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Modulation of Monomer-Polymer Equilibrium of Phosphorylated Smooth Muscle Myosin: Effects on Actin Activation†

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ABSTRACT: Actin activation of the adenosinetriphosphatase (ATPase) of phosphorylated gizzard myosin at low (2 mM) free Mg^{2+} concentration and 50 mM total ionic strength continues to increase on raising the free Ca^{2+} concentration near pCa 3. Similar levels of activity can be obtained by increasing the free Mg^{2+} concentration to a higher (in excess of 4 mM free) concentration. In the presence of micromolar concentrations of free Ca^{2+} and low free Mg^{2+} concentration, the actin-activated adenosine 5'-triphosphate (ATP) hydrolysis exhibits an initial rapid rate which progressively slows to a final, lower but more linear rate. In the presence of high divalent cation concentrations, the fast rate of ATP hydrolysis is maintained during the entire ATPase assay. The ionic conditions which favor the slow rate of ATP hydrolysis are correlated with increased proportions of folded myosin monomers while higher rates of ATP hydrolysis are correlated with increased levels of aggregated myosin. Elevating the thin filament proteins to saturating concentrations does not abolish the change in ATPase rate or the final distribution of myosin aggregates and monomers; however, the stability of the myosin aggregates is enhanced by the presence of thin filament proteins in low divalent cation conditions. The nonlinear profile of the actin-activated ATP hydrolysis in low divalent cation concentrations is eliminated by utilizing nonfilamentous, phosphorylated heavy meromyosin. The data presented indicate that Ca^{2+} and Mg^{2+} alter monomer-polymer equilibrium of stably phosphorylated myosin. The alteration of monomer-polymer equilibrium by Ca^{2+} at low Mg^{2+} concentration modulates ATPase rates.

Actin activation of the Mg·ATPase[1] activity of smooth muscle myosin requires the phosphorylation of the 20 000-dalton light chains of myosin by myosin light chain kinase (Gorecka et al., 1976; Chacko et al., 1976; Sobieszek & Small, 1977). Studies using purified phosphorylated myosin demonstrate that the maximal activation of Mg·ATPase by actin requires Ca^{2+} and tropomyosin (Chacko et al., 1977; Rees & Frederickson, 1977; Chacko & Rosenfeld, 1982; Nag & Seidel, 1983). The level of Ca^{2+} concentration required for actin in the presence of tropomyosin to maximally activate the Mg·ATPase of phosphorylated myosin (Chacko et al., 1986) is higher than that required for myosin light chain kinase activity

† This work was supported by National Institutes of Health Grant HL 22264, NSF Grant PCM 83-09139, and NRSA Grant HL 07051 to W.S.F.

[1] Abbreviations: ATP, adenosine 5'-triphosphate; ATPase, adenosinetriphosphatase; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HMM, heavy meromyosin; PMSF, phenylmethanesulfonyl fluoride; FPLC, fast protein liquid chromatography; P_i, inorganic phosphate; *s*, sedimentation coefficient; TCA, trichloroacetic acid.

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(Walsh et al., 1982). The requirements for Ca^{2+} and tropomyosin for maximal actin activation of phosphorylated myosin are observed irrespective of the source of tropomyosin or actin (Heaslip & Chacko, 1985; Seidel et al., 1986). A recent report by Miyata and Chacko (1986) confirms that the binding of smooth muscle tropomyosin to smooth muscle actin is dependent on Mg^{2+} and ionic strength but not on Ca^{2+}.

Activation by Ca^{2+}, subsequent to phosphorylation, is considered a modulation of the actomyosin system which is already activated by the calcium–calmodulin-dependent phosphorylation (Dabrowska et al., 1978; Chacko et al., 1985a,b; Seidel et al., 1986). Calcium binding by myosin may be a possible mechanism by which Ca^{2+} modulates the actin activation of phosphorylated myosin since it is well established that the binding of Ca^{2+} to Ca^{2+}-specific sites on the myosin molecule is the mechanism which regulates the actin–myosin interaction and contraction in molluscan muscle (Kendrick-Jones et al., 1976; Szent-Györgyi et al., 1973). Although Ca^{2+} binding by myosin is associated with the modulation of actin-activated ATP hydrolysis (Chacko & Rosenfeld, 1982), the full activation of reconstituted actomyosin, in low free Mg^{2+} concentration buffers, requires a free Ca^{2+} concentration higher than that required for calcium binding by myosin (Rosenfeld & Chacko, 1981; Ikebe & Hartshorne, 1985).

The Ca^{2+} and Mg^{2+} requirements for filament formation by smooth muscle myosin are known to be different from those of striated muscle myosin (Wachsberger & Pepe, 1974; Trinick & Cooper, 1980; Megerman & Lowey, 1981). The formation and stabilization of myosin filaments in the presence of Mg·ATP depend on the state of myosin phosphorylation (Suzuki et al., 1978, 1981; Kendrick-Jones et al., 1982) and on ionic conditions that alter the conformation of the myosin monomers (6 S or 10 S) (Onishi & Wakabayashi, 1982; Trybus et al., 1982; Trybus & Lowey, 1984; Ikebe & Hartshorne, 1985). Furthermore, the conformation of the myosin molecule modifies the aggregation properties of the myosin (Craig et al., 1983).

In this paper, we demonstrate, using actomyosin reconstituted with stably phosphorylated myosin, actin, and tropomyosin from smooth muscle, that the increased activity in the presence of high Ca^{2+} concentration in low free Mg^{2+} concentration is associated with an increase in the amounts of aggregated myosin and a decreased level of myosin in folded monomeric form. Furthermore, the monomer–polymer equilibrium is altered during the course of the ATPase assay in low divalent cation concentration. The change in the rate of ATP hydrolysis associated with divalent cation induced monomer–polymer equilibrium of intact myosin is eliminated by utilizing nonfilamentous heavy meromyosin.

