J. Biochem. 126, 787-797 (1999)

The Modulatory Effect of MgATP on Heterotrimeric Smooth Muscle Myosin Phosphatase Activity[1]

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Received July 1, 1999; accepted August 17, 1999

Regulation of the enzymatic activity of heterotrimeric smooth muscle myosin phosphatase (SMMP) by MgATP was examined using phosphorylated myosin (P-myosin), heavy meromyosin (P-HMM), subfragment-1 (P-S1), and 20 kDa myosin light chain (P-MLC_{20}) as substrates. The activity toward P-myosin and P-HMM was dose-dependently reduced by MgATP, whereas that toward P-S1 or P-MLC_{20} was unchanged. The reduction was mainly due to a decrease in the affinity of SMMP for the substrate with the unchanged maximum activity. This regulation is entirely new in the respect that the responsible molecule is the substrate, not SMMP. Because P-myosin derived from myosin stored in 50% glycerol at −20°C was insensitive to MgATP, the proper integrity of P-myosin is required. Coexisting myosin did not affect this regulation, but it inhibited the SMMP activity in the absence of MgATP. With P-myosin, the enzyme activity was biphasically steeply dependent on the ionic strength. This requires that determinations are conducted with a fixed ionic strength. The Q_{10} value was about 2, which was quite similar to that for myosin light chain kinase. These results suggest that the rate of dephosphorylation of P-myosin is lowered at rest, but that it may reach a value comparable to the rate of phosphorylation of myosin in the sarcoplasm with the increased level of P-myosin during muscle activation. This regulation by MgATP may underlie the "latch mechanism" in some respects.

Key words: MgATP, myosin, myosin phosphatase, protein phosphatase-1 (PP1), smooth muscle.

Smooth muscle contraction is primarily regulated through phosphorylation and dephosphorylation of the 20 kDa myosin light chain (MLC_{20}) (*1, 2*). The phosphorylation of MLC_{20} is performed by MLC_{20} kinase (MLCK), which is regulated in a Ca^{2+}/calmodulin-dependent manner. The properties of MLCK including activation by Ca^{2+}/calmodulin have been studied in detail (*3, 4*). On the other hand, a number of candidates for the smooth muscle myosin phosphatase (SMMP) involved in myosin dephosphorylation have been reported (*5*), and there was no consensus regarding the identity of SMMP until Alessi *et al.* (*6*) first isolated a heterotrimeric enzyme composed of 130, 37, and 20 kDa subunits. This phosphatase was extracted from smooth muscle myofibrils, and was able to effectively dephosphorylate phosphorylated heavy meromyosin (P-HMM) or phosphorylated myosin (P-myosin), as well as phosphorylated MLC_{20} (P-MLC_{20}), which has been preferentially used as the substrate for SMMP (*6-8*). These are the main reasons why this trimeric phosphatase is likely to be the primary SMMP *in vivo*. The 37 kDa subunit of the enzyme is a catalytic one which has been reported to be the δ-isoform (PP1Cδ) (*8*), also referred to as the β-isoform (*6*), of protein phosphatase type 1 (*9*). The other two subunits, especially the larger one, were found to be regulatory or modulatory. The large 130 kDa subunit has the ability of targeting the catalytic subunit to myosin (*8, 10*), which is responsible for specific intracellular localization(s) (*11*) and enhancement of the myosin dephosphorylating activity of PP1Cδ (*6, 12*). Due to these properties, the subunit has been named the myosin phosphatase target(ing) subunit (MYPT) (*13*).

Some nucleotides and their analogs have been found to affect the isometric force at a given Ca^{2+} concentration, and to cause changes in Ca^{2+} sensitivity in permeabilized or skinned smooth muscle preparations (*2, 13*). It has been suggested that the inhibition or activation of SMMP activity may be the mechanism underlying these effects (*14-20*). GTP, especially GTPγS, enhances the force development under certain conditions in skinned and permeabilized smooth muscle (*21-23*). The alleged mechanism was the phosphorylation of MYPT by a kinase (Rho-kinase)

[1] This work was supported in part by the Fujisawa Foundation.

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Abbreviations: AMPCPOP, adenosine 5′-(α,β-methylenetriphosphate); AMPOPCP, adenosine 5′-(β,γ-methylenetriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); DTT, dithiothreitol; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HMM, heavy meromyosin; Mes, 2-morpholinoethanesulfonic acid; MLC_{20}, 20 kDa myosin light chain; MLCK, myosin light chain kinase; Mops, 3-morpholinopropanesulfonic acid; Mopso, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; P-myosin (HMM, S1, and MLC_{20}), phosphorylated myosin (HMM, S1, and MLC_{20}); PP1, protein phosphatase type 1; PVDF, polyvinylidine difluoride; S1, subfragment 1; SDS, sodium dodecyl sulfate; SMMP, smooth muscle myosin phosphatase; TCA, trichloroacetic acid.

through the monomeric GTP-binding proteins (*14-17*). Morimoto and Ogawa (*18*) reported that acidic ADP treatment enhanced the force development in Triton X-100 skinned rabbit aorta, ascribing the effect to the direct inhibition of SMMP activity by acidic ADP. ATPγS, which is an analog of ATP, also enhanced the force output in α-toxin-permeabilized rabbit portal vein (*19*), and the phosphorylation of MYPT by an unidentified kinase, which resulted in reduced SMMP activity, was assumed to be the mechanism for this enhancement (*19, 20*). Furthermore, Lee *et al.* (*24*) recently reported that cyclic GMP decreased the isometric force. The activation of SMMP was claimed to be the likely explanation for this, although the details of the mechanism at the protein level have not been determined. Thus, these nucleotides are directly or indirectly involved in the modulation of the enzyme activity of SMMP, and are thought to be the regulators in smooth muscle contraction and relaxation. For other possible regulation of SMMP, dissociation of MYPT by arachidonic acid (*25*), which causes a reduction of SMMP activity, and involvement of a phosphorylation-dependent inhibitory protein, CPI17 (*26*), have been suggested.

The regulation of the enzyme activity mentioned above is due to modulation of the heterotrimeric phosphatase. In this report, we will show that the enzymatic activity is also regulated through the MgATP-induced conformation change of a substrate: this reagent decreases the affinity of the heterotrimeric SMMP for P-myosin or P-HMM, but not for its fragments, phosphorylated subfragment-1 (P-S1) or P-MLC_{20}. This modulation is favorable for the regulation of smooth muscle contraction. Part of the results have been presented in an abstract form (*27*).

EXPERIMENTAL PROCEDURES

Materials—AMPCPOP and AMPOPCP (Li-salt forms) were transformed to the K-salt forms using a 0.24×5.6 cm AG 50W-X8 column (Bio-Rad), followed by neutralization with KOH. A stock solution of vanadate (Vi) was prepared from sodium orthovanadate (*28*). The solution was adjusted to pH 9.0 with HCl, boiled to remove an orange-colored complex, and then cooled. This procedure was repeated until a clear solution at pH 9.0 was obtained.

Protein Preparations—All protein preparation procedures were performed at 0-4°C unless otherwise stated.

