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Filament Assembly and Regulation of the Actin-Activated ATPase Activity of Thymus Myosin[†]

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ABSTRACT: The effects of light chain phosphorylation on the actin-activated ATPase activity and filament assembly of calf thymus cytoplasmic myosin were examined under a variety of conditions. When unphosphorylated and phosphorylated thymus myosins were monomeric, their MgATPase activities were not activated or only very slightly activated by actin, but when they were filamentous, their MgATPase activities were stimulated by actin. The phosphorylated myosin remained filamentous at lower Mg^{2+} concentrations and higher KCl concentrations than did the unphosphorylated myosin, and the myosin concentration required for filament assembly was lower for phosphorylated myosin than for unphosphorylated myosin. By varying the myosin concentration, it was possible to have under the same assay conditions mostly monomeric myosin or mostly filamentous myosin; under these conditions, the actin-activated ATPase activities of the filamentous myosins were much greater than those of the monomeric myosins. The addition of phosphorylated myosin to unphosphorylated myosin promoted the assembly of unphosphorylated myosin into filaments. These results suggest that phosphorylation may regulate the actomyosin-based motile activities in vertebrate nonmuscle cells by regulating myosin filament assembly.

Vertebrate smooth muscle and nonmuscle myosins contain two 200 000-Da heavy chains and two pairs of light chains, M_r 20 000 and 17 000. Phosphorylation of the 20 000-Da light chains (LC20)[1] is thought to play a central role in the regulation of smooth muscle contraction and nonmuscle contractility (Kendrick-Jones & Scholey, 1981; Sellers & Adelstein, 1987). In vitro, this phosphorylation affects both the actin-activated ATPase activities of these myosins (Sobieszek & Small, 1977; Sherry et al., 1978; Trotter & Adelstein, 1979; Sellers et al., 1981; Fechheimer & Cebra, 1982; Scholey et al., 1982; Wagner et al., 1985; Wagner & Vu, 1986) and their assembly into filaments (Suzuki et al., 1978; Scholey et al., 1980; Citi & Kendrick-Jones, 1986; Wagner & Vu, 1987). We have previously shown that the actin-activated ATPase activities of filamentous, unphosphorylated, and phosphorylated calf thymus myosins have about the same maximum rates, V_{max} (Wagner & George, 1986), but phosphorylation does cause a 15-20-fold decrease in K_{app}, the actin concentration required to achieve $^1/_2V_{max}$. Thus, at low actin concentrations phosphorylation causes a large increase in ATPase activity, but at high actin concentrations unphosphorylated and phosphorylated thymus myosins have comparable ATPase activities. Similar results were obtained with skeletal muscle and thymus actins. In the presence of either skeletal muscle or thymus tropomyosin, phosphorylation causes no change in the V_{max} of the actin-activated ATPase activity of filamentous thymus myosin and only a 3-5-fold decrease in K_{app}. This decrease in K_{app} appears to be too small for this phosphorylation to be an effective regulatory system.

The observation that the actin-activated ATPase activities of unphosphorylated and phosphorylated thymus myosins have the same V_{max} was made under conditions in which both the unphosphorylated and the phosphorylated myosins were greater than 90% filamentous (Wagner & George, 1986). These conditions were chosen to avoid the complication of comparing the actin-activated ATPase activities of monomeric and filamentous myosins. When the assays were performed

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[1] Abbreviations: ATPase, adenosinetriphosphatase; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; LC20, 20 000-dalton myosin light chain.

in 150 mM KCl and 10 mM $MgSO_4$, the MgATPase activity of the unphosphorylated myosin even at very high tropomyosin–actin concentrations was less than one-third that of the phosphorylated myosin (Wagner & George, 1986). Since under similar conditions LC20 phosphorylation has been shown to stabilize thymus myosin filaments (Scholey et al., 1980; Craig et al., 1983), this difference in ATPase activity may reflect the different states of assembly of unphosphorylated and phosphorylated thymus myosins under these conditions. This suggests that phosphorylation might regulate the actin-activated ATPase activity of thymus myosin by regulating its filament assembly. In contrast, on the basis of experiments with gizzard smooth muscle heavy meromyosin, it has been suggested that regulation of the actin-activated ATPase activities of nonmuscle myosins is independent of myosin filament assembly (Sellers et al., 1981; Citi & Kendrick-Jones, 1986).

In this paper we have examined the possibility that filament assembly regulates the actin-activated ATPase of thymus myosin by determining the filament assembly and actin-activated ATPase activity of thymus myosin under a variety of conditions. Both the unphosphorylated and the phosphorylated myosins needed to be filamentous for their MgATPase activities to be activated by actin. Under conditions where phosphorylation increased filament formation, there were large increases in actin-activated ATPase activity upon LC20 phosphorylation, but under conditions where phosphorylation had only a small effect on filament formation, it had only a small effect on actin–activated ATPase activity.

