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Myosin Assembly Critical for the Enzyme Activity of Smooth Muscle Myosin Phosphatase: Effects of MgATP, Ionic Strength, and Mg^{2+}

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We suggested that an assembled form of phosphorylated myosin (P-myosin) might exhibit higher affinity for smooth muscle myosin phosphatase (SMMP) than dissociated P-myosin on the basis of the effect of MgATP [Sato and Ogawa (1999) *J. Biochem.* 126, 787–797]. To further deepen our understanding, we examined the SMMP activity and P-myosin assembly with various ionic strengths and Mg^{2+} concentrations, with and without MgATP, all of which are well known to be critical for myosin assembly. The structure of myosin molecules was directly observed by electron microscopy using a rotary shadowing procedure, which was found to be consistent with the sedimentation assay. We found that the SMMP activity was always high when P-myosin was assembled. MgATP, which disassembled P-myosin mostly into a folded conformation, in contrast, decreased the enzyme activity. We also found that glycerol had a dissociating action on P-myosin, primarily dissociating it into an extended conformation, resulting in reduced SMMP activity, and that increases in the ionic strength and Mg^{2+} (>5 mM) inhibited SMMP. These results indicate that myosin assembly is essential for SMMP activity.

Key words: myosin, myosin assembly, myosin phosphatase, protein phosphatase-1, smooth muscle.

Smooth muscle contraction is primarily regulated through phosphorylation of the 20 kDa myosin light chain (MLC_{20}) by Ca^{2+}/calmodulin-dependent MLC_{20} kinase and dephosphorylation by smooth muscle myosin phosphatase (SMMP) (*1*, *2*). Whereas regulation of MLC_{20} kinase activity has been studied in detail, attention has only recently been focused on SMMP. It is now accepted that SMMP is heterotrimeric, being composed of 130, 37, and 20 kDa subunits: the 37 kDa subunit is the catalytic one identified as the PP1Cδ (or β) isoform of protein phosphatase type 1 (*3*), and the 130 kDa one is a regulatory subunit which can bind myosin (*4*), and is sometimes referred to as the myosin phosphatase target(ing) subunit (MYPT) (*5*). The function of the 20 kDa subunit has not been fully elucidated.

It has been reported that the activity of SMMP can be modulated through the following mechanisms: dissociation of the 37 kDa catalytic subunit from MYPT by arachidonate (*6*); phosphorylation of MYPT by Rho-kinase, which is activated by small G-protein, Rho A (*7*); and binding of CPI-17, a phosphorylation-dependent inhibitory protein, to SMMP (*8*). These mechanisms underlie the change in the Ca^{2+} sensitivity of the contractile apparatus caused by several agonists (*9–11*).

Sato and Ogawa (*12*) reported that MgATP dose-dependently reduced SMMP activity toward P-myosin. This inhibition was largely caused by the decreased affinity of the enzyme for the substrate, but there was no change in V_{max}. This modulation of the enzyme activity by MgATP was observed to a lesser extent with P-heavy meromyosin, but there was no change with P-subfragment 1 or P-MLC_{20}. They also showed that this modulation of the enzymic activity by MgATP paralleled the extent of P-myosin sedimentation after centrifugation at 150,000 ×*g* for 10 min, concluding that assembly formation of P-myosin might be critically important for the SMMP activity.

To deepen our understanding of the mechanism underlying the effect of MgATP on SMMP activity, in this study, we examined the effects of the ionic strength and Mg^{2+} on the enzyme activity of SMMP and on P-myosin assembly, because these factors were well known to be critical for myosin assembly. The protein concentration was also a critical factor for the polymerization (*13*). In this respect, it should be stated that the experiments were carried out with a low concentration of P-myosin (0.2 μM or less), at which the effect of MgATP was clearly observed. The structure of P-myosin was observed by electron microscopy in this study. Because we found that glycerol, which has often been used as a stabilizer of myosin, had a serious impact on the myosin structure, we devised a new procedure to avoid it. The electron microscopic findings were also strengthened by the results of sedimentation analysis with a centrifuge. Part of this study has been presented in abstract form (*14*).

MATERIALS AND METHODS

Protein Preparations—Phosphatase-free myosin from bovine stomach or chicken gizzard and MLC_{20} from bovine stomach myosin, and their phosphorylated forms (P-myosin

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Abbreviations: MLC_{20}, 20 kDa myosin light chain; Mopso, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; P-myosin (MLC_{20}, heavy meromyosin, subfragment 1), phosphorylated myosin (MLC_{20}, heavy meromyosin, subfragment 1); SMMP, smooth muscle myosin phosphatase; UP-myosin, unphosphorylated myosin; Γ/2, ionic strength.

and P-MLC_{20}) were obtained, respectively, as described previously (*12*). The phosphorylation levels of the preparations were as follows: bovine stomach P-myosin, 91.5 ± 0.4% (mean ± SE, n = 12); P-MLC_{20}, 93.0 ± 0.4% (n = 12); and chicken gizzard P-myosin made from myosin stored at −80°C and −20°C in 50% glycerol, 94.7 ± 0.7% (n = 7) and 89.9 ± 0.7% (n = 8), respectively. SMMP was purified from chicken gizzard (*12*). Small aliquots of these proteins were frozen rapidly in liquid nitrogen, and then stored at −80°C until use.

Measurement of Phosphatase Activity—Assaying of phosphatase activity was carried out as previously described in detail (*12*). Briefly, the enzyme activity under the standard conditions was determined at 25°C in a solution containing 20 mM Mops/KOH (pH 7.0 at 25°C), 0.5 mM DTT, 0.2 μM bovine stomach P-myosin, 0.16 nM SMMP, 5 mM $MgCl_2$, and an appropriate amount of KCl to adjust the ionic strength to 0.15 M, in the presence and absence of 0.1 mM ATP, unless otherwise indicated. For the determinations with P-MLC_{20}, the concentrations of the substrate and enzyme were increased to 1 μM and 1.6 nM, respectively.