Myosin was purified from bovine stomach or chicken gizzard according to the method of Ebashi (*29*), and then subjected to hydroxylapatite column chromatography (*30*) to render it completely free from phosphatase activity. HMM (*31*), S1 (*31*), and MLC_{20} (*32*) from bovine stomach myosin were prepared as described in each reference. MLCK was usually obtained from bovine stomach (*33*), but also from chicken gizzard in some cases. Calmodulin from bovine brain was isolated as described (*34*). Small aliquots of the proteins were frozen rapidly in liquid nitrogen, and then stored at −80°C until use. Sucrose was added to most purified proteins, except calmodulin or (P-)MLC_{20}, to the final concentration of 10% (w/v) to avoid denaturation during freezing. Chicken gizzard myosin was stored at −20°C in 50% glycerol in the early stage of the experiments, but later at −80°C as usual.

SMMP was purified from chicken gizzard by the method of Alessi *et al.* (*6*) with slight modifications. In brief, muscles were homogenized with a Polytron (PT 10-35; Kinematica GmbH; blade, 20 mm in diameter) in a low salt solution and then washed twice. Crude phosphatase was extracted with a solution containing 0.3 M NaCl without a detergent. The extract was precipitated by the addition of polyethyleneglycol 6000 at the final concentration of 15%. The pellet after centrifugation was dissolved in a solution containing 0.15 M NaCl, and then the clarified supernatant was subjected to anion-exchange column chromatographies on DEAE-Sephacel and Mono Q HR 5/5 in turn. The fractions showing SMMP activities were pooled, and then applied to a cation-exchange column of SP-Sepharose Fast Flow. Finally, the enzyme was purified by affinity column chromatography on thiophosphorylated MLC_{20}-Sepharose 4B (*35*). We obtained 0.44 mg of purified SMMP from 40 g of gizzards with a yield of 19%. This result may suggest that the intracellular content of SMMP is about 1 μM, which is consistent with the values obtained under similar assumptions (*6, 7, 10*).

The purified chicken gizzard SMMP gave three bands corresponding to apparent molecular masses of 130, 37, and 20 kDa in a molar ratio of 1:1:1 on SDS-PAGE. The partial amino acid sequence of the 37 kDa band material was coincident with R190-F224 of the PP1Cδ isoform according to Sasaki *et al.* (*36*) except for C201, which was unidentified in our determination. Western blot analysis of the 130 kDa band material with anti-MYPT and anti-MLCK antibodies clearly showed that there was no contaminating MLCK in the 130 kDa band material for SMMP, at variance with the conclusion of Sobieszek *et al.* (*37*). A similar result was obtained with partially purified SMMP from bovine stomach (data not shown).

Preparation of Phosphorylated Proteins—Myosin, HMM, or S1 (1 mg/ml each) was phosphorylated for 10 min at 25°C in a solution containing 20 mM Mopso/KOH (pH 7.0 at 25°C), 5.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.3 μM calmodulin, 20 nM MLCK, 0.2 mM ATP/Tris, 0.5 mM dithiothreitol (DTT), 1 μg/ml leupeptin, and an appropriate amount of KCl to adjust the ionic strength, which was set at 0.07 M (myosin), 0.06 M (HMM), or 0.04 M (S1). Isolated MLC_{20} (1 mg/ml) was phosphorylated with 50 nM instead of 20 nM MLCK under the above conditions except for the ionic strength of 0.065 M. The reaction was terminated by the addition of 1 mM EGTA, and the medium was cooled in ice; the phosphorylated proteins were then purified by column chromatography. Phosphorylated myosin was purified on a 1.6×5 cm hydroxylapatite column as described (*30*). The eluate from the column was pooled and dialyzed thoroughly against a solution containing 50 mM NaCl, 5 mM sodium bicarbonate, 1 mM EDTA, and 0.5 mM DTT. The dialyzate was precipitated with 20 mM $MgCl_2$. After being kept on ice for 10 min, the precipitate obtained on centrifugation at $27,000 \times g$ for 10 min was resuspended in 1 mM sodium bicarbonate, 1 mM DTT, and 2 μg/ml leupeptin, and then dissolved in 0.3 M KCl, final concentration. The other phosphorylated proteins were purified on a Mono Q HR 5/5 column equilibrated with 20 mM Tris/HCl (pH 7.5 at 4°C), 0.1 mM EDTA, and 1 mM DTT. The column was washed with the same solution, and then developed with a linear gradient of NaCl to 0.5 M. The extent of phosphorylation of MLC_{20} in substrates was determined by densitometry of the two-dimensional electrophoresed gels as follows: bovine stomach P-myosin,

96.1±0.5% (mean±SE, $n=8$); P-HMM, 78.9±2.0% ($n=4$); P-S1, 93.9±0.8% ($n=5$); and P-MLC_{20}, 93.4±0.7% ($n=7$); and chicken gizzard P-myosin stored at −80°C and −20°C in 50% glycerol, 95.9±0.3% ($n=4$) and 89.9±0.7% ($n=8$), respectively.

To obtain [^{32}P]P-myosin from bovine stomach, 0.2 mM [γ-^{32}P]ATP (61.1 Bq/pmol) was used instead of non-radioactive ATP. The amount of ^{32}P incorporated into [^{32}P]P-myosin was 1.83 mol P/mol myosin.

Measurement of Phosphatase Activity—Assaying of phosphatase activity was typically carried out at 25°C in a solution composed of 20 mM Mopso/KOH (pH 7.0 at 25°C), 0.5 mM DTT, 0.2 μM bovine stomach P-myosin, 0.16-0.32 nM SMMP, 1 mM EDTA (or, alternatively, 5 mM $MgCl_2$), and an appropriate amount of KCl to adjust the ionic strength to 0.15 M. For determinations with P-MLC_{20}, however, 1.6 nM SMMP was used. The reaction medium without the enzyme had been preincubated for 2 min at 25°C. The reaction was usually started by the addition of phosphatase, and terminated at a given time (within 3 min) by the addition of an equal volume or more of 10% (w/v) trichloroacetic acid (TCA). The precipitated proteins were washed twice with 1 ml of 100% acetone to remove TCA. The resulting precipitate was dried and then dissolved in 12.5 μl of a lysis buffer [9.5 M urea, 2% (w/v) Nonidet P-40, 2.8% (w/v) Pharmalyte (pH 4.5-5.4), and 5% 2-mercaptoethanol] for two-dimensional gel electrophoresis (*38*). After centrifugation to remove insoluble materials, the supernatant was loaded onto isoelectric focusing gels, which were made in 0.1×9 cm disposable 75 μl micropipettes (Drummond Scientific), and then electrophoresed at 300 mV for 12-15 h in the first dimension. Five millimolar NaOH (anode) and 10 mM glutamic acid (cathode) were used as the electrode solutions. Then the gels were treated for 5 min with the sample buffer for SDS-PAGE, and developed on an SDS-polyacrylamide gel (4% stacking gel and 15% separating gel) in the second dimension. After staining with Coomassie Brilliant Blue, the densities of the spots of unphosphorylated and phosphorylated MLC_{20} were measured with a MasterScan Interpretive densitometer (Scanalytics, Billercia, MA, USA). The phosphorylation level of MLC_{20} was calculated with the following formula: phosphorylation level (%) = 100×(total density of MLC_{20} − density of unphosphorylated MLC_{20})/total density of MLC_{20} (*18*). The total density of smooth muscle MLC_{20} was corrected for the density of non-muscle MLC_{20} (3.9%). We confirmed the linear relationship between the densitometry and the phosphorylation level using 20 μg total myosin, which comprised a mixture of various ratios of P-myosin and unphosphorylated myosin. The time course of dephosphorylation was found to follow an exponential curve. The apparent rate constants were proportional to the amount of the phosphatase present, giving rise to a slope of 2.9 $min^{-1}\cdot nM^{-1}$. Similar results were obtained using [^{32}P]P-myosin, with a slope of 3.0 $min^{-1}\cdot nM^{-1}$, as mentioned later. The phosphatase activity can be approximated to be linear during the initial phase of myosin dephosphorylation when the remaining fraction of P-myosin is 50% or higher. Thus, the enzyme activity was thereafter determined within this range where the reaction was regarded to proceed linearly. All activities were corrected for the density without the enzyme.

