Effects of Phosphorylation, Calcium Ion, and Tropomyosin on Actin-Activated Adenosine 5′-Triphosphatase Activity of Mammalian Smooth Muscle Myosin†

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ABSTRACT: Actomyosin isolated from bovine stomach muscle contains the endogenous light-chain kinase and phosphatase. Myosin can be separated from other proteins by gel filtration on a Sepharose 4B–agarose column. The amount of phosphate covalently bound to the 20 000-dalton light chains of purified myosin can be controlled by phosphorylation or dephosphorylation using the endogenous enzymes prior to column purification. The purified myosin can serve as a substrate for exogenously added light-chain kinase and phosphatase, but the myosin itself is free of the activities for both enzymes. The adenosine 5′-triphosphatase (ATPase) activity of myosin was activated by rabbit skeletal muscle actin only when the 20 000-dalton light chain was phosphorylated. The level of activation correlated with the amount of phosphate bound to the light chain. The maximum activation by pure actin was observed when the molar ratio of myosin to actin was 1:20. The activation was dependent on the amount of phosphate bound to the myosin light chain at all levels of actin concentrations. The actin-activated ATPase activity of stomach muscle myosin is not dependent on Ca^{2+} concentration once the myosin is phosphorylated and is free of kinase and phosphatase activity. The actin-activated ATPase activity was higher when the actin was complexed with tropomyosin. The highest level of activation was obtained when the myosin was fully phosphorylated and the actin was complexed with tropomyosin at a molar ratio of 1:6 (Tm/A). The potentiation of actin-activated ATP hydrolysis by tropomyosin is not dependent on Ca^{2+}. These data indicate that tropomyosin plays a major role in the actin-activated ATP hydrolysis by smooth muscle myosin in the absence of other regulatory proteins.

Published reports show that a Ca^{2+}-dependent kinase that specifically phosphorylates the 18 000–20 000-dalton myosin light chain and a phosphatase that dephosphorylates the light chain are present in muscle and nonmuscle cells (Perry et al., 1975; Morgan et al., 1976; Frearson & Perry, 1975; Adelstein & Conti, 1975). Morgan et al. (1976) demonstrated that phosphorylation has no significant effect on the actin activation of skeletal muscle myosin. Pemrick recently reported that the ATPase activity of actomyosin from skeletal muscle was higher when the myosin was phosphorylated (Pemrick, 1978). However, the role of phosphorylation and dephosphorylation in the regulation of actin-activated ATPase[1] activity of skeletal muscle myosin is not clear. In this contractile system, the ATP hydrolysis by actomyosin is regulated by Ca^{2+} through the troponin–tropomyosin system (Ebashi & Endo, 1968; Fuchs & Briggs, 1968). Evidence for a similar type of regulation is lacking in smooth muscle. On the other hand, in smooth muscle similar to the nonmuscle system (Adelstein & Conti, 1975) the phosphorylation–dephosphorylation system appears to play a role in the regulation of actin-activated ATP hydrolysis (Gorecka et al., 1976; Sobieszek & Small, 1977; Chacko et al., 1976). While the myosin ATPase is not altered by phosphorylation, the actin-activated ATPase activity is increased 5–10-fold by phosphorylation of vas deferens myosin (Chacko et al., 1977). The Ca^{2+}-dependent phosphorylation was also essential for the actin activation of the ATPase activity of myosin from gizzard (Aksoy et al., 1976; Sobieszek & Small, 1977).

Ebashi and his colleagues reported that the actomyosin ATPase in smooth muscle is regulated by Ca^{2+} through leiotonin A and leiotonin C (Mikawa et al., 1977a,b). This mode of regulation described by Ebashi and co-workers requires tropomyosin in addition to the leiotonin, but it does not require phosphorylation of the myosin.

Hence, there appears to be in general two proposed mechanisms for Ca^{2+} regulation in smooth muscle: (1) a phosphorylation-independent regulation by the leiotonin system requiring tropomyosin; (2) regulation by myosin phosphorylation. It is not known what role the tropomyosin plays in the second mechanism. The purpose of this investigation is to determine the effect of phosphorylation, Ca^{2+}, and tropomyosin on the actin-activated ATPase activity of smooth muscle myosin isolated from mammalian smooth muscle.