Materials and Methods

Phosphorylated myosin, actin, and tropomyosin were extracted from chicken gizzard and purified according to the methods previously described (Heaslip & Chacko, 1985; Chacko, 1981). The procedures for myosin preparation included slow precipitation of myosin filaments by dialysis of pooled fractions of the myosin peak from Sepharose 4B-CL column chromatography in 20 mM imidazole hydrochloride (pH 7.0), 10 mM $MgCl_2$, and 1 mM DTT. The filaments were collected by centrifugation (48000*g*, 20 min) and dissolved in and dialyzed overnight against 0.4 M KCl, 20 mM imidazole or MOPS, 4 mM EDTA, 2 mM DTT, 0.5 mM PMSF, 0.001 mM antipain, and 0.01% NaN_3 at pH 7.0. The concentrated, stock, myosin was then dialyzed exhaustively against the same buffer without EDTA, centrifuged at 48000*g* for 20 min, and stored at 4 °C for a maximum of 1 week. Unlike the previous report (Heaslip & Chacko, 1985), EGTA was eliminated from all buffers used for the myosin preparation. Heavy meromyosin (HMM) was prepared by chymotryptic digestion of the phosphorylated myosin and purified according to Kaminski and Chacko (1985).

Protein concentrations were determined according to Lowry et al. (1951) using bovine serum albumin as a standard. Protein purity was assessed by electrophoresis on 1% SDS–7.5% polyacrylamide gels (Fairbanks et al., 1971). The extent of myosin 20K light-chain phosphorylation was determined as previously described (Heaslip & Chacko, 1985) and in all experiments, included in this paper, was in excess of 95%.

The ATPase assays were carried out in 20 mM MOPS (free acid), adjusted to pH 7.0 with KOH, 2 mM Mg·ATP (disodium salt), 2.5 mM DTT, 0.1 mM EGTA, and total and free Mg^{2+} and Ca^{2+} concentrations (Cl_2^- salts) as indicated. The total ionic strength of 0.05 M was maintained with KCl. Ca-EGTA buffer compositions were calculated from the programs of Fabiato and Fabiato (1979) with absolute stability constants obtained from Martell and Smith (1974). Proton constants were adjusted to mixed constants corresponding to the 0.05 M ionic strength. The resulting free calcium concentrations were monitored with a Ca^{2+}-specific electrode constructed in a manner similar to that described by Affolter and Sigel (1979) and calibrated according to Bers (1982). Total ionic strengths of the buffers were monitored by conductance. Actin (0.3 mg/mL), tropomyosin (0.162 mg/mL, and phosphorylated myosin (0.168 mg/mL) or HMM (0.09 mg/mL), except when noted, were added in sequence to the ATPase assay buffer and incubated for 5 min at 25 °C. The assays were initiated with the addition of ATP and gently mixed for 15 s prior to removing aliquots at the indicated times. P_i was measured according to the method of Martin and Doty (1949).

Myosin, actin, and tropomyosin were assayed for kinase and phosphatase activities as previously described (Heaslip & Chacko, 1985) and found to be negative.

In order to quantitate the sedimentable myosin present during the ATPase assays under various ionic conditions, samples (0.45 mL) were removed from the reaction mixture at the indicated time points and immediately centrifuged (15000*g*, 1 min). The TCA precipitates of the separated supernatants and pellets were resuspended in SDS loading buffer, placed in boiling water for 1 min, and subsequently loaded onto 4–12% SDS–PAGE slabs. The amount of myosin heavy chain was estimated by densitometric analysis of the stained gels (Haselgrove et al., 1985). All gels of the supernatant fractions demonstrated less than 2% of total myosin heavy chain at time zero (in the absence of ATP), and thus, the pellets at time zero represent the maximum myosin content of the assays.

The effect of Ca^{2+} and ionic strength on myosin conformation was determined by liquid chromatography (FPLC, Pharmacia) of purified phosphorylated myosin on an HR 10/30 Superose 6 column operated with a column flow rate of 0.5 mL/min and 0.5 MPa excess pressure at room temperature. Fifty-microliter samples of the stock myosin solution at a concentration of 10 mg/mL were applied to the column preequilibrated with buffers identical with those utilized for the ATPase assays. Myosin retained in the column was eluted with the same buffer containing added KCl to a final concentration of 0.4 M. The calibration curve for K_d vs. [KCl] was carried out in a similar fashion with step gradients between the low- and high-salt buffers with other ionic conditions identical with those of the ATPase buffer, prepared as de-