Electron Microscopy—During the course of experiments, we noted that glycerol had a potent dissociating effect on assembled myosin (see Fig. 6A). After the myosin had been adsorbed on mica, however, such an effect was no longer observed. To avoid this effect of glycerol, the method of Mabuchi (*15*) was modified as described below. Usually, myosin was incubated in a reaction medium with no glycerol, where SMMP was omitted from the reaction medium for the enzyme assay for 2 min at room temperature, and diluted 2–50-fold with the same medium (2–50 μg/ml P-myosin). An aliquot of the solution was immediately placed on a freshly cleaved mica plate (5 × 10 mm), and allowed to stand for 30 s. The solution was then sandwiched gently with another mica plate to squeeze out the excess protein solution (*16*). The mica plate on which the specimen was adsorbed was thoroughly washed with the reaction medium (three times, 50 μl each time), and then treated with a fixing solution (2% uranyl acetate in 30% glycerol) for 30 s (twice, 50 μl each time), followed by 3 brief washes (50 μl each) with a rinsing solution (0.1 M ammonium acetate in 50% glycerol). The sandwich procedure was again carried out with a new mica plate, and the first mica plate on which myosin had been fixed was immediately placed in a JFD 9000 (JEOL) to expose it *in vacuo*. After 10–15 min, shadowing with platinum-carbon was performed at an angle of 7.5° (at 50 A and 2 kV for 25 s), followed by reinforcing with carbon at an angle of 90°. Myosin molecules were then observed under an electron microscope (100S, JEOL). The film of the electron microscope was scanned with a film scanner (LS-4500AF, Nikon), and processed with Adobe Photoshop (version 5.0.2), or Deneba Canvas (version 6.0.1).

Sedimentation Analysis of Myosin—A sedimentation assay was carried out to determine the extent of myosin assembly (*17*). The conditions were similar to those for measurement of phosphatase activity except that the enzyme was omitted. After incubation for 2 min at 25°C, the mixture was centrifuged at 2,000 ×g for 10 min in a Himac CF15D (Hitachi-koki) or at 150,000 ×g for 10 min in a TL-100 (Beckman Instruments). The protein concentration of the resulting supernatant was determined according to the method of Bradford (*18*), the amount of the sedimented protein being calculated.

Protein Assay—The protein concentration of SMMP was determined by means of the amido black dye-binding assay (*19*) with bovine serum albumin as a standard. The concentrations of myosin ($E^{1\%}$ = 5.2 at 280 nm) (*20*), MLC_{20} ($E^{1\%}$ = 3.37 at 277 nm) (*21*), calmodulin ($E^{1\%}$ = 2.00 at 277 nm) (*22*), and BSA ($E^{1\%}$ = 6.3 at 280 nm) (*23*) were determined by spectrophotometric measurements.

Statistical Analysis—As can be seen in Fig. 4, the statistical significance of the results was determined by one-way repeated-measure analysis of variance with adjustment by means of Fisher's PLSD method using StatView (version 5.0, SAS Institute). A probability of less than 0.05 was considered significant. Unless otherwise indicated, statistical probability was determined by means of Student's t-test.

RESULTS

Effect of the Ionic Strength on SMMP Activity—SMMP activity toward P-MLC_{20} decreased almost linearly with an increase in ionic strength from 0.05 to 0.32 M, as shown in Fig. 1A, and this relationship was not affected by MgATP. On the other hand, the ionic strength dependence of SMMP activity toward P-myosin was obviously different from that toward P-MLC_{20}. In the absence of ATP, the SMMP activity toward P-myosin biphasically depends on the ionic strength with the peak activity at 0.11 M, and the relationship is steeper than that with P-MLC_{20} (Fig. 1B). MgATP shifted this relationship to a lower ionic strength range with a slight decrease in the peak activity. We previously showed that the primary effects of MgATP were a decrease in the affinity of SMMP for P-myosin without a change in the V_{max} value and an increase in the cooperativity of P-myosin dependence (*12*), concluding that these effects were probably due to the conformation change(s) of P-myosin caused by MgATP. This conclusion is consistent with the results presented here. The results in Fig. 1A might represent the effect of the ionic strength itself on the SMMP activity, and Fig. 1B would show additional effects of the ionic strength on P-myosin (see also "DISCUSSION"). To confirm this hypothesis, we examined SMMP activity and the state of P-myosin molecules in the presence of various ionic strengths, with and without MgATP.

Effect of the Ionic Strength on P-Myosin Assembly—We examined the assembly of P-myosin at ionic strengths of 0.15, 0.11, and 0.07 M by electron microscopy. The ionic strength of 0.15 M was adopted because it was the standard for the enzymic activity in the previous experiments and because it was very similar to the myoplasmic ionic strength. Figure 1B shows that the enzyme activity was about half the peak value without MgATP and that it was reduced to very little in the presence of 0.1 mM MgATP. With 0.11 M, the enzymic activity in the absence of MgATP was around the peak, whereas it was about 30–40% of the peak in the presence of MgATP. With 0.07 M, the activity was reduced to about 80% of the peak activity in the absence of MgATP, whereas it was unchanged by 0.1 mM ATP and was around the peak of the ionic strength-dependent values in the presence of 0.1 mM MgATP. Please note that these determinations were carried out with a low concentration of P-myosin (0.2 μM), where the inhibitory effect of MgATP on SMMP activity could be clearly observed. To date, we have had a great deal of difficulty in observing the