Phosphatase activity was also determined using [^{32}P]P-myosin. The reaction was stopped by the addition of 10% (w/v) TCA and 5 mg/ml bovine serum albumin (BSA). The radioactivity of the supernatant obtained on centrifugation was determined by Cherenkov counting with a LS3801 scintillating counter (Beckman Instruments). Assays under the standard conditions using [^{32}P]P-myosin gave almost the same results as those on two-dimensional gel electrophoresis using P-myosin, as described for the relevant experiments.

When the kinetic parameters for various kinds of substrates were to be determined, the concentrations used were as follows: P-myosin (0.2-2 μM), P-HMM (0.2-2.7 μM), P-S1 (0.2-10.8 μM), and P-MLC_{20} (0.8-52.4 μM). The highest concentration of each substrate was at the upper limit, which was set by keeping the ionic strength of the medium constant. Aliquots of samples which contained nearly equal amounts of MLC_{20} were subjected to two-dimensional gel electrophoresis. The results were best fit to the equation, $V = V_{max}\cdot C^{n_H}/(K_m^{n_H} + C^{n_H})$, where V, C, V_{max}, K_m, and n_H are the activity, the substrate concentration, the maximum activity, the apparent dissociation constant and the Hill coefficient, respectively. n_H was usually unity except in the case where P-myosin and MgATP were present. V_{max} was assumed to be unchanged by MgATP with bovine stomach P-myosin.

Sedimentation Assaying of Myosin—Sedimentation assaying was carried out to determine the extent of myosin assembly as previously described (*39*) in a solution with the same composition as that used for the determination of SMMP activity except that the enzyme was omitted. After incubation for 2 min at 25°C, the mixture was centrifuged in a TL-100 (Beckman Instruments) at 150,000×g for 10 min. The protein concentration of the resulting supernatant was determined by the Bradford method (*40*), the amount of sedimented protein being calculated.

Protein Assay—The protein concentrations of MLCK and SMMP were determined by means of the amido black dye-binding assay according to Kaplan and Pedersen (*41*), using BSA as a standard. The concentrations of myosin, MLC_{20}, calmodulin and BSA were determined by spectrophotometric measurements using the following % absorption coefficients ($E^{1\%}$): 5.2 at 280 nm for myosin (*42*); 3.37 at 277 nm for MLC_{20} (*32*); 2.00 at 277 nm for calmodulin (*43*); and 6.3 at 280 nm for BSA (*44*). The protein concentrations of HMM and S1 were densitometrically determined on Coomassie Brilliant Blue-stained SDS-polyacrylamide gels in terms of myosin, which would give an equivalent density for MLC_{20}. The molar concentrations of proteins were determined with the following molecular weight values: SMMP (170,000); myosin (500,000); HMM (334,000); S1 (130,000); and MLC_{20} (20,000).

Antibody Production and Affinity Purification—Polyclonal antibodies against chicken gizzard MYPT (anti-MYPT) and bovine stomach MLCK (anti-MLCK) were raised in male Japan White rabbits. Antibody production and affinity purification of the sera were carried out as described (*45*). The purified antibodies were concentrated with Centriprep 30 (Amicon) after the addition of BSA, followed by the addition of 0.1% sodium azide. Small aliquots of the concentrated antibodies were quickly frozen in liquid nitrogen and stored at −80°C.

SDS-PAGE and Western Blot Analysis—The protein composition was analyzed on a 5-15% polyacrylamide

linear gradient slab gel (*46*). The gel was stained with Coomassie Brilliant Blue R-250, and destained with 10% acetic acid. When Western blot analysis was performed, the separated proteins on a SDS-polyacrylamide gel were transferred to a PVDF membrane (*47*). After processing the membrane by the routine procedure, we detected positive bands according to the method involving 3,3′-diaminobenzidine (*48*).

RESULTS

General Properties of SMMP as an Enzyme—Because the enzymatic activity may be affected by the ionic strength, pH, temperature and so on, we examined the effects of these common factors on SMMP activity.

Ionic Strength—The activity was strongly dependent on the ionic strength of the medium (Fig. 1A). The maximum activity was observed around the ionic strength of 0.1 M, and the activity markedly decreased on either side of this optimum. When the ionic strength was higher than 0.21 M, however, the activity gradually decreased, being about 16% at 0.21 M and 5% at 0.49 M. Standard determinations were thereafter performed with the constant ionic strength of 0.15 M, which is similar to that of the cytoplasm, at which the activity was about 60% of the maximum level.

pH—The pH dependence of SMMP activity was so broad that pH 6.0-7.2 was optimal. While the activity decreased sharply in the more acidic range to pH 6, it gradually decreased in the more alkaline range to pH 7.2 (data not shown). The standard condition is pH 7.0.

Temperature—The Arrhenius plot was linear between 15 and 40°C, giving an activation energy of 10.4 kcal/mol (Fig. 1B). The Q_{10} value between 25 and 40°C was then calculated to be about 2. This value was quite similar to that for skeletal muscle PP1C (*49*), but it was different from the value of about 5 obtained for myosin-associated protein phosphatase, an alleged protein phosphatase preparation, as reported by Mitsui *et al.* (*49*). A large Q_{10} value has also been reported for the dephosphorylation rate in α-toxin permeabilized smooth muscle (*49*). Because it is well known that SMMP is vulnerable to cleavage by proteinases (*6*), their enzyme specimen was quite likely somewhat modified during the purification steps or during the preparation of the permeabilized smooth muscle. This inference is supported by the finding that their purified phosphatase, in contrast to the crude one, did not bind to myosin (*35*). It should be noted that the Q_{10} value of 2 for SMMP is very similar to that for chicken gizzard MLCK (*50*).