Experimental Procedures

Preparation of Actomyosin. Myosin was isolated according to the method described previously by Chacko et al. (1977) from either frozen or fresh smooth muscle removed from the abomasal portion of bovine stomach. The muscle was minced and washed in washing buffer containing 60 mM KCl, 20 mM imidazole–HCl (pH 6.9), 2 mM (ethylenedinitrilo)tetraacetic acid (EDTA), and 10 mM dithiothreitol (DTT). The washed pieces of muscle chilled in ice were homogenized with a polytron (Brinkman Instruments). The homogenized material was sedimented at 48000*g* for 15 min. The supernatant was discarded. The residue was again homogenized in 20 times the volume of the tissue in extraction buffer, 60 mM KCl, 2 mM EDTA, 20 mM imidazole–HCl (pH 7.1), 10 mM ATP, and 10 mM DTT, and sedimented at 48000*g* for 20 min to yield a cloudy supernatant and a pellet. The supernatant was collected and made 10 mM with respect to $MgCl_2$, and the pH was raised to 7.5. It was then fractionated immediately after addition of ATP (10 mM) into 0–35% and 35–70% fractions by the addition of a saturated ammonium sulfate solution in 10 mM EDTA (pH 7.0). The 0–35% fraction containing predominantly actin was discarded. The 35–70%

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[1] Abbreviations used: ATPase, adenosine 5′-triphosphatase; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

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fraction (crude actomyosin) containing myosin, trace amounts of actin and tropomyosin, light-chain kinase, and phosphatase was dissolved in high-salt buffer containing 0.8 M KCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 5 mM DTT to give a protein concentration of 10–15 mg/mL and dialyzed overnight against low-salt buffer (40 mM KCl, 15 mM imidazole–HCl (pH 7.5), 1 mM EDTA, and 5 mM DTT). The crude actomyosin was used for phosphorylation, dephosphorylation, and purification of myosin.

Preparation of Phosphorylated and Dephosphorylated Myosin. The time required for maximal phosphorylation of the myosin was determined for each preparation as described (Chacko et al., 1977). The dialyzed actomyosin containing endogenous kinase and phosphatase was incubated in 15 mM $MgCl_2$ and 0.1 mM $CaCl_2$ at 23 °C for 10 min prior to phosphorylation with [γ-^{32}P]ATP. The residual phosphate bound to myosin is removed by the endogenous phosphatase during this incubation. The phosphorylation was started by adding 2.5 mM ATP mixed with sufficient [γ-^{32}P]ATP (10–30 Ci/mmol) so as to give 200 000–500 000 cpm in 5 μL of the final mixture. Aliquots of 30–50 μL containing 0.1–0.2 mg of protein were removed at varying intervals (30 s–20 min) and mixed with 1 mL of 10% Cl_3CCOOH and 2% $Na_4P_2O_7$. The ^{32}P incorporated into the myosin was determined as described below for the assay for phosphatase activity.

The unphosphorylated myosin was made by purifying the actomyosin without phosphorylation. The maximally phosphorylated myosin was prepared by incubating the actomyosin for the time required for maximal incorporation of ^{32}P into myosin. Myosin with lower degrees of phosphorylation was obtained by stopping the phosphorylation at an earlier time than that required for the maximal incorporation of ^{32}P into the myosin. Dephosphorylated myosin was prepared by first allowing actomyosin to phosphorylate and then dialyzing it against the same buffer in the absence of ATP at 4 °C for 4–6 h. The sample was then removed from the dialysis bag and incubated for 15 min at 23 °C to allow the endogenous phosphatase to remove the bound phosphate from the myosin. All samples were made 1 M with respect to KCl and were chilled in ice. This prevented further phosphorylation and dephosphorylation of the myosin prior to loading into the columns.

Myosin was purified from the actomyosin either in phosphorylated or in dephosphorylated forms as described by Adelstein & Conti (1975). A 1.5 × 90 cm or 2.5 × 90 cm column of Sepharose 4B CL equilibrated and eluted with 0.8 M KCl, 1 mM EDTA, 20 mM Tris-HCl (pH 7.5), and 5 mM DTT was used for gel filtration. Prior to application to the columns, 2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetracetic acid (EGTA), 10 mM Mg, and 10 mM ATP were added to the samples. Addition of EGTA prevented further phosphorylation of the myosin by nonradioactive ATP added to the sample prior to loading into the column.

Preparation of Partially Purified Myosin Light-Chain (LC) Kinase. The kinase was prepared from the 35–70% ammonium sulfate fraction. This fraction was subjected to isoelectric precipitation by lowering the pH to 6.4. The resulting precipitate was sedimented by centrifugation, and the supernatant was collected. When purified myosin or isolated light chain was incubated with this supernatant in the presence of Ca^{2+} and MgATP, the 20 000-dalton light chain became phosphorylated. The phosphorylated state was stable, indicating the low level of phosphatase activity in this preparation.