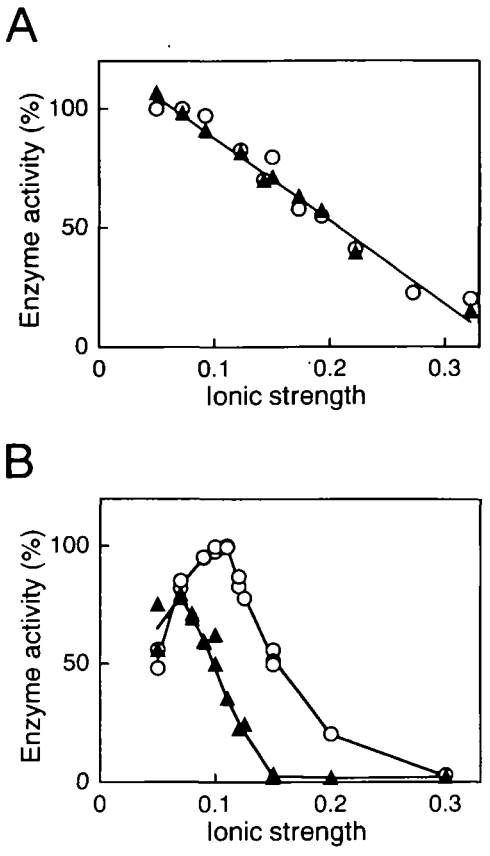

Fig. 1. **Ionic strength dependence of SMMP activity with different substrates, P-MLC$_{20}$ (A) and P-myosin (B).** A, SMMP activity toward P-MLC$_{20}$. Each assay was performed at 25°C in a solution containing 20 mM Mopso/KOH (pH 7.0), 5 mM $MgCl_2$, 0.5 mM DTT, 1 μM bovine stomach P-MLC$_{20}$, and 1.6 nM chicken gizzard SMMP, with various ionic strengths obtained with KCl (Γ/2 = 0.050–0.32 M), in the presence (closed triangles) and absence (open circles) of 0.1 mM MgATP. B, SMMP activity toward P-myosin. The assay was performed in a similar medium to that in (A), except that 0.2 μM bovine stomach P-myosin and 0.16 nM chicken gizzard SMMP were used (Γ/2 = 0.050–0.30). Activities were evaluated as described under "MATERIALS AND METHODS," and were expressed as values relative to those at the ionic strength of (A) 0.050 M without MgATP (100% = 1.1 μmol/min/mg protein) and (B) 0.11 M without MgATP (100% = 14.8 μmol/min/mg protein).

myosin structure, including the dissociated and assembled forms, by means of the rotary shadowing procedure (*24*), especially at a specific ionic strength. The disassembling action of shearing stress on spraying is one well known reason. Another reason is the inevitable use of glycerol, with the assumption that the reagent should be inert as to myosin assembly, although some investigators were suspicious of its effect (*25*). As shown later, glycerol was found to have a dissociating action on myosin assembly (see Fig. 6A). To avoid this with the modified method of Mabuchi (*15*), we modified the procedure so as to gently adsorb the myosin molecules onto mica in the absence of glycerol, followed by fixation and staining in the presence of glycerol. Figure 2 shows electron micrographs of P-myosin with various ionic strengths in the presence and absence of MgATP. In the absence of MgATP at the ionic strength of 0.15 M, most of the P-myosin molecules formed small homogeneously sized filaments, which were composed of dozens of P-myosin molecules (Fig. 2A). The longitudinal length of each filament was 0.56 ± 0.06 μm (mean ± SD, n = 108), which was considerably shorter than the 2.2 μm in rabbit portal vein smooth muscle myosin (*26*). The presence of 0.1 mM MgATP caused almost complete dissociation of this assembly (Fig. 2D). The dissociated P-myosin showed several types of conformations (Fig. 2D): extended (4.1%), bent at about 50 nm (14.9%) or 100 nm (13.6%) from the tip of the myosin tail, folded (54.3%), and unclassified including myosin with a short or zigzag tail (13.2%) and so on (n = 1,033), according to the classification of Onishi and Wakabayashi (*27*). At the ionic strength of 0.11 M, which was the optimum for the enzyme activity in the absence of MgATP, the dimensions of P-myosin filaments were further increased, as shown in Fig. 2B. Electron micrography of P-myosin in the presence of 0.1 mM MgATP revealed a mixture of assembled myosin similar to those shown in Fig. 2A and dissociated myosin, as shown in Fig. 2D (Fig. 2E). At the ionic strength of 0.07 M, where the enzyme activity of SMMP overshot to about 80% of the peak activity, the myosin filaments were much more associated, forming gigantic bundles, in the absence of MgATP (Fig. 2C). In the presence of 0.1 mM MgATP, on the other hand, associated P-myosin appeared very similar to that shown in Fig. 2A (Fig. 2F). In summary, lowering of the ionic strength promoted the assembly of P-myosin, and MgATP stimulated disassembly. It was a surprise to us that such a huge assembly as that shown in Fig. 2C was a good substrate, although access to this huge substrate by the enzyme may be limited to some extent, probably because of steric hindrance. This may explain why the enzyme activity was decreased in a medium of an ionic strength lower than 0.11 M in the absence of ATP.

Similar results were obtained with UP-myosin, although the effect of MgATP was very marked (data not shown, but refer to Fig. 3A). Only a few UP-myosin filaments were observed on electron microscopy in the presence of MgATP, even at the ionic strength of 0.05 M. UP-myosin at the ionic strength of 0.15 M was fully dissociated in the presence of MgATP, being largely composed of the folded conformation (68.6%), as in the case of P-myosin. We cannot definitely conclude that the fraction of the folded form of UP-myosin is higher than that with P-myosin, although it seems to be so. Thus, UP-myosin molecules were inclined to have a folded structure in the presence of MgATP, this being consistent with the results obtained in earlier investigations (*25, 28*).

These electron microscopic observations were supported by the results of sedimentation assaying of P-myosin under similar conditions (Fig. 3): in the absence of MgATP, P-myosin at the ionic strength of 0.15 M was sedimented on centrifugation for 10 min at 150,000 ×g, but not at 2,000 ×g, whereas P-myosin at the ionic strength of 0.07 M was fully sedimented on centrifugation at 2,000 ×g for 10 min. Note that about 40% of the P-myosin at the ionic strength of 0.11 M was in the supernatant after centrifugation at 2,000 ×g for 10 min, but was almost completely sedimented on centrifugation at 150,000 ×g for 10 min. This indicated that P-myosin assemblies at 0.11 M were certainly greater in size than those at 0.15 M, but not as great as those at 0.07 M. In the presence of MgATP, the results of sedimentation of P-myosin support the conclusions drawn from the electron