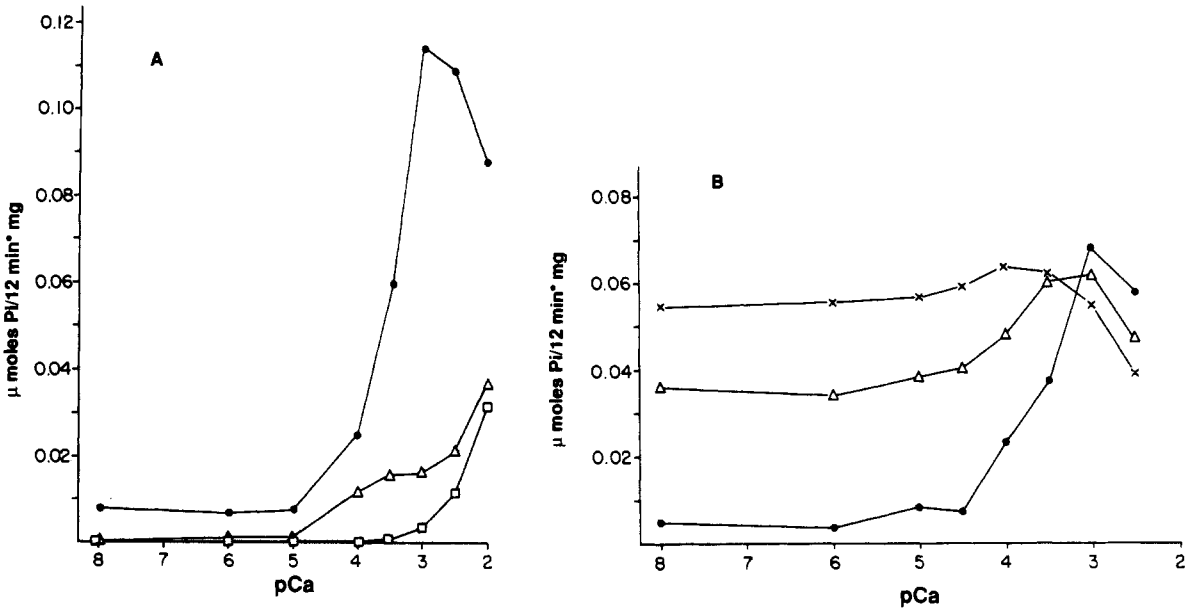

FIGURE 1: Calcium and magnesium dependence of the ATPase of phosphorylated gizzard myosin. Symbols represent P_i liberated per milligram of myosin for the first 12 min of assay of (A) myosin alone (□), myosin reconstituted with actin (△), and myosin reconstituted with actin plus tropomyosin in 2 mM free Mg^{2+} (●) and (B) separate assays of myosin reconstituted with actin plus tropomyosin at 2 mM (●), 3 mM (△), or 4 mM (×) free Mg^{2+}. Other assay conditions were as described under Materials and Methods.

scribed above, at pH 7.0. K_d was calculated as $(V_e - V_o)/(V_i - V_o)$; V_o was determined with Blue Dextran, and V_i was determined with ATP. The protein concentration in the eluted fractions were determined by the method of Bradford (1976).

RESULTS

Ca^{2+} Activation of the Actomyosin ATPase. Figure 1A depicts the effect of free Ca^{2+} concentration on the low-salt Mg·ATPase activity of phosphorylated myosin alone and myosin reconstituted either with smooth muscle actin or with smooth muscle actin plus tropomyosin in 2 mM free Mg^{2+} buffers. The ATPase activity of myosin alone increased gradually on raising the Ca^{2+} concentration to pCa 3. On raising the Ca^{2+} concentration above pCa 3, the ATPase activity increased steeply. Actin activated the ATPase of myosin at pCa between 6 and 3.5; at the highest Ca^{2+} concentrations, the magnitude of activation was diminished. Tropomyosin potentiated the actin-activated ATPase at all Ca^{2+} concentrations; however, the enhanced activity decreased on raising the Ca^{2+} concentrations higher than pCa 3.

The effect of Mg^{2+} concentration on Ca^{2+} activation of the phosphorylated myosin reconstituted with actin and tropomyosin is shown in Figure 1B. The activity increased on raising the free Mg^{2+} concentration at lower free Ca^{2+} concentration. The stimulation of actin-activated ATPase activity by Ca^{2+} was less remarkable at higher Mg^{2+} concentrations. The ATPase activity was inhibited as the concentration of total divalent cations rose above 4.1 mM. At 2 mM free Mg^{2+}, a divalent cation concentration above 4.1 mM was reached between pCa 3 and 2.5, and at 4 mM free Mg^{2+}, the divalent cations reached 4.1 at pCa 4. The stimulation of the ATPase activity by divalent cation was much greater when the concentration was increased by raising Ca^{2+} rather than Mg^{2+} concentration. The maximum activity was reached at a significantly lower total divalent cation concentration in 2 mM free Mg^{2+} at pCa 3 than that in 4 mM free Mg^{2+} at pCa 4 (3.0 vs. 4.1 mM total free divalent cations).

Time Course of ATP Hydrolysis. The ATPase activities in Figure 1A,B were determined from the P_i liberated between 15 s and 12 min. The rate of P_i liberation between 6 and 12 min was subsequently found to be lower than that of the earlier time points. Figure 2A depicts the P_i liberated at various time periods after the initiation of the ATPase assay at 2 mM free Mg^{2+}. The P_i liberated in the pCa 4 and 8 buffers had an initial fast phase followed by a slow phase. In pCa 3 buffers, the initial fast rate was sustained throughout the entire course of the ATPase assay. The initial rates at all three Ca^{2+} concentrations were very similar; however, all activities for pCa 4 were lower than the corresponding data for pCa 3, and likewise, all pCa 8 data were lower than pCa 4 data.

The effect of raising Mg^{2+} concentration on the linearity of the reactions was determined for pCa 8, 4, and 3 at 3 mM free Mg^{2+} (Figure 2B). The ATP hydrolysis at pCa 8 and 4 was faster and more constant in 3 mM than in 2 mM free Mg^{2+} (compare panels A and B of Figure 2). While the total divalent cation concentration at pCa 4 in 3 mM free Mg^{2+} was slightly higher than at pCa 3 in 2 mM Mg^{2+} (3.1 vs. 3.0 mM), the reaction was not completely linear. At pCa 3 with 3 mM free Mg^{2+}, the reaction was essentially linear, and the overall hydrolysis rate was similar to that observed with 2 mM free Mg^{2+} at pCa 3. In agreement with the data from Figure 1B, maximum ATPase was attained at lower concentrations with Ca^{2+} than with Mg^{2+}. While linear rates were observed at high pMg, the specific activity was the same as that observed at lower total divalent cation concentration when high Ca^{2+} concentration was utilized. The small difference in P_i liberation at the first time points of the assays observed in Figure 2B may have been caused by the initial high free divalent cation concentration (5–6 mM total) prior to the addition of ATP at the beginning of the assay.