ATPase Assay. Myosin ATPase activities were determined in 0.5 M KCl and either in 2 mM EDTA or in 10 mM $CaCl_2$ with a myosin concentration of 6–12 μg/mL. Actin-activated ATPase activity was measured in the presence of 10 mM imidazole–HCl (pH 7.2), 2 mM ATP, 2.6 mM $MgCl_2$, and either 0.1 mM $CaCl_2$ or 2 mM EGTA. A final concentration of 0.1–0.2 mg/mL myosin was used for the actin-activated ATPase activity. The concentrations of actin and tropomyosin varied with experiments. Samples were incubated at 37 °C, and aliquots were removed at zero time and at two additional times to ascertain the linearity of phosphate release. Inorganic phosphate was measured by the method of Martin & Doty (1949).

Kinase Assay. Kinase activity was assayed by using [γ-^{32}P]ATP and smooth muscle light chain as previously described by Daniel & Adelstein (1976).

Phosphatase Assay. Phosphatase activity was determined by the release of ^{32}P from myosin or isolated light chain which were previously phosphorylated by smooth muscle kinase in the presence of [γ-^{32}P]ATP. The samples to be tested for the phosphatase activity were incubated in 15 mM imidazole–HCl (pH 7.2), 20 mM KCl, 10 mM $MgCl_2$, and 5 mM DTT at 23 °C for 5–30 min. The reaction was terminated by adding 50% trichloroacetic acid (Cl_3CCOOH) and 10% $Na_4P_2O_7$ to a final concentration of 10% and 2%, respectively. The samples were then heated to 90–95 °C for 20 min, cooled on ice, and filtered through a millipore filter (0.45 μm) with a millipore manifold (Millipore). The filters were washed 4 times with cold 5% Cl_3CCOOH and 1% $Na_4P_2O_7$. The protein was trapped on the filter, and the radioactivity remaining bound to it was determined by counting the filter in a Beckman 2000LS liquid scintillation spectrometer using Ready-Solve HP (Beckman) scintillation cocktail.

Phosphate bound to myosin was also determined by Na-DodSO_4–polyacrylamide gel electrophoresis. For this purpose, the reaction was terminated by adding guanidine hydrochloride to a final concentration of 5 M. These samples were dialyzed for 8–12 h against 0.2 M NaCl and against water at room temperature to remove the excess ATP and NaCl. After dialysis, the sample was dried in a freeze dryer and electrophoresed, and the bound ^{32}P determined as described below.

Gel Electrophoresis. Freeze-dried samples were electrophoresed on 1% Na$DodSO_4$–7.5% polyacrylamide gels under the conditions of Fairbanks et al. (1971). After electrophoresis, the gels were stained in 30% CH_3OH, 8.4% CH_3COOH, and 0.03% Coomassie Brillant Blue R. The gels were destained with the same solution without the dye and scanned at 584 nm by using a Transidyene gel scanner–integrator (Transidyene, Ann Arbor, MI). The amounts of protein under each band were estimated from the area under each peak by using a standard curve obtained by plotting the amounts of light chain against the areas obtained after densitometric scans of the stained gels. The gels were sliced into 2-mm pieces. Each slice was crushed in scintillation vials and counted in a scintillation counter by using Ready-Solve HP scintillation fluid. The counts from the slice containing the light chain were standardized to the amount of protein or area under the 20 000-dalton light chain band. The amount of phosphate bound to this light chain was calculated.

Preparation of Other Proteins. Rabbit skeletal muscle actin was prepared as outlined by Spudich & Watt (1971). Tropomyosins from smooth and skeletal muscle was prepared by the methods of Sobieszek & Small (1976) and Eisenberg & Kielley (1974). All the proteins were assayed for the biological activity by monitoring their effect on ATPase activity with purified skeletal muscle myosin. These were all found to be free of kinase and phosphatase activity. Protein concentrations

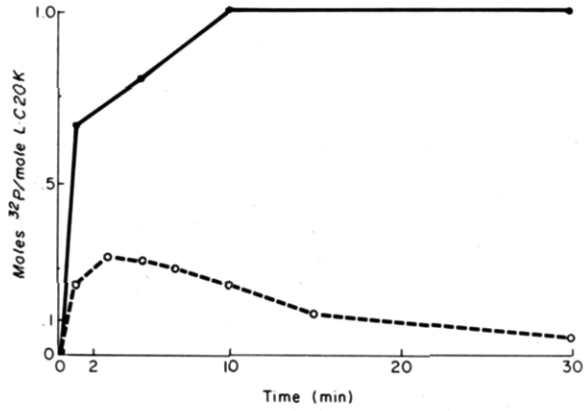

FIGURE 1: Effect of ATP concentration on the time course of phosphorylation of bovine stomach muscle myosin. The 35–70% ammonium sulfate fraction was dialyzed against 15 mM imidazole–HCl (pH 7.3), 60 mM KCl, and 2.5 mM DDT. The samples were incubated in 15 mM $MgCl_2$ and either in 0.1 mM ATP (dotted line) or in 2.5 mM (solid line) ATP. Ca^{2+} was present in both cases.

were determined by the procedure of Lowry et al. (1951) with bovine serum albumin as a standard.