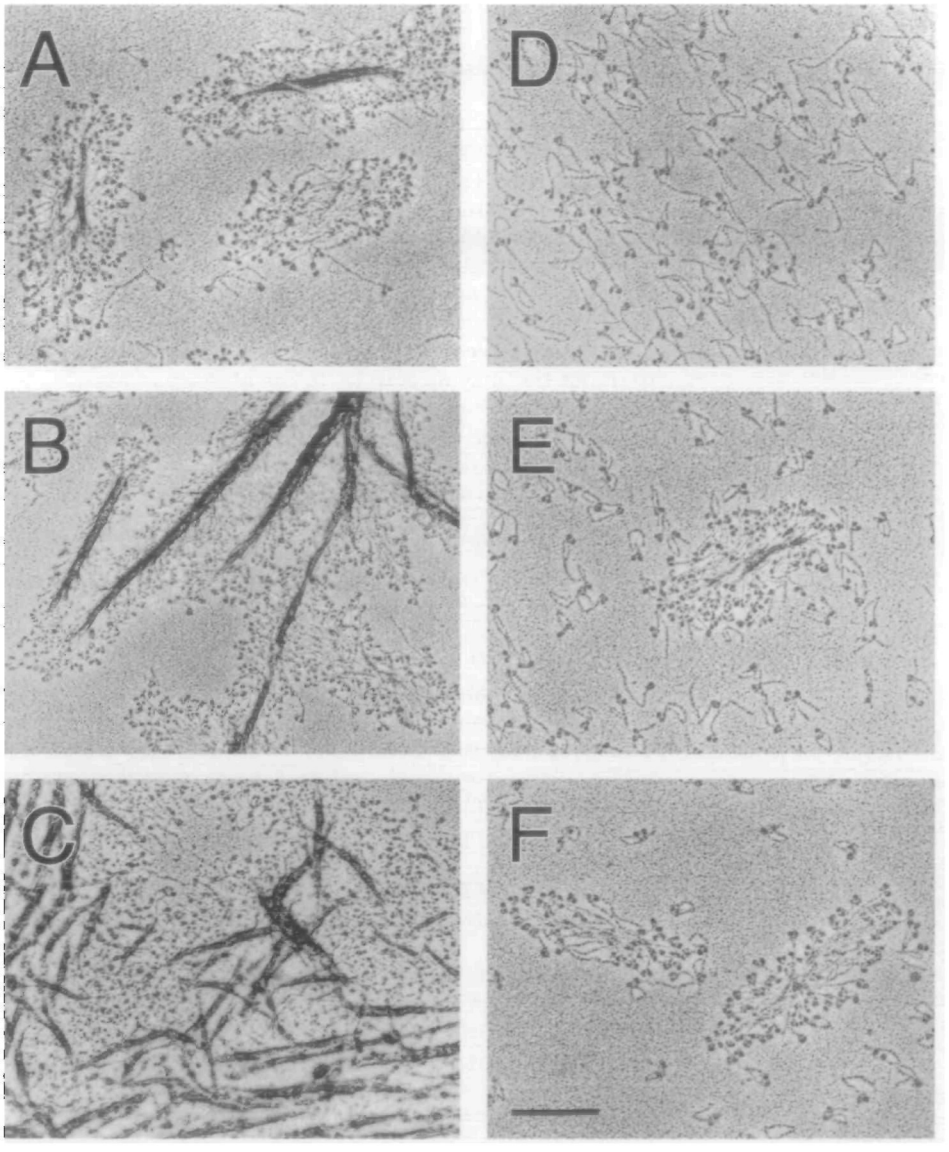

Fig. 2. **Rotary shadowed images of P-myosin at various ionic strengths.** The structure of bovine stomach P-myosin was observed by electron microscopy at the ionic strengths of 0.15 M (A, D), 0.11 M (B, E), and 0.070 M (C, F). P-myosin in each solution, which was similar to that in Fig. 1B except for the omission of SMMP, was incubated for 2 min at room temperature in the presence (D, E, F) and absence (A, B, C) of 0.1 mM MgATP. P-myosin was then adsorbed onto mica, rotary-shadowed, and observed under an electron microscope as described under "MATERIALS AND METHODS." Scale bar, 200 nm.

microscopic observations (Figs. 2, D–F, and 3). In the presence of MgATP, little of the P-myosin was precipitated at 0.15 M after centrifugation at 150,000 ×*g* for 10 min, indicating that most of the P-myosin was dissociated. The sediment fraction on centrifugation increased as the ionic strength decreased to 0.05 M, indicating that the fraction of assembled myosin increased. Sedimentation assaying by centrifugation at 150,000 ×*g* for 10 min was also carried out using UP-myosin. The result in the absence of MgATP was similar to that in the case of P-myosin (data not shown). In the presence of 0.1 mM MgATP, however, little UP-myosin was sedimented over the range of $\Gamma/2$ = 0.05–0.3 M (Fig. 3A), indicating that UP-myosin was more dissociable than P-myosin in the presence of MgATP, consistent with the results reported (*28*). The difference from the results of Suzuki *et al.* (*28*) on closer comparison may be due to the difference in protein concentrations used in the experiments, because the concentration they used was 10 times ours.

Furthermore, we performed a series of similar experiments with P-myosin in the presence of 1 mM EDTA instead of 5 mM $MgCl_2$, and obtained similar results, but the magnitudes of the effects of ionic strength and ATP were much weaker (data not shown). These results may indicate that Mg^{2+} promotes myosin assembly and that ATP has a weaker effect than MgATP in the dissociation of myosin filaments (see later).

Assembly of P-Myosin from Myosin Stored at −20°C—We previously reported that the effect of MgATP on SMMP activity depended on the P-myosin preparation. Unlike P-myosin from unfrozen, fresh myosin or myosin which had been rapidly frozen and stored at −80°C, P-myosin from myosin stored at −20°C in 50% glycerol was insensitive to the inhibitory effect of MgATP. Consistently, this ATP-resistant P-myosin was sedimented in the presence of MgATP on centrifugation at 150,000 ×*g* for 10 min (*12*). We examined the two P-myosins by electron microscopy. In contrast to dissociable P-myosin in the presence of MgATP, as shown in Fig. 2D ($\Gamma/2$ = 0.15 M), the ATP-insensitive P-myosin mostly formed filaments, as shown in Fig. 2A, irrespective of the absence or presence of MgATP, although it showed weak signs of dissociation in the presence of