In order to ensure that the time-dependent loss of ATPase rate in low Ca^{2+} was not due to denaturation during ATPase assay, the actomyosin was incubated in pCa 8 buffer (2 mM free Mg^{2+}), and free Ca^{2+} concentration was increased during the ATPase assay. Aliquots were taken at intervals during ATPase assay for determination of the P_i liberated in a fashion identical with the previously described experiments. After removal of the aliquots for the 4-min time points, additions calculated to raise free Ca^{2+} concentration to pCa 3 while maintaining the specified buffer conditions were delivered to

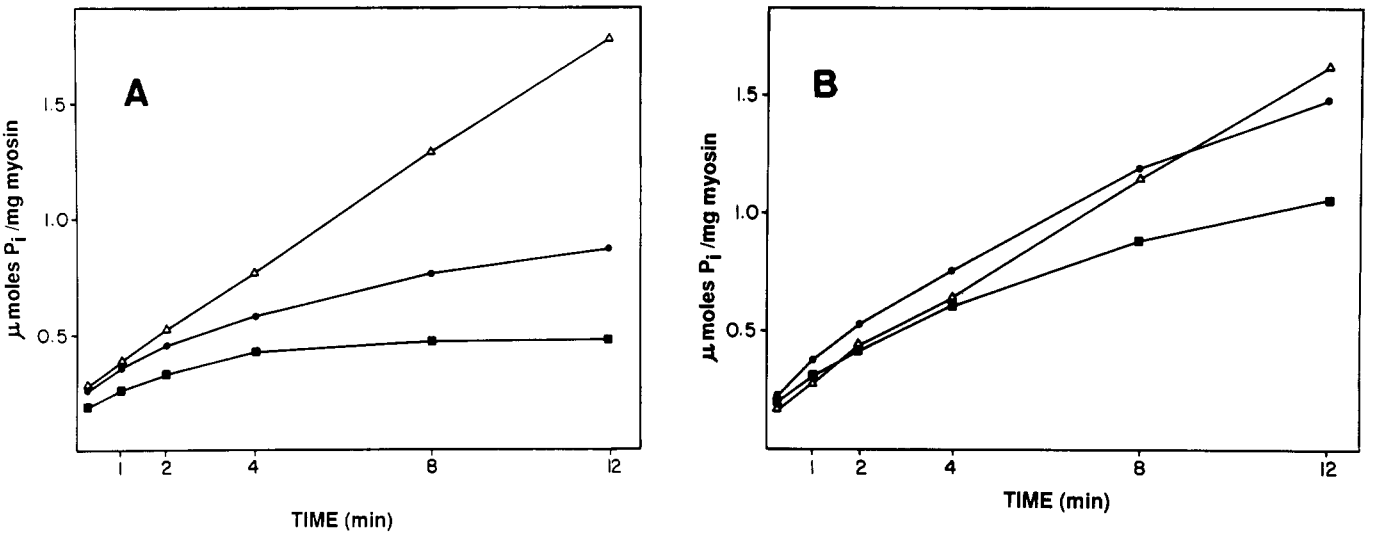

FIGURE 2: Effect of Ca^{2+} and Mg^{2+} on the time course of ATP hydrolysis by phosphorylated gizzard myosin reconstituted with actin plus tropomyosin. Symbols represent P_i liberated per milligram of myosin in pCa 3 (△), pCa 4 (●), and pCa 8 (■) in the presence of 2 mM (A) or 3 mM (B) free Mg^{2+}. Other assay conditions were as described under Materials and Methods.

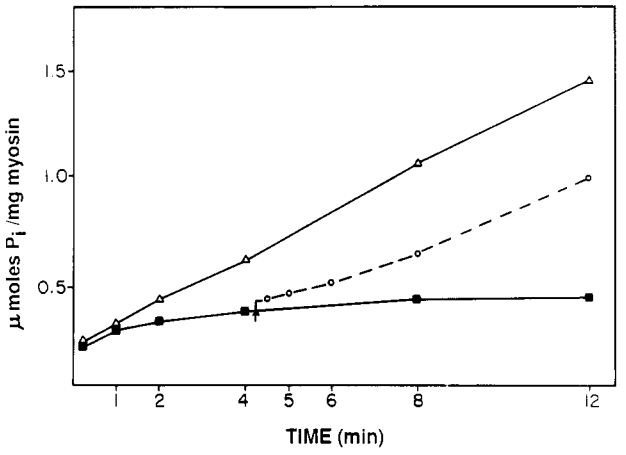

FIGURE 3: Effect of added calcium on the time course of the ATPase of phosphorylated gizzard myosin reconstituted with actin plus tropomyosin in 2 mM free Mg^{2+}. Symbols represent P_i liberated per milligram of myosin in pCa 3 (△, ○) and pCa 8 (■). The arrow indicates $CaCl_2$ and ATP additions (in order to maintain 2 mM Mg·ATP) to the pCa 8 assay, after the 4-min time point was obtained, that increased the buffer composition to that of the pCa 3 assay (○). Other assay conditions were as described under Materials and Methods.

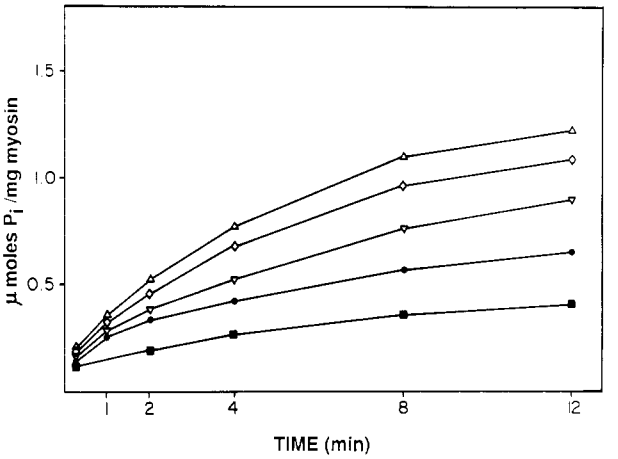

FIGURE 4: Effect of increased tropomyosin concentration on the time course of the ATPase of phosphorylated gizzard myosin reconstituted with actin (A) plus varying ratios of tropomyosin (TM) in 2 mM free Mg^{2+} at pCa 4: 0:1 (■), 1:12 (●), 1:9 (▽), 1:6 (◇), and 1:3 (△) TM:A. Other assay conditions were as described under Materials and Methods.

the assay, and the assay was continued. As shown in Figure 3, raising the Ca^{2+} concentration from pCa 8 to pCa 3 caused an increase in the ATP hydrolysis rate which, after a lag phase, approached the rate of P_i liberation observed in the pCa 3 control.