Results

(*1*) *Effect of Ca^{2+} and ATP Concentration on Phosphorylation.* The crude actomyosin containing myosin, kinase, and phosphatase was incubated with [γ-^{32}P]ATP mixed with cold ATP (2.5 mM) either in the presence of 0.1 mM $CaCl_2$ or in the presence of 2 mM EGTA. When Ca^{2+} was present in the incubation mixture, the phosphorylation reached its peak (1 mol of P_i/mol of LC) in 1–7 min and maintained at steady state for 15 min. In the presence of EGTA the incorporation of phosphate was considerably lower (0.05 mol of P_i/mol of LC), indicating the requirement for Ca^{2+} for the activation of the LC kinase.

Figure 1 shows the effect of ATP concentration on the initial level of phosphorylation and the maintenance of the phosphorylated state. The degrees of phosphorylation at the peaks were different for low (0.1 mM) and high (2.5 mM) concentration of ATP. The incorporation of radioactive phosphate into the LC was 1 mol of P_i/mol of LC in 2.5 mM ATP while it was only 0.2 mol of P_i/mol of LC in 0.1 mM ATP. Furthermore, a difference was noticed also in the maintenance of myosin in the phosphorylated state. After reaching the peak of phosphorylation, the amount of phosphate incorporated into myosin declined immediately in low (0.1 mM) ATP concentration. Addition of more ATP (0.1 mM) to this mixture will cause the phosphorylation to reach again the original peak only to fall again with time (not shown). Hence, the phosphorylation–dephosphorylation cycle can be repeated.

(*2*) *Effect of Phosphorylation on Actin-Activated ATPase Activity.* The myosin was purified from 35–70% ammonium sulfate fractions which were either dephosphorylated or phosphorylated prior to application of the sample to the columns. The purified dephosphorylated or unphosphorylated myosin incubated with [γ-^{32}P]ATP and Ca^{2+} fails to incorporate ^{32}P into light chain, indicating the absence of kinase activity. Similarly, phosphorylated myosin remains in the phosphorylated state during the ATPase assay (maximum 60 min) since it is free of phosphatase activity. Presence of traces of kinase and phosphatase activities are occasionally found in some column fractions of myosin; therefore, it is necessary to determine the activities of these enzymes for each column prior to the utilization of the myosin for ATPase assay. The myosin fractions containing traces of phosphatase and kinase activities are usually obtained from overloaded columns (over 12 mg for 1.5 × 90 cm column). Excessive amounts of actin in the actomyosin fraction also favors the incomplete separation of actin from myosin. In those instances, the phosphatase and kinase activities are more prominent in the actomyosin peak.

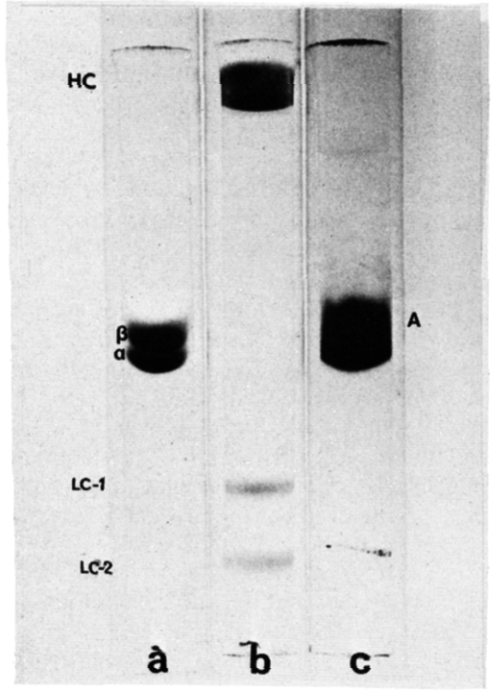

FIGURE 2: 7.5% $NaDodSO_4$–polyacrylamide gel electrophoresis of myosin, actin, and tropomyosin. Gel a shows the α and β subunits of smooth muscle tropomyosin. Gel b shows the heavy chain (HC), 20000-dalton light chain (LC-1), and 16000-dalton light chain (LC-2) of column-purified bovine stomach muscle myosin. Gel c is that of rabbit skeletal muscle actin.

The actin-activated ATPase activity of myosin purified without prior phosphorylation is low [0.007 μmol of P_i/(mg min)]. Addition of partially purified kinase to the myosin produces a 20-fold increase in the actin-activated myosin ATPase activity. However, the increase in ATPase activity is observed only in the presence of Ca^{2+}. The $NaDodSO_4$–polyacrylamide gel electrophoresis and elution of ^{32}P from the LC indicate that the increase in ATPase activity in the presence of the partially purified kinase is associated with covalent binding of phosphate to the myosin light chain.