Divalent Cations—Because the sensitivity to divalent cations can be used as a criterion for the classification of protein phosphatases (type 1 *vs.* types 2A, 2B, and 2C), we examined the effects of divalent cations on the enzyme activity of SMMP. Neither Mg^{2+} (0.1-5 mM), Ca^{2+} (0.1 μM-5 mM), Mn^{2+} (1 μM-1 mM), Co^{2+} (0.01-1 mM), nor Ni^{2+} (0.01-0.1 mM) had any significant effect, whereas Zn^{2+} and Cd^{2+} inhibited the enzyme activity at relatively low concentrations. The IC_{50} values were determined to be about 70 and 5 μM, respectively. With P-MLC_{20} as a substrate, however, the IC_{50} values increased to 200 and 40 μM, respectively. This indicated that the effects of Zn^{2+} and Cd^{2+} on the SMMP activity were, in part, ascribable to the conformational change in P-myosin that might be induced by these cations.

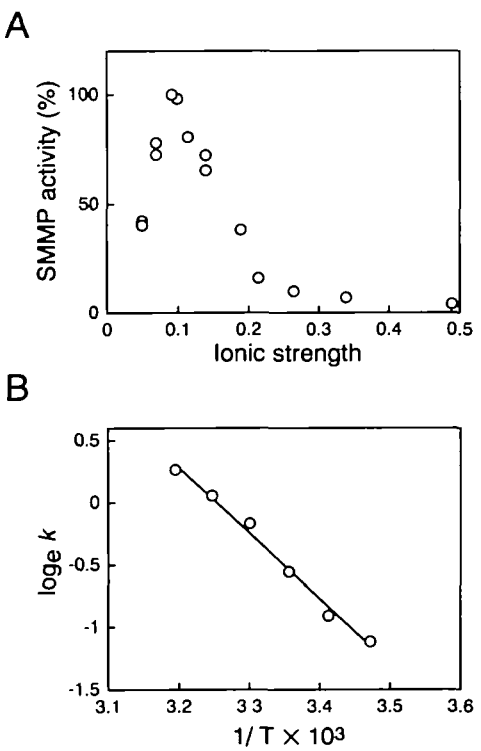

Fig. 1. **Effects of the ionic strength and temperature on SMMP activity.** A: Effect of the ionic strength on SMMP activity. Each assay was performed at 25°C in a solution of 20 mM Mopso/KOH (pH 7.0), 1 mM EDTA, 0.5 mM DTT, 0.2 μM bovine stomach P-myosin, 0.32 nM chicken gizzard SMMP, and various ionic strengths, adjusted with KCl (0.050-0.49 M). Activities were determined as described under "EXPERIMENTAL PROCEDURES," and are expressed relative to that at the ionic strength of 0.091 M (100%=8.1 μmol/min/mg protein). B: Arrhenius plot for SMMP activity. The assay was performed in medium similar to that in (A), but with a constant ionic strength of 0.15 M. The temperature is indicated in the figure (15, 20, 25, 30, 35, and 40°C). The slope shown gives an activation energy (E_A) of 10.4 kcal/mol. T, absolute temperature; k, apparent rate constant of dephosphorylation.

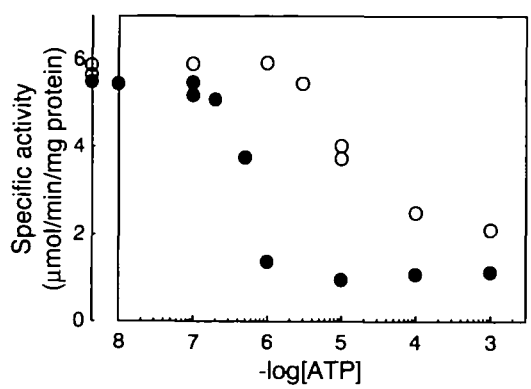

Fig. 2. **Effect of ATP on SMMP activity.** The assay was carried out under the following conditions for 1 min at 25°C: 20 mM Mopso/KOH (pH 7.0), 0.5 mM DTT, 0.2 μM bovine stomach P-myosin, 0.16 nM chicken gizzard SMMP, and an appropriate amount of KCl to keep the ionic strength constant (I=0.15 M), containing 0-1 mM ATP in the presence of 1 mM EDTA (○) or 5 mM $MgCl_2$ (●). Activity was determined as described under "EXPERIMENTAL PROCEDURES."

Okubo *et al.* (*51, 52*), in contrast, reported that their myosin-bound phosphatase, which was a dimer consisting of the N-terminal half of the 130 kDa regulatory subunit (MYPT) and PP1Cδ, was activated by Co^{2+} or Mn^{2+}. Interestingly, according to Zhang *et al.* (*53*), the PP1Cδ isoform expressed in *Escherichia coli* also required Mn^{2+} for the enzyme activity. Because our SMMP was not dependent on Mn^{2+} or Co^{2+} for the activity (at least up to 1 mM), these findings may indicate that the Mn^{2+} or Co^{2+} dependence of SMMP is determined by the coexisting modulatory subunits.

Inhibitors—Protein phosphatase can be characterized as to its sensitivity to its inhibitors, okadaic acid and microcystin-LR, which reduce the activity in a reversed S-shaped dose-dependent manner. The IC_{50} values for okadaic acid and microcystin-LR giving half the maximal inhibition were 16 and 0.16 nM, respectively, when the reaction was started by the addition of P-myosin. When the reaction was started by the addition of SMMP, the IC_{50} values were 37 and 8 nM, respectively. This difference can be accounted for by the very slow interaction between SMMP and inhibitors. These results show that the heterotrimeric SMMP is functionally classified as protein phosphatase type 1. This conclusion corresponded to the results as to the primary amino acid sequence, that was reported previously (*6*).

Effect of ATP on SMMP Activity towards P-Myosin—As shown in Fig. 2, ATP concentration-dependently inhibited SMMP activity toward 0.2 μM bovine stomach P-myosin in the presence of 1 mM EDTA (open circles) or 5 mM $MgCl_2$ (filled circles). The IC_{50} values for free ATP and MgATP were determined to be about 10 and 0.6 μM, respectively (Fig. 2 and Table I). MgATP was about 20 times more potent than free ATP. These findings were also observed with [^{32}P]P-myosin. We also examined the inhibitory effects of phosphate compounds including nucleotides and V_i with 0.2 μM bovine stomach P-myosin as the substrate (Table I). Among the ATP analogs, AMPCPOP was potent, although the IC_{50} value was larger. AMPOPCP, however, did not show effective inhibition, nor was inhibition by ATPγS or MgATPγS (up to 1 mM each) observed. This indicates that the mechanism for the inhibition by MgATP cannot be due to the phosphorylation of MYPT (see "DISCUSSION"). The inhibition by ITP or GTP was much less than that by ATP. Free ADP, MgADP, and free AMP also showed much weaker effects on SMMP activity. Cyclic AMP (up to 1 mM) or cyclic GMP (up to 0.1 mM) by itself did not inhibit SMMP activity. Furthermore, the IC_{50} values for PP_i, P_i, and V_i were much higher than that for ATP. These results indicate that MgATP is specifically effective in inhibiting the enzyme activity of SMMP.