The effect of thin filament protein concentration on the linearity of the ATPase assay was determined in another series of experiments. The effect of tropomyosin concentration on the initial and final rates of actin-activated ATP hydrolysis by actomyosin was determined at pCa 4 (Figure 4). The myosin and actin concentrations were kept constant to obtain a myosin:actin ratio of 1:20 while the tropomyosin to actin ratio was varied. The ATP hydrolysis showed a decrease in rate with respect to time at all tropomyosin:actin ratios, although the overall levels of activity increased with saturating amounts of tropomyosin. The specific activities during the later time points were similar at 1:3 and 1:6 ratios of tropomyosin to actin. Similar data were obtained by increasing the ratios of myosin to actin (as high as 1:40) and did not reveal any trend toward increased linearity (not shown). Therefore, the concentration of the thin filament proteins did not have a major

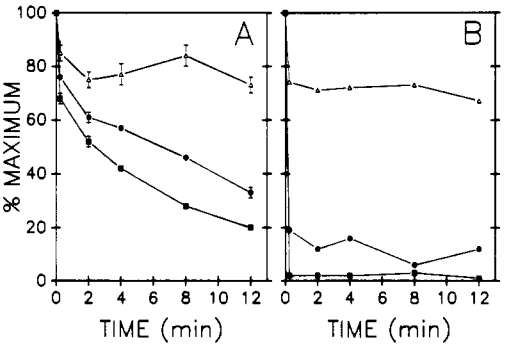

FIGURE 5: Effect of pCa on the change in sedimentable myosin during the time course of the ATPase reactions at 2 mM free Mg^{2+}. Maximum sedimentable myosin at zero time is equal to the total myosin in the assays (0.168 mg/mL) and is identical for all pCa's. (A) +Actin, +tropomyosin. Symbols represent pCa 3 (△), pCa 4 (●), and pCa 8 (■) ± SEM for $n \geq 5$. (B) A control for myosin alone; symbols as above. Other assay conditions were as described under Materials and Methods.

effect on the nonlinear nature of the ATPase reaction.

Effects of Ca^{2+} on the Amount of Myosin in Actomyosin Aggregates. The effect of Ca^{2+} on the formation and stability of the aggregates of myosin and actomyosin was determined

(see Materials and Methods) during the time course of ATPase assays containing 2 mM free Mg^{2+} (Figure 5). Prior to the addition of ATP, all myosin in pCa 3, 4, and 8 buffers was found in the pellet. The amount of the myosin in the pellet decreased very rapidly after addition of ATP. The rate of decrease and the final percentage of sedimentable myosin in each assay were dependent on the pCa and the presence or absence of thin filament proteins. A constant 70–80% sedimentation of the myosin was maintained for all time points in the pCa 3 assays. In contrast, assays at pCa 4 and 8 (Figure 5A) showed a decrease in sedimentable myosin with time, and the rate of decrease of myosin in the pellet was diminished by the presence of Ca^{2+}. A control experiment, with the same concentration of myosin, in the absence of actin and tropomyosin (Figure 5B) showed that the minimum level of sedimentable myosin, for each pCa, was reached within the time required to obtain the first data point after the addition of ATP. The presence of thin filament proteins increased the recovery of myosin in the pellet for assays at pCa 4 and 8; however, the final amounts of myosin recovered in these assays approached the minimum level observed in the control assay for each pCa. The linear rates of ATP hydrolysis in pCa 3 buffers (Figure 2A) were associated with the constant amounts of pelleted myosin (Figure 5A); conversely, nonlinear ATP hydrolysis in pCa 4 and 8 correlated with decreasing sedimentable myosin. Increasing the free Ca^{2+} concentration during the course of the ATPase assays, similar to the experiment in Figure 3, increased the myosin content in the pellet. The increase in the sedimenting myosin by raising the Ca^{2+} concentration may be compared to the recovery of the ATPase rates observed in Figure 3. Exact correlations between specific activity and percent aggregation of myosin were difficult since simultaneous measurements could not be made. The data at the 4-min time points in Figures 2A and 5A indicated a maximum specific activity of 0.12 μmol of P_i/(mg·min) (Figure 2A, pCa 3) maintained with 77% sedimenting myosin. The 57% sedimentation at pCa 4 corresponded to a specific activity between 0.061 (2–4 min) and 0.046 μmol of P_i/(mg·min) (4–8 min), and the 42% sedimentation value for pCa 8 corresponded to a rate between 0.048 (2–4 min) and 0.011 μmol of P_i/(mg·min) (4–8 min). The expected values of the rates for pCa 4 and 8, based on a positive linear correlation between activity and percent sedimentation, were 0.089 and 0.065, respectively. Thus, the rate of decrease in ATPase appears to precede the rate of myosin disassembly.