For determination of the effect of light-chain phosphorylation on the actin activation of myosin ATPase activity, purified myosins (Figure 2) at various levels of phosphorylation were prepared by gel filtration. These myosins were reconstituted with pure rabbit skeletal muscle actin (Figure 2) at molar ratio of 1:20 (M/A). In Figure 3 the actin-activated ATPase activities of myosins phosphorylated at varying degrees are plotted against the amount of phosphate bound per mole of 20000-dalton light chain. The actin-activated ATPase activity is dependent on the amount of phosphate bound to the light chain. The highest level of ATPase activity is obtained when 1 mol of phosphate is bound/mol of light chain. Furthermore, once the myosin is phosphorylated, the actin-activated ATPase activity is not dependent on Ca^{2+}.

(*3*) *Potentiation of Actin-Activated ATPase Activity by Tropomyosin.* Phosphorylated and unphosphorylated myosin obtained by gel filtration and the actin used for actin activation are free of tropomyosin (Figure 2). The addition of pure tropomyosin to the reconstituted actomyosin potentiates the ATPase activity when the myosin is phosphorylated. Addition of tropomyosin has no effect on the actin activation of unphosphorylated myosin. Figure 4 shows the effect of increasing

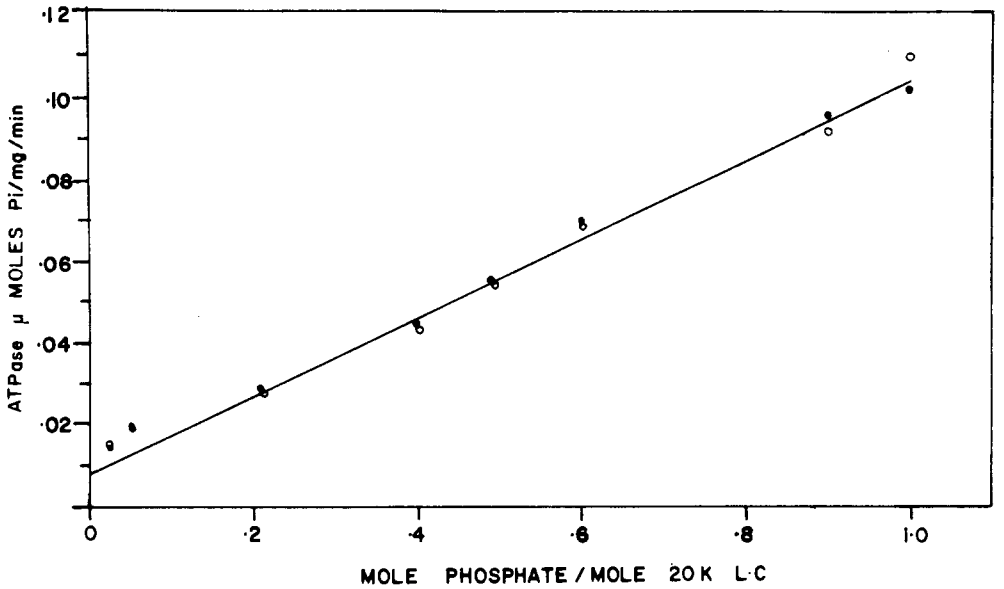

FIGURE 3: Effect of phosphorylation on 20 000-dalton light chain (20K LC) on actin-activated ATPase activity of purified myosin. The assay conditions are described under Experimental Procedures, Myosin, 0.2 mg; myosin/actin molar ratio, 1:20; $CaCl_2$, 0.1 mM; EGTA, 2 mM. The actin-activated ATPase activity depends upon the amount of phosphate bound to myosin light chain. The fully phosphorylated myosin (1 mol of phosphate/mol of LC) has the highest ATPase activity. The ATPase activities in the presence of Ca^{2+} (closed circle) and EGTA (open circle) are similar.

