphorylation of myosin heavy chain catalyzed by PKC was recently reported in both intact human platelets and purified platelet myosin, the extent of heavy-chain phosphorylation of purified myosin was small under our experimental conditions, so the effect of heavy-chain phosphorylation on the conformational change or actin-myosin interaction was not studied in this study. We tried to use the same conditions for PKC phosphorylation of purified platelet myosin as those reported by Kawamoto et al., and the extent of heavy chain phosphorylation was also small. One possible explanation is that the putative myosin binding proteins in intact platelet cytoplasm or purified protein(s) may alter the environment of the heavy-chain phosphorylation sites.

One of the striking findings is that sequential phosphorylation was achieved (3 mol of phosphate/mol of LC20) by MLC kinase plus PKC. One mole of phosphate was maximally phosphorylated by MLC kinase, and 2 mol of phosphate by PKC (Fig 1). These results suggest that phosphoamino acids of the two kinases are different. In a preceding study, Ikebe and Reardon reported that in bovine platelet myosin, the phosphorylation site was serine-19 by MLC kinase and threonine-9 and serine-1 or serine-2 by PKC, although additional phosphate was incorporated under certain experimental conditions. These sites are identical with the phosphorylation sites of smooth muscle HMM. Independently, Kawamoto et al. by comparing two-dimensional tryptic mapping with that in gizzard smooth muscle LC20, reported that serine-1, serine-2, and threonine-9 of LC20 of purified human platelet myosin was phosphorylated by PKC, whereas there was very little phosphorylation of threonine-9 in intact platelets after treatment with TPA. Although amino acid sequences of human platelet myosin have not been reported yet, phosophoamino acids of human platelet myosin by both kinases are expected to be the same as those of bovine platelet myosin and gizzard myosin.

The analytical ultracentrifugation study showed that human platelet myosin forms 10s conformations at low (ie, 0.1 mol/L KCl) ionic strength and 6s conformations at high (ie, 0.4 mol/L KCl) ionic strength. These results were consistent with those described previously by Takashima et al. Conformational change was also reported with a vertebrate nonmuscle myosin from brush border, thymus, or bovine erythrocytes. We further showed that these conformational changes are paralleled by enzymatic activities, ie, Ca2+ and Mg2+-ATPase activities using gel filtration and viscosity methods (Figs 2 through 5). The enzymatic activity that is subject to regulation in vivo is the actin-activated ATPase activity of myosin. The major contribution derived from the experiments in the absence of actin discussed above is the realization that two conformations of myosin possess different enzymatic properties. This property was previously shown with gizzard smooth muscle myosin and bovine erythrocyte myosin, and recently with bovine platelet myosin. Thus, the ‘shape-activity’ hypothesis for smooth muscle myosin developed by Ikebe and Hartshorne may be extended to some of the nonmuscle myosins.

There are apparent differences in the effects of phosphorylation on biochemical properties of platelet myosin between the two kinases. One difference is the effect of phosphorylation on the 10s to 6s transition. MLC kinase phosphorylation shifted the equilibrium toward 6s, ie, at 0.1 mol/L KCl viscosity and Mg2+-ATPase activity of phosphorylated myosin are about the same as that at 0.22 mol/L KCl and 0.25 mol/L KCl of dephosphorylated myosin, respectively. In contrast, PKC phosphorylation had no effect. Moreover, MLC kinase phosphorylation increased the actin-activated ATPase activity about 15-fold, whereas PKC phosphorylation had no effect (Table 1). To confirm the effects of phosphorylation on conformational change at 0.2 mol/L KCl, intrinsic tryptophane fluorescence, another tool for evaluating the conformational change, was monitored (Fig 5). Shimizu previously showed that the addition of ATP to bovine platelet myosin caused enhancement of the intrinsic tryptophane fluorescence. Our results confirmed this finding, and further showed that the phosphorylation of myosin by MLC kinase, but not that by PKC, favors the transition from 10s to 6s.

The second difference is that PKC phosphorylation, which itself had no effect on actin-activated Mg2+-ATPase activity of platelet myosin, inhibited the ATPase activity of platelet myosin prephosphorylated by MLC kinase. It was
previously reported that actin-activated ATPase activity of smooth muscle HMM was inhibited by PKC phosphorylation. One obvious concern is whether or not this inhibition is related to the inhibition of the 10S to 6S conformational change. KCl-dependence of viscosity and Mg$^{2+}$-ATPase activity were not influenced by PKC phosphorylation, and additional change was not observed in myosin prephosphorylated by MLC kinase (Figs 4 and 5). In addition, analytical ultracentrifugation showed that the sedimentation coefficient at 0.2 mol/L KCl was almost the same for MLC kinase phosphorylation and MLC kinase plus PKC phosphorylation (Table 1). These results were in contrast to those reported by Umekawa et al., who showed that the PKC phosphorylation of smooth muscle myosin prephosphorylated by MLC kinase shifted the myosin conformation from 6S to 10S, by analytical ultracentrifugation and electron microscopy. In contrast, Ikebe and Reardon reported recently that no change in the conformation of bovine platelet myosin was observed after additional phosphorylation by PKC to the MLC kinase phosphorylated myosin, judging from the KCl-dependence of viscosity and ATPase activity, although the sedimentation coefficient data were not shown. In this study, the Vmax of actin-activated ATPase is markedly increased with prephosphorylated myosin by MLC kinase. On the other hand, additional phosphorylation by PKC increases the Ka without altering Vmax. These results together indicate that PKC phosphorylation did not inhibit actin-activated Mg$^{2+}$-ATPase activity by inhibiting the conformational change of platelet myosin.

One possible explanation for the inhibition of ATPase activity is that additional phosphorylation by PKC alters the affinity for actin, probably by altering the subtle conformation at the actin binding site on the myosin head.

ACKNOWLEDGMENT

We thank Drs Shigeru Sasakawa and Yoshihide Ishikawa (the Japan Red Cross Blood Center) for providing platelet concentrates.

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Effect of phosphorylation of myosin light chain by myosin light chain kinase and protein kinase C on conformational change and ATPase activities of human platelet myosin

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