In order to differentiate between a decrease in activity due to a decrease in myosin aggregate structure and a decrease in activity due to divalent cation effects on the active sites, the ATPase assay was carried out with nonfilamentous HMM. Maximum activity of acto-HMM in smooth muscle was reported (Kaminski & Chacko, 1984) near 1 mM free Mg^{2+}. Unlike assays with the intact myosin molecule, the assays with HMM demonstrated linear rates of ATP hydrolysis at all Ca^{2+} concentrations in 1 mM free Mg^{2+} (Figure 6) and in 2 mM free Mg^{2+} (not shown). The maximum specific activity of the HMM in 1 mM free Mg^{2+}, expressed per mole of active site, was similar to the linear rates observed in Figure 2 at pCa 3 for the intact myosin molecule [0.41 vs. 0.46 mol of P_i/(mol·s)]. The maximum stimulation of acto-HMM ATPase by free divalent cations (1 mM free Mg^{2+}, pCa 4) occurred at a much lower total concentration than for intact myosin (1.1 vs. 3.0–4.1 mM). Acto-HMM activity at pCa 3 in 1 mM free Mg^{2+} was notably lower than at pCa 4 (Figure 6) as were all assays conducted at 2 mM free Mg^{2+}. These results indicated that the divalent cation requirements for maintaining myosin filament structure and actomyosin ATPase were 3 times larger than the divalent cation requirements for maximum acto-HMM activity. Hence, the major effect of the divalent cations on actomyosin ATPase was related to the support of myosin filament structure.

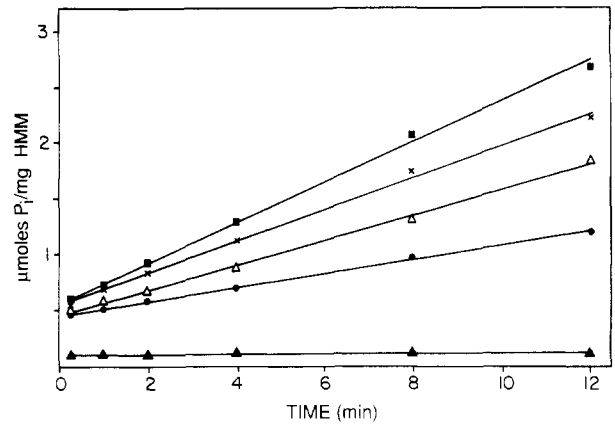

FIGURE 6: Time course of HMM ATPase. Symbols represent P_i liberated per milligram of HMM of (▲) HMM alone at pCa 4, (●) HMM + actin at pCa 4, (■) HMM + actin + tropomyosin at pCa 4, (△) HMM + actin + tropomyosin at pCa 8, (×) HMM + actin + tropomyosin at pCa 3. HMM concentration was 0.09 mg/mL. Free Mg^{2+} concentration was 1 mM with an HMM:actin ratio of 1:40 and an actin:tropomyosin ratio of 3:1. Other assay conditions were as described under Materials and Methods.

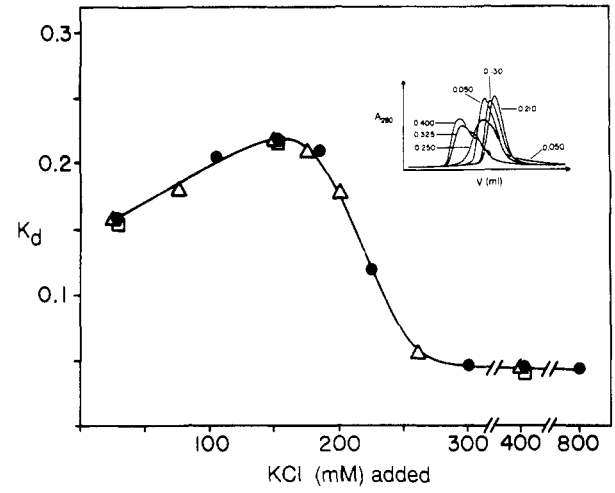

FIGURE 7: Elution of phosphorylated myosin from the Superose 6 column at varing KCl concentrations. Symbols represent determinations from different preparations. The inset is a composite of the results from one set of experiments of elution profiles of phosphorylated myosin from the Superose 6 column. The lines represent the absorbance at 280 nm of identical loadings of myosin to the preequilibrated column run at the indicated total ionic strengths (molar). Other conditions and calculations are as described under Materials and Methods.

Effect of Ca^{2+} on the Conformation and Distribution of Myosin Monomers and Polymers. The influence of the buffer conditions used for ATPase assays on the conformational state of the myosin molecules was investigated by gel filtration. Stock myosin was subjected to gel filtration on a Pharmacia FPLC HR 10/30 Superose 6 column equilibrated with buffers identical with those used for ATPase assays. The elution profile of fully phosphorylated gizzard myosin at different ionic strengths in pCa 8 buffers is shown in Figure 7. At high ionic strength, where the myosin molecules do not form filaments (Megerman & Lowey, 1981) and exist as extended monomers (Trybus & Lowey, 1984), the elution was very near the V_o of the column (K_d = 0.04). As the ionic strength decreased, the

Table I: Effect of Ca^{2+} on the Elution of Phosphorylated Myosin from the FPLC Column[a]

pCa	K_d	% monomer
8	0.159 ± 0.003 (6)	75 ± 8 (6)
4	0.160 ± 0.005 (6)	45 ± 10 (6)
3	0.193 ± 0.013 (8)	16 ± 11 (5)

[a] Conditions: Buffers, operation of the column, determination of protein concentration, and calculation of K_d are as outlined under Materials and Methods. Percent monomer was determined from the combined protein values contained in the eluted peak fractions expressed as a percentage of the total myosin loaded. The values are listed ± standard deviation with the number of experiments in parentheses.

relative size of the eluted myosin decreased until a minimum size distribution (K_d = 0.21) was reached near 200 mM total ionic strength. Further reduction in the ionic strength resulted in increasing the size of the eluted myosin molecule with the final K_d = 0.16 at 0.05 M, the ionic strength used for ATPase assays. The inset of Figure 7 is a composite tracing of the A_{280} recorder responses from one set of experiments. In contrast to symmetrical elution profiles reported for unphosphorylated myosin (Cross & Sobieszek, 1985), the elution of phosphorylated myosin was asymmetric at both extremes of ionic strength with symmetrical, highly homogeneous, peaks found only near 200 mM total ionic strength. Also notable was the tendency for prolonged tailing of the peaks at low ionic strengths.