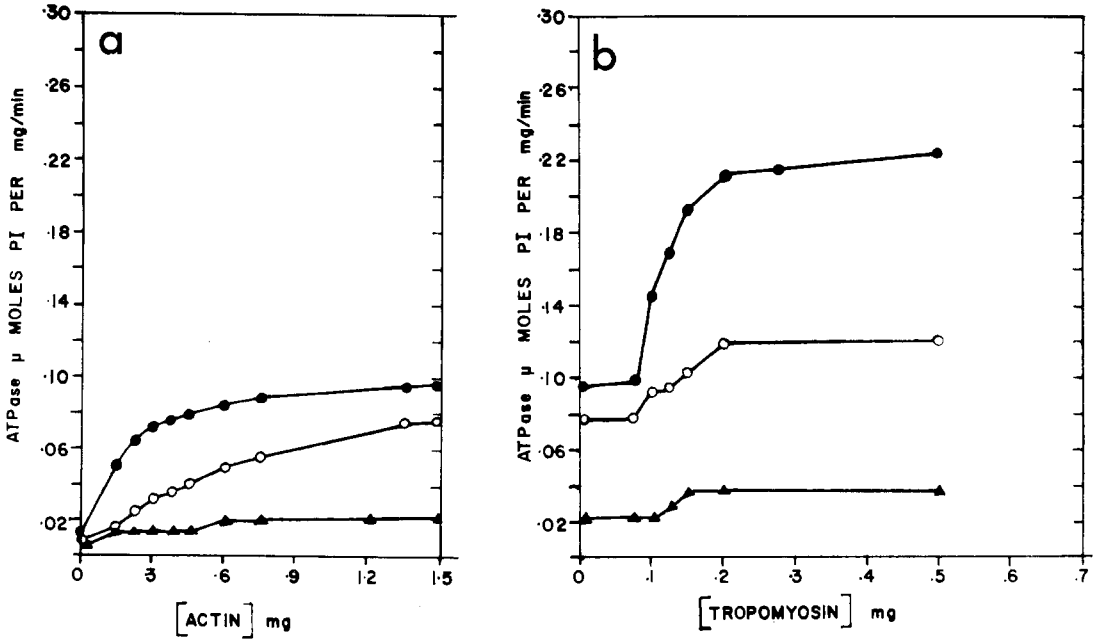

FIGURE 4: Effect of increasing the amounts of actin (a) and tropomyosin (b) on actin-activated ATP hydrolysis. Assay conditions: myosin, 0.25 mg; actin, concentrations shown in the graph; $MgCl_2$, 2.6 mM; ATP, 2 mM; KCl, 20 mM; $CaCl_2$, 0.1 mM; imidazole–HCl (pH 7.2), 10 mM; temperature 37 °C. The molar ratio of myosin to actin was 1:40 when tropomyosin was added to the reconstituted actomyosin. The myosins used in this experiment had KEDTA- and Ca^{2+}-stimulated ATPase activities of 1.2–1.4 μmol of P_i/(mg min) and 0.5–0.65, respectively. (●) Phosphorylated myosin, 0.9 mol/mol of light chain; (○) phosphorylated myosin, 0.5 mol/mol of light chain; (▲) dephosphorylated myosin, 0.05 mol/mol of light chain still bound to the myosin.

amounts of actin (a) and tropomyosin (b) on the ATPase activity of myosin. Increasing the actin concentration is associated with an increase in ATPase activity until the molar ratio of myosin to actin becomes 1:20. No remarkable increase in the ATP hydrolysis is obtained by further increase in actin concentration. If tropomyosin is added to the reconstituted actomyosin, a further activation of ATPase is observed (Figure 4b). This potentiation of actin-activated ATPase activity is increased with increasing concentration of tropomyosin until the molar ratio of actin to tropomyosin reaches 6:1. The degrees of potentiations are approximately the same irrespective of the levels of phosphorylation of the myosin light chain. However, the highest ATPase activity is exhibited by the maximally phosphorylated myosin, reconstituted with actin (molar ratio of M/A, 1:20) and tropomyosin (molar ratio of A/Tm, 6:1). The potentiation can be obtained either by adding tropomyosin to the reconstituted actomyosin or by adding actin–tropomyosin complex to the myosin. The activation of the ATPase activity of phosphorylated myosin by increasing concentration of actin is compared with the potentiated activities obtained with increasing concentration of actin complexed with tropomyosin in Figure 5. With both pure actin and actin complexed with tropomyosin, the ATPase activity increased gradually until a molar ratio of M/A reached 1:15. An increase in the molar ratio of M/A to 1:30 with pure actin gave only slight increase in ATPase. However,