To deepen the understanding of the mechanism of the inhibition by MgATP, similar experiments with various amounts of P-myosin were carried out. The phosphatase activity in the absence of MgATP was dependent on the amount of P-myosin, which showed a Michaelis-Menten type relationship (filled circles in Fig. 3). This is consistent with the notion that the two heads of myosin are independent of and homogeneous with each other. The K_m and V_{max} values obtained were 0.45 μM (0.9 μM in terms of the concentration of the head) and 25.2 μmol/min/mg protein, respectively, in the medium with an ionic strength of 0.15 M at pH 7.0 and 25°C (Tables II and III). The activity in the

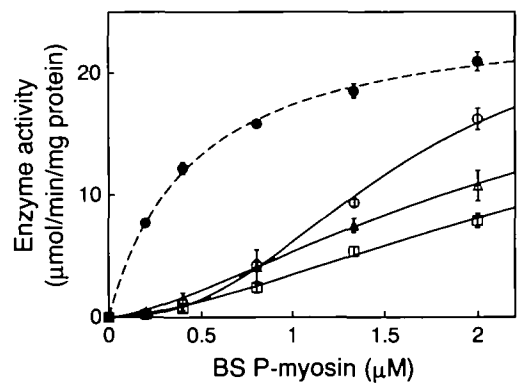

Fig. 3. **SMMP activity toward bovine stomach P-myosin in the presence and absence of MgATP.** The assay was carried out at 25°C in a solution of 20 mM Mopso/KOH (pH 7.0), 5 mM $MgCl_2$, 0.5 mM DTT, 0.2-2 μM bovine stomach P-myosin, 0.16 nM chicken gizzard SMMP, and an appropriate amount of KCl (I=0.15 M), in the presence of 0 μM (●), 10 μM (○), 0.1 mM (△), or 1 mM (□) MgATP. The results in the absence of MgATP (broken line) were best fit by a Michaelis-Menten type equation, whereas those in its presence (solid lines) were accounted for by Hill equations with appropriate K_m and n_H values under the assumption of an unchanged V_{max} value (see "EXPERIMENTAL PROCEDURES" and Table II). Error bars indicate SE of three or four determinations or the half-range of the deviation for duplicate determinations; no error bars are shown in cases where the deviation is within a symbol.

TABLE I. **Inhibitory effects of phosphate compounds including nucleotides and V_i.** Assays were carried out as shown in Fig. 2, with various concentrations of the reagents indicated in place of ATP. The concentration ranges of the reagents used are indicated in parentheses when effects were not detectable (ND). cAMP, cyclic AMP; cGMP, cyclic GMP.

Reagent	IC_{50} (mM)	
	+1 mM EDTA	+5 mM $MgCl_2$
ATP	0.01	0.0006
AMPCPOP	0.02	0.002
AMPOPCP	ND (10 μM-1 mM)	ND (10 μM-1 mM)
ATPγS	ND (10 μM-1 mM)	ND (10 μM-1 mM)
ITP	0.3-0.5	~0.01
GTP	0.2-0.3	Erratic[a]
ADP	2-3	~0.1
AMP	>10	–
cAMP	ND (0.1 μM-1 mM)	–
cGMP	ND (0.1 μM-1 mM)	–
PP_i	2-3	–
P_i	>10	–
V_i	2	–

[a]No effect at 10 μM, but half the maximum at 100 μM or higher.

TABLE II. **Effects of MgATP on K_m and the Hill coefficient of SMMP for P-myosin.** The assay was carried out as described in Fig. 3. The parameters for the apparent dissociation constant (K_m) and Hill coefficient (n_H) were obtained from the best-fit curve under the assumption of an unchanged V_{max} (25.2 μmol/min/mg protein). The data are means ± SE (number of determinations) or half-ranges of the deviation for duplicate determinations.

[ATP] (μM)	K_m (μM)	n_H
0	0.45±0.04 (3)	1.0
10	1.60±0.07 (2)	2.39±0.03 (2)
100	2.4 ±0.2 (2)	1.5 ±0.1 (2)
1000	3.2 ±0.1 (2)	1.57±0.06 (2)

TABLE III. **Enzymatic properties of SMMP with various kinds of substrates in the presence and absence of MgATP.** Assays were carried out as described in Figs. 3 and 4. The data are means±SE (number of determinations) or half-ranges of the deviation for duplicate determinations. For details, see "EXPERIMENTAL PROCEDURES."

Substrate	−ATP			+0.1 mM ATP		
	V_{max} (U/mg protein)[b]	K_m (μM)	n_H	V_{max} (U/mg protein)[b]	K_m (μM)	n_H
P-myosin[a]	25.2±0.6(3)	0.91±0.08(3)	1.0	25.2[c]	4.8±0.5(2)	1.5
P-HMM[a]	20.1±1.0(2)	2.9 ±0.6(2)	1.0	22.3±0.7(2)	5.3±0.1(2)	1.0
P-S1	34.5±0.6(2)	5.0 ±0.6(2)	1.0	36.2±0.1(2)	5.3±0.4(2)	1.0
P-MLC_{20}	5.0±0.4(2)	5.3 ±0.3(2)	1.0	5.1±0.4(2)	5.7±0.3(2)	1.0

[a]Expressed in terms of single heads. [b]1 U = μmol dephosphorylated MLC_{20}/min. [c]Assumed to be unchanged.

presence of 10 μM MgATP (open circles), however, showed cooperative dependence on the P-myosin concentration. As the concentration of MgATP increased, the apparent affinity for P-myosin decreased further, although the dependence on the P-myosin concentration appeared less steep (Fig. 3). MgATP markedly inhibited SMMP activity with a low concentration of P-myosin (*e.g.*, 0.2 μM, as shown in Fig. 2), but the extent of the inhibition was much less with a high concentration of the substrate (*e.g.*, 2 μM). Because the enzyme activity with 2 μM P-myosin in the presence of 10 μM MgATP came close to the value in the absence of MgATP, V_{max} was likely to be unchanged regardless of the presence of MgATP. Under the assumption of an unchanged V_{max}, the kinetic parameters for the results in Fig. 3 were simulated according to the Hill equation as described under "EXPERIMENTAL PROCEDURES." The evaluated K_m and Hill coefficient values were 1.6 μM and 2.4 in the presence of 10 μM MgATP; 2.4 μM and 1.5 in the presence of 0.1 mM MgATP; and 3.2 μM and 1.6 in the presence of 1 mM MgATP, respectively (Table II). The K_m values gradually increased with an increase in the MgATP concentration. The Hill coefficient values were 1.5-2.4 in the presence of MgATP, which was larger than unity in the absence of MgATP. The larger Hill coefficient value indicates that the two heads of myosin may not be independent of each other in the presence of MgATP. Similar results were obtained with [^{32}P]P-myosin. In the absence of MgATP, K_m and V_{max} were 0.49 μM and 21.1 μmol/min/mg protein, respectively, following Michaelis-Menten kinetics. In the presence of 10 μM MgATP, K_m increased to 1.2 μM with a Hill coefficient of 1.7, the V_{max} value being unchanged.

Experiments with Myosin Fragments—Because the results mentioned above suggest that the conformation change(s) in myosin molecules may be critical for the activity of SMMP, we examined the effect of MgATP on the enzymatic activity toward myosin fragments.