Table I summarizes the elution data from a number of similar experiments carried out to quantitate the conformation and monomer distribution characteristics of myosin in the ATPase buffers with respect to pCa. In low free Ca^{2+} (pCa 8), the majority of loaded myosin elutes as a single peak with slight tailing. In control experiments, conducted in the absence of ATP, no myosin eluted from the column at pCa 8. When ATP was added, the entire amount of loaded myosin eluted gradually. In the presence of 2 mM Mg·ATP, myosin eluted with a similar K_d both at pCa 4 and at pCa 8; however, only half of the loaded myosin eluted with pCa 4 buffer. At pCa 3, only a small amount of the loaded myosin eluted from the column, and the K_d was increased. Full recovery of the retained myosin, as fractions of a single sharp peak, was obtained with a step gradient wash with buffer containing 0.4 M KCl.

Discussion

This investigation to characterize the effects of divalent cations on the actomyosin ATPase is different from previously published reports (Seidel et al., 1986; Ikebe & Hartshorne, 1985; Heaslip & Chacko, 1985; Chacko et al., 1986) in the following aspects: (1) utilization of stably phosphorylated myosin and HMM with gizzard actin and gizzard tropomyosin in the absence of other proteins which bind to the contractile proteins (caldesmon, calmodulin, kinase, etc.); (2) stringently controlled ionic conditions in the ATPase assay buffers, particularly Mg·ATP, pCa, pMg, and the total ionic strength; (3) increased tropomyosin concentration to achieve stoichiometric binding of tropomyosin to actin as determined by Miyata and Chacko (1986); (4) more frequent determinations of the P_i liberated during the course of the ATPase assays; and (5) quantitation of the aggregate state of myosin during the time course of the assays. This experimental approach enabled us to evaluate the effect of varying low levels of free divalent cations on the modulation of the rates of ATP hydrolysis in relation to alterations in myosin monomer–polymer equilibrium during the ATPase assay.

The increase in the ATPase activity of myosin on raising Ca^{2+} to nonphysiological concentrations, shown in Figure 1, is similar to the observation of Ikebe et al. (1983) in which the divalent cation concentration was elevated by increasing free Mg^{2+} concentration. Clearly, high concentrations of free divalent cation (Ca^{2+} or Mg^{2+}), although nonphysiological, permit maximal ATPase activity of reconstituted actomyosin at low ionic strengths at pH 7. While in the current studies at 2 mM free Mg^{2+}, the Mg·ATPase of myosin continues to rise on increasing the Ca^{2+} concentration, the tropomyosin-potentiated, actin-activated ATPase activity declines at the very high Ca^{2+} concentrations. The maximum tropomyosin-potentiated, actomyosin activity requires a total free divalent cation concentration between 3 and 4 mM depending on Ca/Mg. Although increasing Mg^{2+} will substitute for Ca^{2+} in activating the actomyosin ATPase, a given level of activity requires substantially higher concentrations of free Mg^{2+} than free Ca^{2+}. The nonlinearity of the assays with low free divalent cation concentration does not greatly alter the appearance of the curves shown in Figure 1 which is determined from the total liberation of P_i between 15 s and 12 min.

The initial phase of the ATPase reaction in 2 mM free Mg^{2+} is rapid, and it is maintained in pCa 3. The low final rates of ATPase activity in pCa 8 are not associated with denaturation of the myosin or the thin filament protein concentration since the low rates can be hastened by increasing the Ca^{2+} concentration during the assays. The fact that Mg^{2+} can also produce a similar effect, though at a higher divalent cation concentration than is required with Ca^{2+}, indicates that the increase in the rate of ATP hydrolysis is not due to an effect on the Ca^{2+}-specific sites but rather on the Ca^{2+}–Mg^{2+} sites which have a slightly higher affinity for Ca^{2+} over Mg^{2+}. The higher affinity of Mg^{2+} for ATP may account for some of the difference in requirements for these two divalent cations. The nonspecific divalent cation binding sites on the myosin that are occupied by Mg^{2+} are more likely to be altered by the addition of ATP.

An earlier report (Strzelecka et al., 1979), using skeletal muscle proteins, demonstrates that the actomyosin ATPase rate decreased during the course of the assay due to the extrusion of actin from the actomyosin gel during the ATPase assay. In the current study, utilizing smooth muscle proteins, changes in the content of actin and tropomyosin in the pellet were observed to be in the same ratio to the myosin content. However, the myosin content, sedimented as actomyosin complex, is higher during the fast rate than in the slow rate of ATP hydrolysis. At pCa 3, when the activity is maintained at the fast rate, the myosin content of the aggregates is much more stable during the assay. Furthermore, increased myosin sedimentation can be induced during assays in low pCa by raising the free Ca^{2+} concentration. The positive correlation of increased ATPase activity and increased amount of myosin molecules in the pellets suggests that a high proportion of myosin molecules are assembled into filaments and are available for interaction with thin filaments.