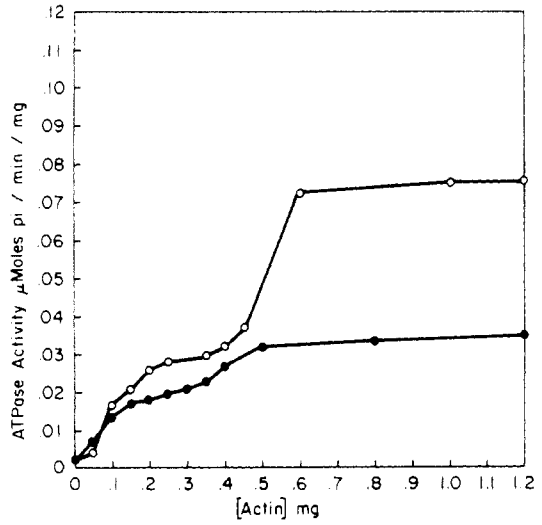

FIGURE 5: Effects of increasing amount of pure actin and actin complexed with tropomyosin on ATP hydrolysis by phosphorylated myosin. Assay conditions: same as described in Figure 4. Phosphorylated (0.6 mol of P_i/mol of LC) myosin (0.2 mg) was reconstituted either with pure actin (●) or with actin complexed with gizzard tropomyosin (○). The G-actin was complexed with tropomyosin at the molar ratio of 1:6 (Tm/A) to obtain the actin–tropomyosin complex. The myosin used for this experiment had KEDTA- and Ca^{2+}-activated ATPase activities of 0.75 and 0.40 μmol of P_i/(mg min), respectively.

if the actin was complexed with tropomyosin (Tm/A = 1:6), subsequent increase in actin showed a remarkable increase in the ATPase activity. The increase in activity leveled off when the molar ratio of myosin/actin reached 1:30. ATPase activity of phosphorylated myosin reconstituted with actin–tropomyosin complex is 3 times greater than that of the same myosin reconstituted with actin which is not complexed with tropomyosin.

The actin-activated ATPase activity of partially phosphorylated myosin is lower than that of fully phosphorylated myosin even in the presence of tropomyosin (Figure 4). Nevertheless, the actin activation of myosin ATPase is greater in the presence of tropomyosin at all levels of the myosin phosphorylation.

Tropomyosin prepared from both skeletal muscle and smooth muscle showed the same effect. The potentiation of actin-activated ATPase activity was not dependent on the Ca^{2+} concentration, since the effect was shown both in the presence of Ca^{2+} (0.1 mM) and in the presence of EGTA (2 mM).

Discussion

The presence of kinase and phosphatase in the crude myosin (35–70% ammonium sulfate fraction) makes this a useful preparation to obtain myosin in either the phosphorylated or unphosphorylated state. However, the presence of these two enzymes during the ATPase assay complicates the system, since the ATP hydrolysis by myosin takes place simultaneously with phosphorylation and dephosphorylation of myosin light chain by the kinase and phosphatase. Furthermore, Ca^{2+} is needed for the activation of the actomyosin, because kinase is dependent on Ca^{2+} for its activation. This makes it difficult to measure the ATPase activity in the absence of Ca^{2+}. Sherry and his collaborators (Sherry et al., 1978) have utilized adenosine 5′-*O*-(3-thiophosphate) [ATP(γ-*S*)] to phosphorylate the myosin prior to ATPase assay, since the thiophosphorylate protein is a poor substrate for phosphatase (Gratecos & Fischer, 1974).

Small & Sobieszek (1977) prepared two forms of actomyosin from pig stomach. One of these actomyosins had negligible Mg–ATPase activity, and its activity was increased in the presence of Ca^{2+} by adding a fraction from myofibril extract. The increase in Mg–ATPase was associated with Ca^{2+}-dependent phosphorylation of the 20 000-dalton light chain. The other form of actomyosin prepared by Small & Sobieszek (1977) had a very high Mg–ATPase activity, but it exhibited little Ca^{2+} sensitivity. They concluded that the latter form of actomyosin was already phosphorylated. The effect of tropomyosin on the actin-activated ATPase activity is difficult to understand in this study since the actomyosin prepared from pig stomach by Small & Sobieszek (1977) also contained tropomyosin. However, the higher level of ATPase activity observed for actomyosins from pig stomach in their study appears to be due to the presence of tropomyosin in the actomyosin.

In this investigation, we have used a simplified system in which the myosin is obtained in either phosphorylated or dephosphorylated form free of kinase and phosphatase. The level of phosphorylation can be controlled by varying the incubation period. Since both kinase and phosphatase are present in the crude actomyosin preparation and the ATP is also hydrolyzed by actomyosin, a high concentration of ATP is used to keep the myosin in a phosphorylated state prior to the application of the sample to the columns. The phosphatase activity is also inhibited by raising the KCl concentration to 1 M. It is also important to check the phosphatase and kinase activity of the purified myosin, since very low activities of these enzymes are occasionally detected in the myosin fractions. Since the unphosphorylated and phosphorylated myosins prepared by this procedure are free of kinase and phosphatase, they should be valuable for the study of the kinetics of actomyosin ATPase. Using this procedure, it is possible to obtain phosphorylated myosin with 1 mol of phosphate/mol of light chain, a level of phosphorylation unable to be achieved by Sherry et al. (1978) in their studies on gizzard myosin.