P-HMM—When P-HMM was used as the substrate, the relationship between the P-HMM concentration and the enzyme activity of SMMP followed Michaelis-Menten type kinetics, irrespective of the presence or absence of 0.1 mM MgATP (Fig. 4A). The V_{max} (μmol/min/mg protein) and K_m (μM) values with P-HMM at 25°C were calculated to be 20.1 and 1.4 (2.9 μM in terms of the head concentration) without MgATP, and 22.3 and 2.6 (5.3 μM head concentration) with 0.1 mM MgATP, respectively (Table III). The K_m value in the presence of 0.1 mM MgATP was about twice as high as that in its absence, indicating that the probable reason for the inhibitory effect of MgATP on P-HMM is an increase in K_m for SMMP. The interaction between the two heads in HMM might be much weaker.

P-S1 and P-MLC_{20}—We also determined the dependence of the enzyme activity of SMMP on the P-S1 concentration (Fig. 4B). The relationships in both the presence and absence of MgATP followed Michaelis-Menten type kinetics. No inhibition by MgATP, however, was observed with P-S1. The V_{max} (μmol/min/mg protein) and K_m (μM) values with P-S1 at 25°C were determined to be 34.5 and 5.0 without MgATP, and 36.2 and 5.3 with 0.1 mM MgATP, respectively (Table III). These values with or without MgATP were not significantly different. A similar experiment was also performed with P-MLC_{20} as a substrate, which also indicated that MgATP did not affect the enzyme activity of SMMP. The V_{max} (μmol/min/mg protein) and K_m (μM) values with P-MLC_{20} were 5.0 and 5.3 without MgATP, and 5.1 and 5.7 with 0.1 mM MgATP, respectively (Table III), and again there was no significant

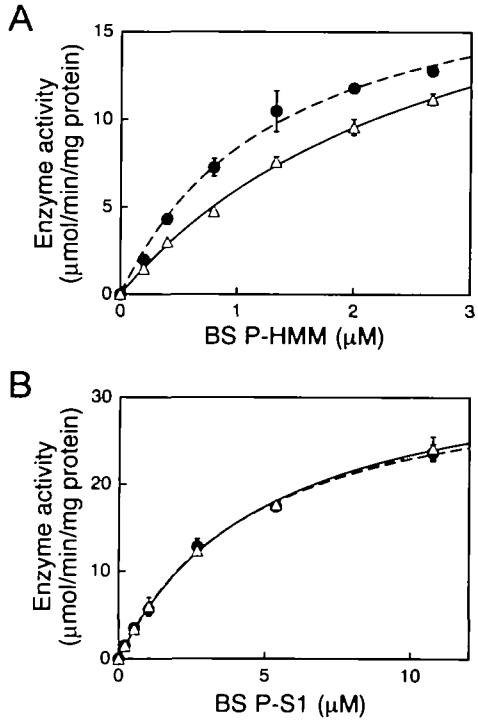

Fig. 4. **SMMP activity toward bovine stomach P-HMM (A) or P-S1 (B) in the presence (△) and absence (●) of MgATP.** The assay conditions are given in the legend to Fig. 3, except for bovine stomach P-HMM (0.2-2.7 μM) and P-S1 (0.2-10.8 μM). The results are best fit to lines with $n_H = 1$, and appropriate values of K_m and V_{max} (Table III) in the presence (solid line) and absence (broken line) of 0.1 mM MgATP. Error bars indicate SE of triplicate determinations or the half-range of the deviation for duplicate determinations; no error bars are shown in cases where the deviation is within a symbol. The result for 0.8 μM P-HMM in the presence of 0.1 mM MgATP is for a single determination.

change in the parameter values with or without MgATP. It should be noted that P-MLC_{20} was the poorest among the four substrates examined with respect to K_m and V_{max}.

In the absence of ATP, the K_m values were in the order of P-myosin < P-HMM < P-S1 = P-MLC_{20}. V_{max} for P-S1 was highest among the four substrates examined, whereas V_{max} for P-MLC_{20} was lowest. These observations suggest that not only the head, but also the rod region of myosin is responsible for the inhibitory effect of MgATP on SMMP activity. The integrity of myosin molecules is crucial for the proper function of SMMP.

Effect of Coexisting Unphosphorylated Myosin—As shown in Fig. 3, 10 μM MgATP reduced the phosphatase activity toward 0.2 μM P-myosin to 20% or less of the control value in the absence of MgATP, whereas the enzyme activity retained with 2 μM P-myosin was as high as about 80% of the control value. The next question was how much the reduction was with 0.2 μM P-myosin in addition to 2 μM unphosphorylated myosin. Determinations by densitometry were impossible because an excess amount of MLC_{20} derived from myosin interfered with the density of P-MLC_{20} derived from P-myosin. Therefore, [^{32}P]P-myosin was exclusively used for these experiments. As shown in Fig. 5, the enzyme activity toward 0.2 μM [^{32}P]P-myosin was 5.68 ± 0.05 μmol/min/mg protein (mean $\pm$ SE, $n=5$) in the absence of MgATP, whereas it was 1.49 ± 0.08 μmol/min/mg protein ($n=5$) in the presence of 10 μM MgATP. When 2 μM unphosphorylated myosin was added in addition to 0.2 μM [^{32}P]P-myosin, the enzyme activities in the presence and absence of 10 μM MgATP were 1.36 ± 0.05 ($n=5$) and 3.97 ± 0.11 ($n=5$) μmol/min/mg protein, respectively. The reduced activity in the presence of 10 μM MgATP was not affected by the presence of 2 μM unphosphorylated myosin.

Unphosphorylated myosin, however, slightly decreased the enzyme activity of SMMP in the absence of MgATP. Since the presence of 1 μM unphosphorylated myosin gave an activity level of 5.0 μmol/min/mg protein, this weak inhibition by unphosphorylated myosin appeared to be concentration-dependent. These findings are consistent with the results reported by Shirazi *et al.* (7). Because similar results were obtained using myosin which had been preincubated with SMMP, the effect of non-radioactive phosphorylated myosin, which might contaminate the myosin preparation used, cannot be the reason for the decrease in the activity (data not shown). This is also supported by the finding that the phosphatase activity in the presence of MgATP was similar with or without unphosphorylated myosin. Ichikawa *et al.* (*12*) showed that SMMP bound to unphosphorylated myosin and that MgATP reduced this interaction, the ability of the enzyme to bind P-myosin being retained. Our results are consistent with their conclusions.