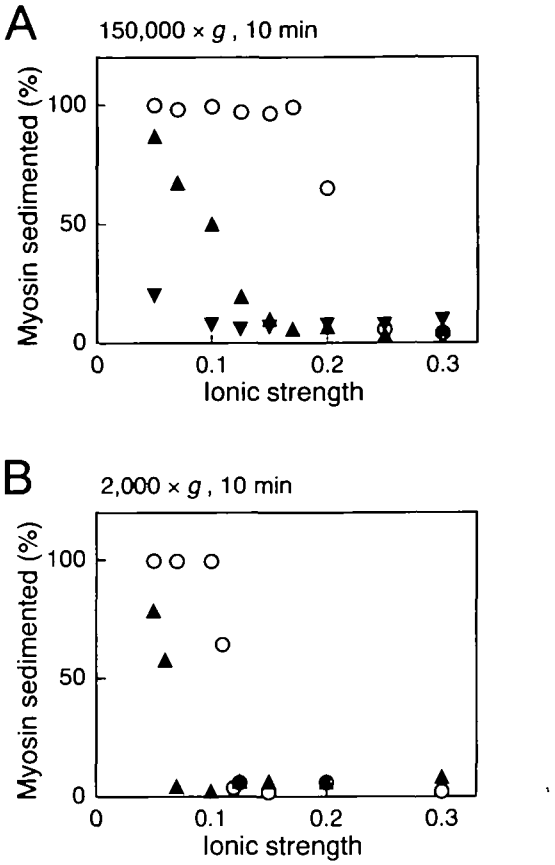

Fig. 3. **Effects of ionic strength on the sedimentation of P-myosin in the presence and absence of ATP.** The assay was carried out at 25°C under conditions similar to those in Fig. 2. The solution was centrifuged at 150,000 ×*g* for 10 min (A) or 2,000 ×*g* for 10 min (B) in the presence (closed triangles) or absence (open circles) of 0.1 mM MgATP. Each myosin concentration in the supernatant was determined according to the method in (*18*), and the amount of myosin sedimented was estimated as described under "MATERIALS AND METHODS." The reversed triangles show the results with UP-myosin in the presence of 0.1 mM MgATP.

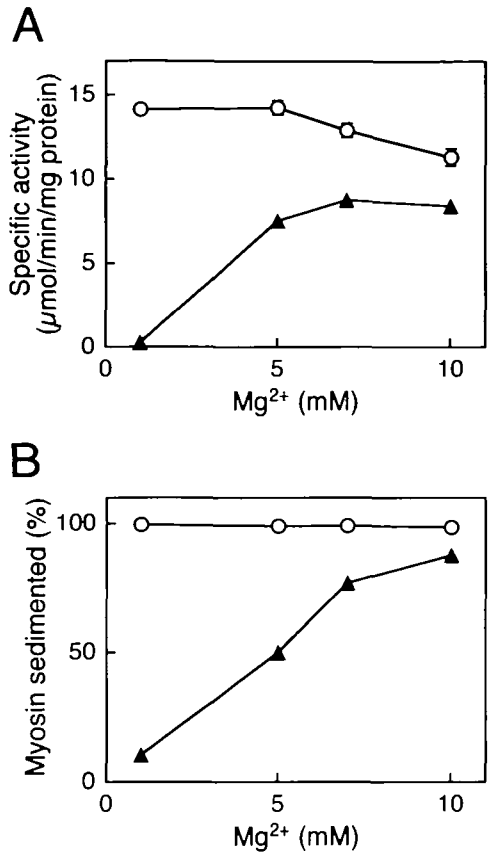

Fig. 4. **Effects of Mg^{2+} on SMMP activity and P-myosin sedimentation in the presence and absence of ATP.** Phosphatase activity (A) and P-myosin sedimentation (B) were determined as shown in Figs. 1 and 3, respectively, in solutions at the ionic strength of 0.10 M containing various concentrations of Mg^{2+}, with (closed triangles) or without (open circles) 0.1 mM ATP. Each result is shown as the mean ± SE (n = 5). Symbols without error bars indicate that the SE was within the size of the symbols. The results were examined by means of Fisher's PLSD post-hoc test. The effects of MgATP on SMMP activity and myosin sedimentation were significant with $p < 0.01$ at each Mg^{2+} concentration. In the absence of MgATP, the enzyme activity was significantly decreased by 7 ($p < 0.05$) and 10 mM ($p < 0.01$) Mg^{2+}. In the presence of MgATP, the enzyme activities with 1 ($p < 0.01$) and 5 mM ($p < 0.05$) Mg^{2+} were significantly lower than those with 7 and 10 mM Mg^{2+}. The extent of P-myosin sedimentation in the presence of MgATP increased as the Mg^{2+} concentration increased up to 10 mM ($p < 0.01$), which is consistent with the ratio of enzyme activity with and without MgATP at each Mg^{2+} concentration.

MgATP (data not shown). The filament lengths at the ionic strength of 0.15 M were 0.56 ± 0.08 μm (mean ± SD, n = 111) without ATP and 0.52 ± 0.09 μm (n = 122) with 0.1 mM MgATP, both of which were similar to the value with bovine stomach P-myosin in the absence of ATP. These results might provide an explanation for why previous investigators failed to show the reduction of the SMMP activity toward P-myosin caused by ATP.

Effect of Mg^{2+}—Because it is well known that a high concentration of Mg^{2+} promotes the myosin assembly, we examined the effects of Mg^{2+} on SMMP activity and P-myosin sedimentation (Fig. 4). On the basis of the results in Fig. 1, the ionic strength was kept at 0.10 M, where the enzyme activity nearly peaked in the presence of 5 mM Mg^{2+} and no ATP. In the absence of ATP, the enzyme activity was unchanged by Mg^{2+} up to 5 mM and it decreased as the Mg^{2+} concentration was increased above 5 mM. Most of the P-myosin was sedimented on centrifugation for 10 min at 150,000 ×*g* irrespective of the Mg^{2+} concentration (1–10 mM), indicating that P-myosin was of the assembled conformation. Therefore, the results in Fig. 4 indicate that Mg^{2+} at over 5 mM dose-dependently inhibits SMMP activity. MgATP decreased both SMMP activity and the fraction of P-myosin sedimented. The extent of inhibition decreased as the Mg^{2+} concentration increased. The ratios of (with ATP)/(without ATP) for SMMP activity and for the sedimented fraction of P-myosin increased in parallel as Mg^{2+} increased to 10 mM.

Figure 5 shows P-myosin conformation observed on electron microscopy under the same conditions as in Fig. 4. In the absence of MgATP, most P-myosin molecules formed filaments at 1 mM Mg^{2+} (Fig. 5A), whereas P-myosin formed large bundles at 7 mM Mg^{2+} (Fig. 5B). These images were similar to those at the ionic strength of 0.15 M and 0.11–0.07 M with 5 mM Mg^{2+}, respectively, as shown in Fig. 2,