The actin-activated ATPase activity of bovine stomach muscle is correlated linearly with the level of phosphate bound to the myosin light chain. The ATPase activity of phosphorylated myosin is activated 4–10-fold by actin, depending upon the level of light-chain phosphorylation. The extent of activation is similar to that reported by us (Chacko et al., 1977) and Small & Sobieszek (1977) for other mammalian smooth muscles. The dephosphorylated myosin and unphosphorylated myosin have a slightly higher ATPase activity in the presence of actin than without actin and this is found to be due to a low degree of residual phosphate still bound to the myosin (S. Chacko, unpublished observation). The actin-activated ATPase activity of fully phosphorylated (1 mol of P_i/mol of LC) myosin is 10 times higher than that of myosin which was not phosphorylated prior to purification. The higher fold activation (20-fold) observed when partially purified kinase was added to the pure myosin during ATPase assay is attributed to the effect of tropomyosin (see below) present in the partially purified myosin.

The actin activation of column-purified phosphorylated myosin is not dependent on Ca^{2+} at all levels of phosphorylation, since the removal of Ca^{2+} did not inhibit the actin-activated ATPase activity. Hence, once the myosin is phosphorylated, the change in Ca^{2+} concentration had no effect on the actin activation of myosin ATPase. In this regard, the myosins isolated from bovine stomach muscle, avian gizzard, and pig stomach appear to be different from the myosins from guinea pig vas deferens and pig carotid. Myosin isolated from carotid and column-purified phosphorylated myosin from guinea pig vas deferens have higher actin-activated ATPase

activity in the presence of Ca^{2+} than in the presence of EGTA (Mrwa & Rüegg, 1975; Chacko et al., 1977).

The ATPase measurements at increasing amounts of actin show that the saturation of the active sites on myosin with actin was completed at a molar ratio of 1:20 (myosin to actin). However, the saturation of active sites by actin did not increase the ATP hydrolysis unless the 20000-dalton light chain was phosphorylated. The level of ATPase activity at all actin concentrations depended upon the degree of light-chain phosphorylation.

The finding that the actin complexed with tropomyosin at a molar ratio of 6:1 (A/Tm) is a better activator of myosin ATPase in low salt suggests that the tropomyosin increases the affinity between actin and myosin resulting in the potentiation of the ATP hydrolysis. Phosphorylation of the 20000-dalton light chain is essential for this effect to occur. Potentiation of actin-activated ATPase activity by tropomyosin has also been reported for rabbit skeletal muscle under conditions that favor rigor complexes (Bremel et al., 1972) and in *Limulus* striated myosin at high ATP concentrations where the rigor complexes were minimal (Lehman & Szent-Gyorgyi, 1972). A recent report by Sellers (1980) indicates that the actin-activated ATPase activity in *Limulus* myosin is dependent on myosin light-chain phosphorylation. However, in rabbit skeletal muscle, the myosin phosphorylation is not a prerequisite either for actin activation of myosin ATPase or for its potentiation by tropomyosin (Bremel et al., 1972). The lack of dependence on Ca^{2+} for the potentiation of actin-activated ATPase activity of smooth muscle myosin strongly suggests that this effect is not due to a kinase or a Ca^{2+} regulatory system like leiotonin (Mikawa et al., 1977a).

The presence of specific kinase and a phosphatase that phosphorylates and dephosphorylates the myosin light chain in bovine stomach muscle suggests the possibility that the phosphorylation–dephosphorylation may be linked to the contraction–relaxation cycle in this smooth muscle. The Ca^{2+} dependence of the kinase further indicates that the Ca^{2+} regulates contraction through phosphorylation. The finding that actin-activated ATPase activity of phosphorylated myosin is higher than that of unphosphorylated myosin supports the possibility that the phosphorylation plays a role in the regulation of contraction in smooth muscle. A recent report by Barany et al. (1979) indicates that the development of tension is associated with phosphorylation of light chain. Hoar et al. (1979) also showed that the tension development in glycerinated smooth muscle strip is correlated with phosphorylation of myosin light chain.

The mechanism by which the tropomyosin potentiates the actin-activated ATPase activity is not clear at the moment. A sequential activation of actomyosin in smooth muscle may be (1) activation of the light-chain kinase by Ca^{2+} and (2) an increase in actin-activated ATPase activity. The potentiation of actin-activated ATPase activity by tropomyosin may simply be due to enhancement of actomyosin complex formation in the presence of tropomyosin. Only the first step in the activation is dependent on Ca^{2+} in the bovine stomach muscle (this report), avian gizzard (Gorecka et al., 1976; Sobieszek & Small, 1977), and pig stomach (Small & Sobieszek, 1977).

In view of the absence of a regulatory system involving troponin in smooth muscle, the role played by tropomyosin in the potentiation of actin-activated ATPase is significant. The amounts of tropomyosin in smooth muscles vary with the source (Cohen & Murphy, 1978). It appears to play a crucial role in the development of tension even in the absence of a troponin system.

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