Similar Modulation by MgATP of SMMP Activity and Myosin Assembly—We have so far examined the enzymatic properties of chicken gizzard SMMP toward bovine stomach P-myosin as a substrate. To determine whether or not the inhibitory effect of MgATP is also observed for P-myosin from another source, we prepared chicken gizzard P-myosin using two distinct procedures. One lot was prepared from myosin which had been stored at -80°C, and the other from myosin stored at -20°C in 50% glycerol. As shown in Fig. 6A, the SMMP activity toward 0.2 μM chicken gizzard P-myosin derived from myosin stored at -80°C was markedly reduced by MgATP (filled circles),

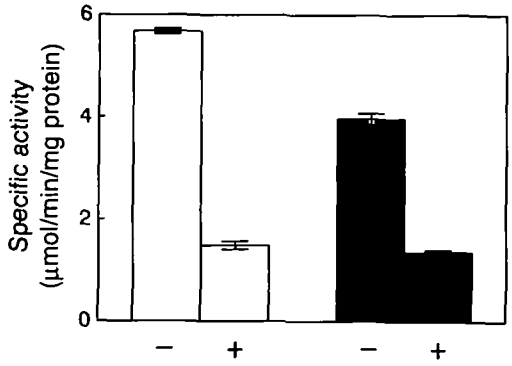

Fig. 5. **Effect of unphosphorylated myosin on SMMP activity.** The assay was carried out at 25°C as in Fig. 3, except for [^{32}P]P-myosin, with (shaded bars) or without (open bars) 2 μM unphosphorylated myosin. (+) and (−) indicate the presence and absence of 10 μM MgATP, respectively. Error bars indicate SE of five determinations. Phosphatase activity was significantly reduced with coexisting unphosphorylated myosin in the absence of MgATP ($p<0.001$), whereas the decreased activity in the presence of MgATP was unchanged by unphosphorylated myosin.

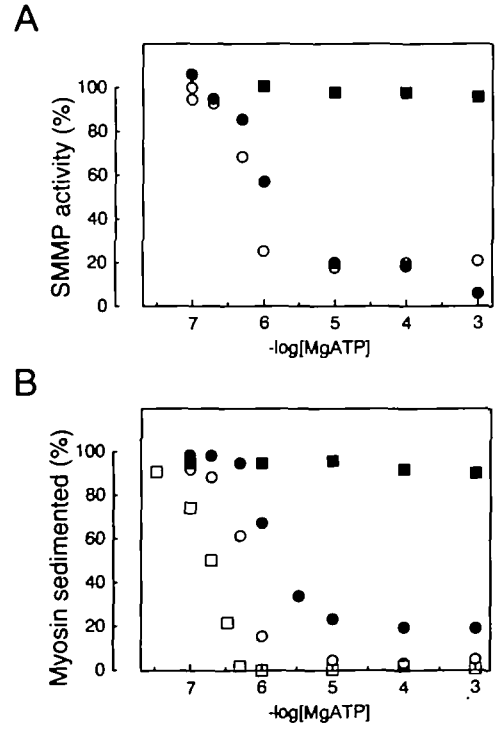

Fig. 6. **Effect of MgATP on SMMP activity (A) or the extent of myosin assembly as determined on sedimentation (B) using distinct myosin sources.** A, SMMP activity. Experiments were carried out as in Fig. 2. In these determinations, bovine stomach P-myosin (○) was prepared from myosin stored at -80°C, whereas chicken gizzard P-myosin was prepared from myosin stored at -80°C (●) and -20°C in 50% glycerol (■). A value of 100% corresponds to 5.5 (○), 4.9 (●), and 3.4 (■) μmol/min/mg protein, respectively. B, sedimentation of myosin. Experiments were performed in the same solution except for the omission of the enzyme. The symbols are the same as in A, except for □, which refers to unphosphorylated bovine stomach myosin.

and the IC_{50} value for MgATP was about 1 μM, which was similar to the value with bovine stomach P-myosin (open circles in Fig. 6A, and also see Fig. 2). These results indicated that the inhibition by MgATP occurred irrespective of the source of P-myosin. Surprisingly, however, no effect of MgATP was observed with the other lot of chicken gizzard P-myosin derived from myosin stored at -20°C in 50% glycerol (filled squares). Despite the marked difference in the results with these gizzard P-myosins, the kinetic parameters in the absence of MgATP were very similar: the V_{max} (μmol/min/mg protein) and K_m (μM) values were 26.3 ± 1.1 ($n=3$) and 0.83 ± 0.03 μM ($n=3$) with the former lot of chicken gizzard P-myosin, and 22.8 ± 0.6 ($n=2$) and 1.04 ± 0.07 ($n=2$) with the latter lot, respectively. These parameters were close to the values with bovine stomach P-myosin although the K_m values with gizzard P-myosin were about twice as high as those with the bovine stomach specimen. Similar results were obtained with a crude SMMP preparation from bovine stomach: the SMMP activity toward P-myosin from bovine stomach myosin was decreased by MgATP.

Because it is well known that MgATP influences the molecular conformation of myosin molecules and their assembly (*54, 55*), we examined the sedimentation of myosin in the presence of various concentrations of MgATP. As shown in Fig. 6B, MgATP concentration-dependently reduced the sedimented fraction of 0.2 μM bovine stomach P-myosin (open circles), closely paralleling the effect on SMMP activity (Fig. 6A) with the same IC_{50} value of 0.6 μM. With unphosphorylated myosin from bovine stomach, similar results were obtained (open squares). The potency of MgATP, however, was threefold that with P-myosin. Chicken gizzard P-myosin derived from myosin stored at -80°C (filled circles) similarly showed marked reduction of the sedimented fraction in the presence of MgATP ($IC_{50}\cong1.3$ μM), and chicken gizzard P-myosin derived from the specimen stored at -20°C (filled squares) was unchanged by MgATP. These findings correspond quite well to the results as to phosphatase activities in Fig. 6A. These lines of evidence indicate that MgATP causes conformational change(s) of myosin molecules, affecting not only the enzyme activity of SMMP but also the extent of myosin assembly. It should be noted that the storage of chicken gizzard myosin at -20°C in 50% glycerol is likely to cause some subtle modification(s) in myosin molecules or the loss of some crucial factors, which may lead to some different conclusions.

DISCUSSION

We have shown that MgATP reduces the affinity of SMMP for P-myosin or P-HMM, but not for P-S1 or P-MLC_{20}. This modulation is entirely new in the respect that the affected molecules are P-myosin (the substrate), not SMMP (the enzyme), which has been the subject of thorough investigations. During the course of our study, we confirmed the previous classification of the heterotrimeric enzyme, which was free from MLCK, as PP1 on structural and functional bases, and the finding that it dephosphorylated not only P-MLC_{20} but also P-myosin. The following new findings are also added to the general characteristics of the dephosphorylation reaction by SMMP. First, the dephosphorylation reaction strongly depends on the ionic strength of the medium. Therefore, we had to compensate for the concentrations of salts which may be carried over when P-myosin or its fragments were used as substrates to keep the ionic strength in the medium constant. In this study, the ionic strength was kept at 0.15 M, which was close to that of the cytoplasm; under this condition, the enzyme activity is about 60% of the value at the optimum ionic strength. Second, the Q_{10} value of the dephosphorylation reaction was about 2, which was similar to that for MLCK, but not 5 (*49*). Finally, and equally important, the kinetic parameters for SMMP differed among the four substrates examined (P-myosin, P-HMM, P-S1, and P-MLC_{20}), all of which followed Michaelis-Menten type kinetics in the absence of MgATP. P-myosin was a suitable substrate with the highest affinity and a large V_{max}. P-MLC_{20} was the poorest substrate among the four substrates examined, showing the lowest affinity and V_{max} values. The V_{max} value of 25 μmol/min/mg protein at 25°C with P-myosin was 4–6 times as high as the results reported by Ichikawa *et al.* (*12, 20*), and more than 20 times the value reported by Shirazi *et al.* (*7*). A likely reason for this discrepancy is the possible increase in the ionic strength in their determinations.