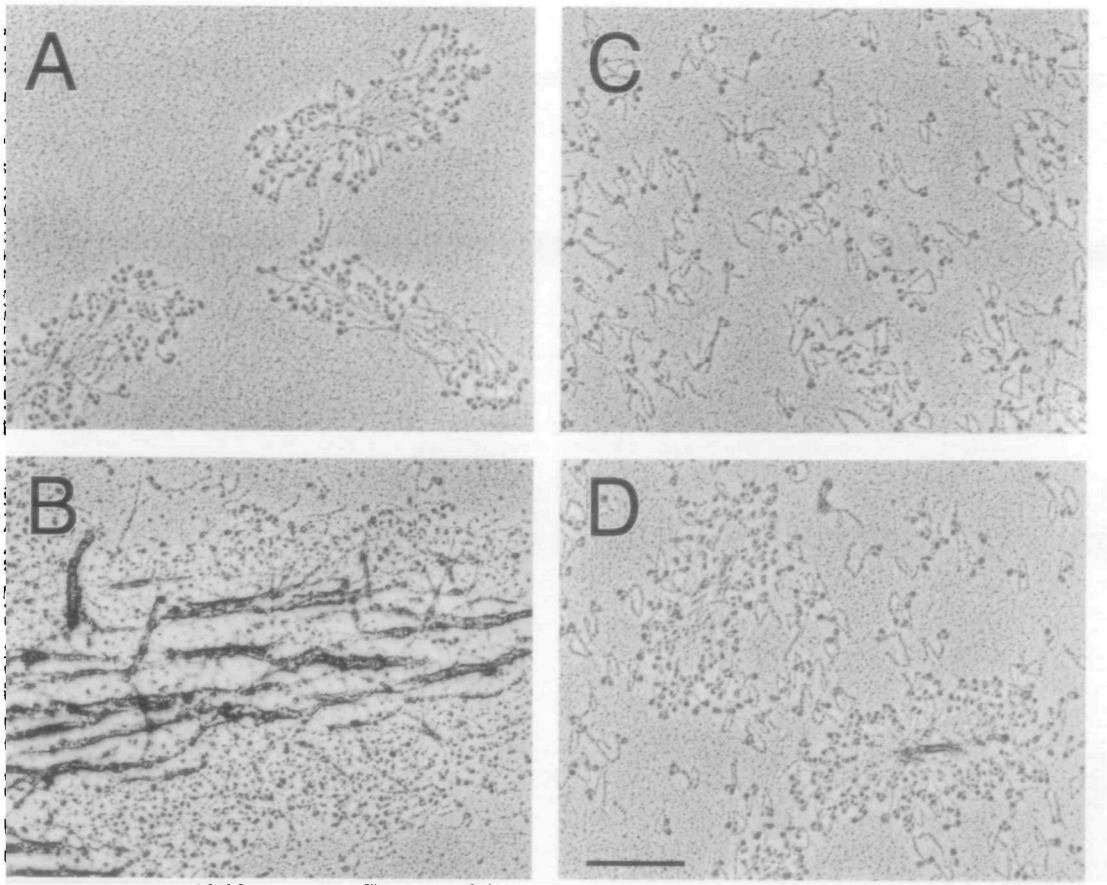

Fig. 5. **Effect of Mg^{2+} on P-myosin assembly in the presence and absence of MgATP.** The conditions of P-myosin solutions were similar to those in Fig. 2 except that the ionic strength was kept constant at 0.10 M, and that the Mg^{2+} concentrations were 1 mM (A, C) and 7 mM $MgCl_2$ (B, D). The solutions in the presence (C, D) and absence (A, B) of 0.1 mM MgATP were treated as described in the legend to Fig. 2 (see also "MATERIALS AND METHODS"). Scale bar, 200 nm.

A–C. In the presence of 0.1 mM MgATP, however, P-myosin with 1 mM Mg^{2+} was almost completely dissociated (Fig. 5C), while numerous filaments were observed with 7 mM Mg^{2+} in contrast to in the case of 1 mM Mg^{2+} (Fig. 5D, see also Fig. 2, D–F). These results are consistent with those in Figs. 2 and 4. Thus, we can conclude that Mg^{2+} stimulated assembly formation of P-myosin and antagonized MgATP-induced P-myosin disassembly. Also of note is that Mg^{2+} at higher than 5 mM inhibits the SMMP activity itself.

Effects of Glycerol on SMMP Activity and P-Myosin Assembly—During the course of this study, we found that a high concentration of glycerol also inhibited the myosin assembly. The electron micrographs in Fig. 6A clearly show that glycerol dose-dependently dissociated the P-myosin assembly and that most of the P-myosin was disassembled in the presence of 50% glycerol. This was confirmed more quantitatively by sedimentation assaying (data not shown). Although more than half the P-myosin dissociated by MgATP took on the folded conformation, most of the myosin molecules dissociated by glycerol took on the extended conformation (72.2%), a minority taking on the folded conformation (panel d in Fig. 6A). To clarify whether the primary mechanism underlying the decrease in SMMP activity caused by MgATP should be ascribed to the simple effect of P-myosin disassembly or the folded conformation, which was alleged to prevent the assembly, we examined the effect of glycerol on SMMP activity. With P-MLC_{20} as the substrate, glycerol reduced SMMP activity in an almost linear manner in relation to its concentration and to a very low level in the presence of 50% glycerol (data not shown). SMMP activity toward P-myosin, however, decreased more steeply, as shown in Fig. 6B. This reduction by glycerol of the enzyme activity is likely to be due to the combination of the effect of the dissociation of P-myosin and the inherent inhibitory effect of glycerol itself. Taking all these findings into consideration, we can conclude that the decrease in the SMMP activity in the presence of 0.1 mM MgATP is simply due to the P-myosin disassembly. In other words, whether in the extended or folded form, dissociated P-myosin gave rise to low enzyme activity. This is also consistent with the hypothesis that the affinity of SMMP for dissociated P-myosin is lower than that for assembled P-myosin.

DISCUSSION

In this study we examined in parallel the extent of P-myosin polymerization and SMMP activity with various ionic strengths and Mg^{2+} concentrations in the presence and absence of 0.1 mM MgATP. The experiments were carried out with a fixed low concentration of P-myosin ($\leq$0.2 μM), where the effect of MgATP was clearly observed. This study, although being qualitative, strengthens our previous