Smooth muscle myosin acquires different conformations depending on the state of light-chain phosphorylation, ionic strength, and other buffer conditions (Onishi & Wakabayashi, 1982; Trybus et al., 1982; Trybus & Lowey, 1985; Cross & Sobieszek, 1985, 1986; Ikebe & Hartshorne, 1985). The elution profile (Figure 7) is in agreement with similar data generated with phosphorylated and unphosphorylated myosins (Cross & Sobieszek, 1985) and centrifugation data (Trybus & Lowey, 1984) in which myosin monomers exist in a distribution between folded monomers (10 S) and antiparallel folded dimers (15 S) at ionic strengths near 50 mM. At ionic strengths greater than 0.3 M, the condition at which the

phosphorylated myosin utilized in this investigation is stored, myosin remains in extended (6S) form (Trybus & Lowey, 1984). However, the high ionic strength prevents the aggregation of myosin molecules into filaments (Trinick & Cooper, 1980; Megerman & Lowey, 1981); hence, the myosin remains entirely in monomeric form. Lowering the ionic strength to 0.05 M, a condition similar to the ATPase assay, converts the extended monomeric myosin into both myosin polymers, which remain at the top of the FPLC column, and to folded (10S and 15S) monomers and dimers that elute in the ATPase buffers. The similarity in the K_d values of the eluted myosin in pCa 4 and 8 was consistent with equal distributions of folded monomers and dimers whereas the significant increase in the K_d for pCa 3 suggested a shift of the myosin equilibrium from dimers toward folded monomers. The amount of myosin retained on the top of the column was positively related to the free Ca^{2+} concentration of the elution buffers. Although the time frames and methodologies of Table I and Figure 5 are different, there is notable agreement that the stabilized aggregate structure of myosin in pCa 3 buffers amounts to 75–80% of the total myosin. The data in Figure 5 and Table I also indicate that the proportion of fully phosphorylated myosin molecules which exist in folded forms and as polymers is strongly influenced by the free Ca^{2+} concentration in these buffers. Additionally, there is no evidence for a significant pool of extended monomers (6 S) existing in these buffers. These results support the recent equilibrium model proposed by Cross et al. (1986).

The present study is in full agreement with a previous report that the folded conformation is associated with decreased ATPase activity (Ikebe et al., 1983). Our result on the effect of free Ca^{2+} concentration on the myosin conformation and the associated rate of actin–activated ATP hydrolysis provides evidence that Ca^{2+} modulates the ATPase activity by altering the monomer–polymer equilibrium. Furthermore, the change in the monomer–polymer equilibrium occurs during the ATPase assay, and the conformation that favors the polymer formation and increased actin-activated ATPase activity is induced by Ca^{2+} and Mg^{2+}. Although it is difficult to determine the shape of the myosin molecules in the myosin filaments and/or actomyosin aggregates observed during ATPase assays, the molecules are expected to be in extended form. The time lag between the disassembly of myosin and the loss in ATPase rate noted in these experiments may indicate a change in the active sites of filamentous myosin to an inactive state prior to filament disassembly.

Both dephosphorylated and phosphorylated myosins are in equilibrium between polymer and monomer depending on the ionic conditions (Trybus & Lowey, 1984). In that study, addition of Mg·ATP depolymerizes all dephosphorylated filaments in 150 mM KCl, but only half of the phosphorylated filaments are disassociated to monomers. Phosphorylated myosin which remains in filamentous form in the presence of Mg·ATP can be obtained in phosphate (10 mM) and citrate buffers (Trybus & Lowey, 1984); however, these conditions are not ideal for ATPase assays. Conditions of high Mg^{2+} concentration and low ionic strength (Ikebe et al., 1984) maintain myosin in stable filamentous form; however, the effect of Ca^{2+} on the actomyosin ATPase is not evident in high Mg^{2+} concentration (Chacko & Rosenfeld, 1982).

In the absence of myosin filament formation, the rate of actin-activated ATP hydrolysis is linear as shown in the experiment with HMM (Figure 6). The 2–3 mM difference in total free divalent cation requirements for maximum activities of HMM and intact myosin agrees with previous reports (Kaminski & Chacko, 1984; Seidel et al., 1986). Specific effects of Ca^{2+} or Mg^{2+} on acto-HMM ATPase cannot be determined from this study since the HMM was not fully saturated with actin. However, the absence of a nonlinear acto-HMM ATPase supports the idea that unstable filament structure is responsible for the declining rates of ATP hydrolysis of intact myosin.

Modulation of the actin-activated ATPase of stably phosphorylated smooth muscle myosin by divalent cations observed in this study may be accounted for as follows: The divalent ion concentration controls the rate at which added ATP dissociates the preassembled thick filaments and also defines the final position of the monomer–polymer equilibrium. At pCa 8, the initial rapid rates of hydrolysis decrease as the quantity of myosin in the polymer form decreases and the amount of, less active, folded monomer increases. The final rates of ATP hydrolysis reflect the active polymer–inactive monomer equilibrium position. Free divalent cation concentrations between 3 and 4 mM (depending on whether Ca^{2+} or Mg^{2+} is used) prevent significant degradation of assembled myosin and thus preserve linear rates of activity.

Increasing evidence suggests that myosin molecules in smooth and striated muscle are thought to be in equilibrium with the thick filaments (Trybus & Lowey, 1986; Saad et al., 1986). Myosin in the intact smooth muscle cell is organized into thick filaments irrespective of the level of phosphorylation and the state of contraction (Somlyo et al., 1981). Future study should address the possibility that the myosin monomer–polymer equilibrium may exist in the intact smooth muscle cell and may be altered by the state of myosin phosphorylation and fluctuations in the divalent cation concentration. A shift in this equilibrium may not be enough to totally dismantle the myosin filaments but sufficient to reduce the number of myosin molecules in the filament form. This possibility may also be relevant to skinned fiber preparations (Hellstrand & Anders, 1985; Paul et al., 1983) in which the alteration of the ionic conditions affects myosin filaments more directly than in the intact cell.

ACKNOWLEDGMENTS

We thank Alisa Belzer for secretarial assistance and Drs. Hidetake Miyata and Richard Heaslip for valuable discussions. The gizzards used for this study were a gift donated by Victor F. Weaver Inc., New Holland, PA.

Registry No. ATPase, 9000-83-3; Mg, 7439-95-4; Ca, 7440-70-2.

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