The SMMP activity toward 0.2 μM P-myosin was reduced in the presence of MgATP. ATP in the presence of 1 mM EDTA was 20-fold less potent than MgATP. MgAMP-CPOP was one-third as potent as MgATP. The other nucleotides were weak or ineffective. The lack of an effect of ATPγS indicates that the underlying mechanism is not due to phosphorylation of some molecules, as postulated in the case of MYPT. The enzyme activity was not affected by MgATP when P-S1 or P-MLC_{20} was used as the substrate. The enzyme, however, showed decreased affinity for P-myosin or P-HMM in the presence of MgATP. Interestingly, P-myosin prepared from chicken gizzard myosin stored in 50% glycerol at -20°C was immune to MgATP, while the substrate prepared from chicken gizzard myosin stored at -80°C was sensitive to MgATP. This distinct behavior of P-myosin was observed not only in the phosphatase activity but also in myosin assembly, as determined by sedimentation assaying of myosin. Some unknown but crucial factor(s) may be missing or a subtle modification of myosin molecules may occur during storage in glycerol.

Ichikawa *et al.* (*12*) reported that SMMP bound unphosphorylated myosin in the absence of MgATP, but less strongly in its presence. SMMP can, however, bind thiophosphorylated myosin even in the presence of MgATP. They also showed that the holoenzyme bound thiophosphorylated MLC_{20} irrespective of the presence or absence of ATP; and consistently reported that the enzymatic activity toward P-myosin or P-MLC_{20} was not affected by MgATP. It should be mentioned that the complex formation on affinity column chromatography, as shown by Ichikawa *et al.* (*12*), does not necessarily indicate the enzyme-substrate complex. With P-MLC_{20}, we confirmed their results (see Fig. 4B). With P-myosin, however, their results were not necessarily compatible with ours. Although the chicken gizzard P-myosin preparation (■) in Fig. 6A was in agreement, another P-myosin preparation (●) as well as bovine stomach P-myosin (○) was affected by MgATP. This clearly shows that the quality of P-myosin is critical for this difference. The results in Fig. 6A suggest that chicken gizzard myosin might be more vulnerable to deterioration

than bovine stomach myosin.

It is interesting that the effect of MgATP differed among the four substrates, although the direct target was P-MLC_{20}. Neither P-S1 nor P-MLC_{20} was affected. P-myosin and P-HMM were differently affected by MgATP (Figs. 3 and 4). These results show that the proper integrity of myosin molecules is indispensable for studying SMMP regulation.

Because our determinations were carried out under conditions near those in the cytoplasm, the *in vivo* activity of dephosphorylation by SMMP can be estimated. The tissue concentration of ATP was reported to be 0.5-2 μmol/g wet weight (*56*), which would correspond to 1.5-6 mM in the myoplasm under the assumption that 50% of the tissue would be the extracellular water space and that about 70% of the cell would be the water-accessible space. The intracellular concentration of MgATP can thus be assumed to be 1.5-6 mM, because most ATP is in the Mg-form. The intracellular concentration of myosin is estimated to be 30-50 μM, the reported levels of phosphorylated myosin being about 10% (3-5 μM) at rest and about 50% (15-25 μM) in the activated state, although the results as to the phosphorylation level varied among the investigators (*4*). Figure 5 shows that the SMMP activity primarily depends on the concentration of P-myosin in the presence of MgATP. The results in the presence of 1 mM MgATP in Fig. 3 and Table II will give rise to the highest estimate of the myoplasmic SMMP activity in the presence of several millimolar MgATP, because the effect of MgATP depends on its concentration up to at least 1 mM. The calculated activity is expected to be about 0.5 V_{max} or less at rest and to be enhanced to near V_{max} in the activated state. Assuming that the molecular mass and the intracellular concentration of SMMP are about 170 kDa and 1 μM, respectively, its total cellular activity of V_{max} (25 μmol/min/mg protein at 25°C) corresponds to about 4.3 μmol/ml/min at 25°C. Then, the apparent rate constant for dephosphorylation in the first-order kinetics would be calculated to be 0.7-1.2 s^{-1} at 25°C. This estimate is similar to the recently reported determinations with permeabilized smooth muscle fibers when corrections were made using $Q_{10}=2$ (*57, 58*). These dephosphorylation rate values are comparable in magnitude to the total cellular phosphorylation rate for MLCK in the presence of sufficient Ca^{2+}: $V_{max}=15$ μmol/min/mg protein at 24°C (*59*) would give rise to 4-6 μmol/ml/min or 1-1.5 s^{-1} under the assumptions that the molecular mass and the intracellular content of MLCK are 130 kDa and 2-3 μM, respectively. The same conclusion will be reached at 37°C, physiological temperature, because the Q_{10} values ($\sim$2) for SMMP and MLCK are almost the same. The similar rate constants for phosphorylation and dephosphorylation may lead to the conclusion that the maximum steady state phosphorylation level of myosin is around 50%, and thus far from 100%. This conclusion is consistent with the results obtained for intact and skinned smooth muscles (*4*). This also suggests that ATP consumption by the phosphorylation-dephosphorylation cycle largely contributes to total ATP hydrolysis during muscle contraction.

In the resting state, where only a minor fraction of myosin is phosphorylated in the presence of a large fraction of unphosphorylated myosin, the dephosphorylating activity of SMMP is expected to be in a reduced state, as mentioned above, partly because of the binding of SMMP to myosin, and partly because of the effect of MgATP on P-myosin (see Fig. 5). When smooth muscles are stimulated, increased [Ca^{2+}] activates the MLCK activity under the condition of reduced activity of SMMP, resulting in the increased fraction of phosphorylated myosin. As the fraction of phosphorylated myosin increases, the SMMP activity will increase, because the inhibiting mechanisms are removed. A sufficient amount of P-myosin will compensate for the reduction in the affinity of SMMP for P-myosin, which is caused by MgATP, and enzymatic activity near V_{max} will be attained. It is quite likely that transient overshooting of the phosphorylation level may occur if the transition of the P-myosin conformation from the lowered to the stimulated state occurs slowly. In the above discussion, we assumed an a priori homogeneous distribution of myosin within smooth muscle cells; the actual localization of myosin may be uneven, however, because it may form bundles. This indicates that the intracellular SMMP activity may be higher than the expected value in the region of more concentrated myosin where the attainable level of P-myosin would be higher, whereas in the less concentrated region the SMMP activity may be lower. This may cause dissociation between the averaged phosphorylation level and the averaged extent of interaction between P-myosin and actin. This means that the activity of SMMP in the presence of MgATP may not be homogeneous spaciotemporarily throughout the cytoplasm, and may explain some aspects of the so-called "latch mechanism."

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