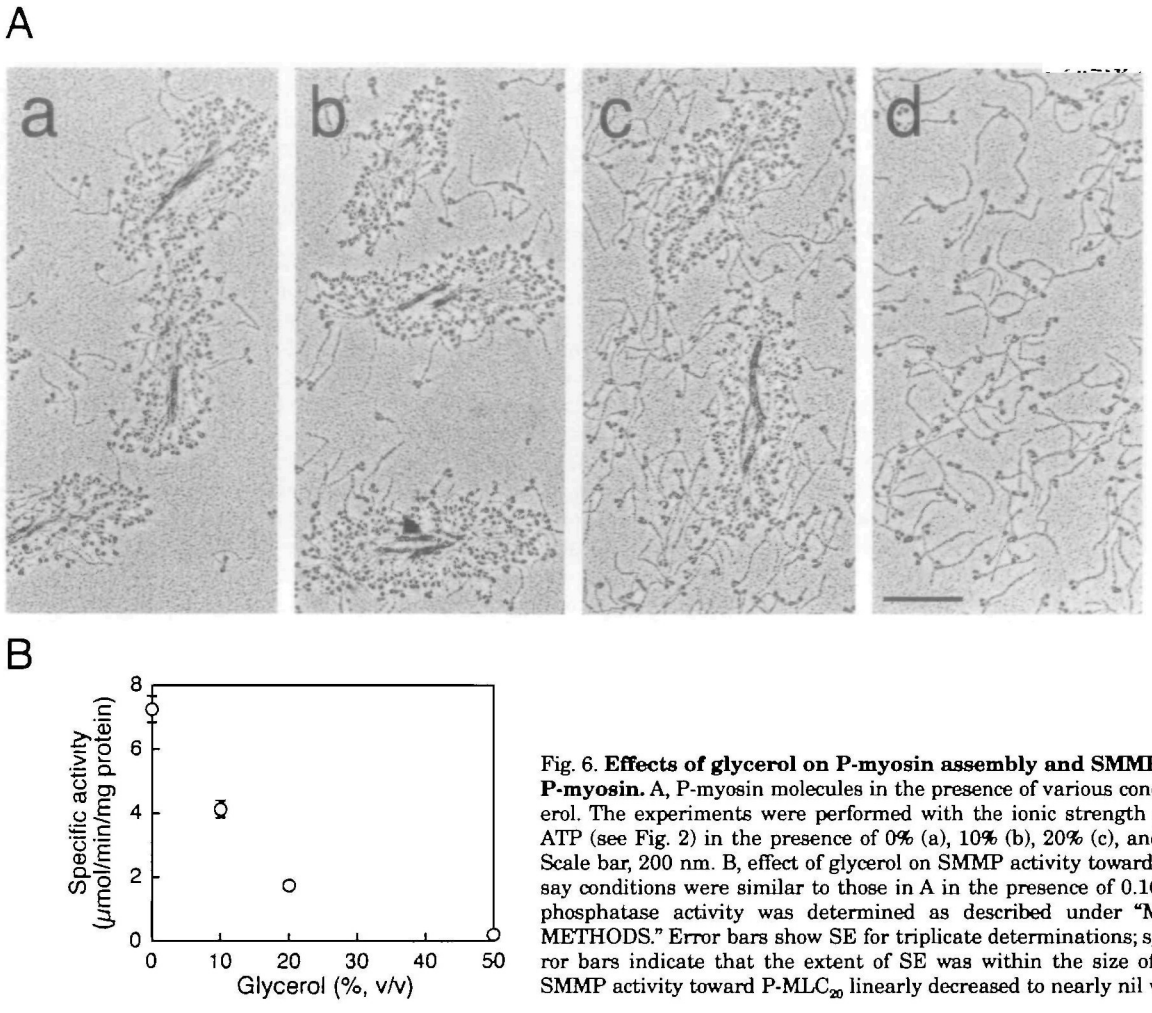

Fig. 6. **Effects of glycerol on P-myosin assembly and SMMP activity toward P-myosin.** A, P-myosin molecules in the presence of various concentrations of glycerol. The experiments were performed with the ionic strength of 0.15 M without ATP (see Fig. 2) in the presence of 0% (a), 10% (b), 20% (c), and 50% (d) glycerol. Scale bar, 200 nm. B, effect of glycerol on SMMP activity toward P-myosin. The assay conditions were similar to those in A in the presence of 0.16 nM SMMP. Each phosphatase activity was determined as described under "MATERIALS AND METHODS." Error bars show SE for triplicate determinations; symbols without error bars indicate that the extent of SE was within the size of the symbols. The SMMP activity toward P-MLC_{20} linearly decreased to nearly nil with 50% glycerol.

conclusion that myosin assembly is crucially important for SMMP activity (*12*), as described below.

For clear observation of a well preserved conformation of myosin molecules including their assembly formation by means of the rotary shadowing procedure under an electron microscope, however, there are some difficulties to overcome. First, the shearing stress on spraying may destroy assembled myosin, which was avoided by using a mica plate to which myosin was adsorbed. Second, we found that glycerol, which was often used as a protein stabilizing agent and essential for maintaining structure during drying *in vacuo*, had a potent dissociating effect on myosin assembly. Trybus and Lowey (*25*), in fact, reported that the state of myosin molecules observed on electron microscopy often differed from that determined on sedimentation velocity analysis. We learned that myosin molecules adsorbed onto mica were resistant to the distorting effect of glycerol.

The electron microscopic findings for dissociated, small-assembled and large-assembled myosins are well consistent with the results of sedimentation analysis, which is much easier. The precipitates obtained on centrifugation at 2,000 ×*g* for 10 min primarily consist of large bundles, as shown in Fig. 2C, but may also contain relatively large bundles, as shown in Fig. 2B. The molecules in the supernatant obtained on centrifugation at 150,000 ×*g* for 10 min correspond to dissociated forms, as shown in Figs. 2D and 5C. The resulting precipitates, on the other hand, contain assembled myosins, as shown in Fig. 2, A–C. The fraction collected with the intermediate centrifugal force (2,000–150,000 ×*g* for 10 min), therefore, may include primarily P-myosin filaments, as shown in Fig. 2A, in the mixture of dissociated and small-assembled myosin depicted in the other panels. It should be mentioned that the intermediate sized assembled myosin shown in Fig. 2B will be included in this fraction. Because the precipitates obtained on centrifugation at 2,000 ×*g* for 10 min were greatly dependent on the ionic strength, as shown in Fig. 3B, the formation of the intermediate sized assembly may be in transition. Figure 3A also shows that UP-myosin was more readily dissociated by MgATP than P-myosin. It has, in fact, been reported that P-myosin was not dissociated by MgATP (*28*). We would like to point out, however, that this might depend on the quality of the P-myosin used. As described here, P-myosin prepared from myosin stored at −20°C in 50% glycerol was insensitive to MgATP, but P-myosin made from fresh myosin and myosin stored at −80°C was sensitive to MgATP. Furthermore, Kendrick-Jones *et al.* (*13*) showed that the critical concentration of P-myosin for its polymerization was very low, but that there was a clear dif-

ference in the critical concentrations with and without MgATP. Therefore, the protein concentration used in the experiments could be another explanation for the discrepancy.

We previously showed that 0.1 mM MgATP increased the K_m value of SMMP for P-myosin from 0.45 to 2.4 μM at the ionic strength of 0.15 M with unchanged V_{max} values. This value in the presence of 0.1 mM MgATP in terms of the myosin head was similar to those with P-subfragment 1 and P-MLC_{20}, which are insensitive to MgATP. Therefore the K_m value of SMMP for dissociated myosin may be about a few μM. At the ionic strength of 0.10 M, the enzyme activity of SMMP was almost the same at 1 and 5 mM Mg^{2+} (Fig. 4). Figures 5 and 2 show that myosin formed small and large assemblies at 1 and 5 mM Mg^{2+}, respectively. This may indicate that there is no difference in the K_m values of about 0.5 μM as well as the V_{max} values for assembled P-myosin, regardless of the extent. Figure 1 shows the very steep ionic strength dependence in the range of 0.10–0.20 M with P-myosin, although the dependence with P-MLC_{20} was shallower. Because there was no marked difference in the enzymic activity or K_m values between small and large assemblies of P-myosin, the results in this figure are mainly due to changes in V_{max}, indicating the effect of ionic strength itself on the enzyme activity, which was distinct from that on P-MLC_{20}. This implies that P-MLC_{20} cannot be an appropriate substitute for P-myosin for determination of the extent of the effect of ionic strength on enzyme activity. This effect of ionic strength on V_{max} may also explain why the SMMP activity in the presence of 0.1 mM ATP at the ionic strength of 0.07 M was higher than that without ATP at the ionic strength of 0.15 M, although the level of assembled P-myosin under the latter condition was similar to or higher that under the former condition.

The transition of dissociated myosin between extended and folded conformations, and their biological implications have attracted the attention of many investigators (*13, 28–30*). In this study, P-myosin dissociated by MgATP showed largely a folded conformation but very little a purely extended conformation (1–4%). This was also the case with UP-myosin. Dissociated P-myosin in the presence of glycerol, in contrast, was largely of the extended conformation (70%). In either case, SMMP showed very weak activity with dissociated P-myosin, implying that there was little difference between the folded and extended conformations as substrates for SMMP. We reported that the K_m values of SMMP for substrates were in the order of P-myosin < P-heavy meromyosin < P-subfragment 1 ≅ P-MLC_{20} (*12*). This indicates that not only the head component but also the rod component of myosin molecules is important for a suitable configuration of the phosphorylated site of P-MLC_{20}, which is the substrate for SMMP. Because the rod component is critically involved in myosin assembly, as shown in Figs. 2 and 5, assembled P-myosin, irrespectively of the size unless it causes steric hindrance, may take on a more suitable configuration than dissociated P-myosin for the enzyme activity, which is represented by a lower K_m. Taking all these findings and discussions into consideration, our conclusions are summarized in Fig. 7. Assembled P-myosin shows higher affinity for SMMP, resulting in higher enzymic activity than that of dissociated P-myosin. The transition between dissociated and associated P-myosin can be affected by the ionic strength, MgATP and Mg^{2+}, as summarized in Fig. 7. The SMMP activity is mainly modulated by myosin association. Ionic strength and Mg^{2+}, however, also have independent impacts. Because these factors including MgATP are kept constant *in situ*, they cannot be involved in modulation of the enzyme activity.

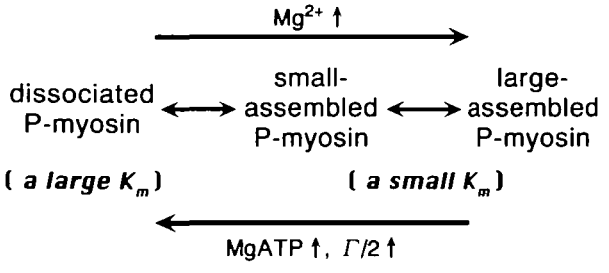

Fig. 7. **Summary of the results obtained in these experiments.** Γ/2, ionic strength. From the previous results (*12*), K_m for dissociated P-myosin in the presence of 0.1 mM MgATP can be estimated to be 2.4 μM. It will be a larger value in the presence of the myoplasmic concentration of MgATP. Figures 5A (1 mM Mg^{2+}) and 2B (5 mM Mg^{2+}) showed small and large assembled P-myosin, respectively. Figure 4 showed that the enzyme activity in the absence of MgATP was unchanged by 1–5 mM Mg^{2+}, indicating a low K_m value [about 0.5 μM (*12*)] with the same V_{max} value. Increases in the ionic strength and MgATP concentration stimulate P-myosin dissociation, and Mg^{2+} promotes P-myosin assembly. The ionic strength (Fig. 1) and Mg^{2+} (>5 mM) (Fig. 4) may also affect V_{max}.

Protein concentrations are also an important factor for this dissociation and association reaction. P-myosin is resistant to the dissociating effect of MgATP, whereas UP-myosin easily dissociates on the addition of MgATP. This suggests that P-myosins may have much higher affinity for association with rods than UP-myosin: phosphorylation of MLC_{20} in the myosin head, in turn, affects reciprocally the rod portion to promote myosin assembly. This may explain why the addition of UP-myosin in the presence of 0.2 μM P-myosin did not decrease the reduction by MgATP of SMMP activity in contrast to the effect of increasing amounts of P-myosin (*12*). This finding leads us to conclude that the fraction of P-myosin is an important factor for the modulation of SMMP activity *in situ*. At rest, where the fraction of P-myosin is about 10%, the SMMP activity may be lower than V_{max}. On activation, where the fraction of P-myosin is increased to 50% or more, the SMMP activity would reach near V_{max} (*12*). This modulation of SMMP activity *in situ* in reference to MLC_{20} kinase activity would be important for considering the phosphorylation level during the process of activation and deactivation of smooth muscles.

Now it is generally considered that myosin *in situ* forms filaments even at rest, and that assembly and disassembly of myosin cannot be important for smooth muscle contraction (*31, 32*). This assembly formation may be due to a high concentration of myosin (30–50 μM) in part and the virtue of several myosin-associating factors in part (*33–36*). It remains to be determined, however, whether the phosphorylatable sites or the neighbor of MLC_{20} on myosin at rest take on the same suitable configuration as those in P-myosin assembly, which were shown here.

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