MATERIALS AND METHODS

Calf thymus myosin was isolated by using a modification (Wagner et al., 1985) of the "ammonium sulfate" procedure of Scholey et al. (1982). When isolated by this procedure, thymus myosin has about 20% phosphorylated LC20. The light chains were dephosphorylated as described previously (Wagner et al., 1985). The dephosphorylated myosin contained less than 5% phosphorylated LC20. Turkey gizzard myosin light chain kinase was isolated as described by Adelstein and Klee (1981). Actin and skeletal muscle tropomyosin were isolated from the acetone powder of rabbit skeletal muscle (Wagner & Weeds, 1977; Smillie, 1982). Calf thymus tropomyosin was isolated by the procedure of Cote and Smillie (1981) for the isolation of platelet tropomyosin. Tropomyosin–actin was prepared by mixing F-actin and either skeletal muscle or thymus tropomyosin at a 4:1 molar ratio. Bovine testis calmodulin was given to us by Dr. Claude Klee of the National Cancer Institute.

Thymus myosin, 3–5 mg/mL, was phosphorylated at 25 °C by 30 nM gizzard myosin light chain kinase in 0.5 μM calmodulin, 10 mM $MgSO_4$, 1 mM ATP, 150 mM KCl, 1.0 mM DTT, 0.1 mM $CaCl_2$, and 10 mM imidazole, pH 7.0. After 10 min, all of the 20 000-Da light chains were phosphorylated (Wagner et al., 1985). The fraction of LC20 phosphorylated was determined by electrophoresis on urea/glycerol polyacrylamide gels (Perrie & Perry, 1970).

The actin-activated ATPase assays of thymus myosin were performed in 0.5 mM DTT, 1 mM EGTA, 2 mM [γ-^{32}P]ATP, 20 mM imidazole, pH 7.0 at 37 °C, and KCl and $MgSO_4$ as given in the text. The ATPase reactions were started by adding 50 μL of myosin to 450 μL of reaction mixture. The myosin was usually 0.3 mg/mL, and the actin and tropomyosin–actin concentrations were varied from 20 to 120 μM. Aliquots were removed after 4, 9, and 14 min, and the amount of inorganic phosphate was determined (Pollard & Korn, 1973). Similar results were obtained by using skeletal muscle and thymus tropomyosins. The MgATPase activity of thymus myosin in the absence of actin varied from 3 to 20 nmol min^{-1} mg^{-1} depending on assay conditions. These rates are given in the figure legends.

The fraction of filamentous myosin was estimated by sedimentation (Kendrick-Jones et al., 1983; Sellers et al., 1983; Wagner et al., 1985; Wagner & Vu, 1987; Citi & Kendrick-Jones, 1986). Myosin was incubated in the same buffers used for the ATPase assays at 37 °C for 5 min, and then 1-mL samples were centrifuged at 37 °C for 15 min at 125000*g* in a Beckman Ti50 rotor. The fraction of myosin remaining in the supernatant was determined by a dye binding assay (Bradford, 1976). The fraction of myosin pelleted under any given set of conditions varied by about 5% from one experiment to the next. In the absence of ATP, greater than 95% of the myosin in 6 mM $MgSO_4$ and 50 mM KCl (filamentous myosin) was pelleted by a 15-min centrifugation at 125000*g*, and only 5–10% of the myosin in 0.6 M KCl (monomeric myosin) sedimented. The fraction of myosin pelleted in the different assay solutions was used as an indication of the fraction of myosin in filaments. The state of myosin filament assembly was checked by electron microscopy. Samples of myosin (10 μL) in the buffers used for the ATPase assays were placed on electron microscope grids. After 10 s, the samples were washed with 10 drops of the same buffer but without ATP and stained with 1% uranyl acetate (Scholey et al., 1980, 1982). This technique can be used to determine whether a sample of myosin contains filaments, but it cannot be used to quantitate the fraction of filamentous myosin.

Sedimentation velocity measurements were carried out in a Beckman Model E ultracentrifuge at 25 °C using the UV absorption optical system. In agreement with published data (Scholey et al., 1982), thymus myosin in 0.6 M KCl, 10 mM $MgCl_2$, 0.2 mM DTT, and 10 mM imidazole, pH 7.0, had a sedimentation coefficient of 5.9 S, and unphosphorylated thymus myosin in 150 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 0.2 mM DTT, and 10 mM imidazole, pH 7.0, had a sedimentation coefficient of 11.2 S.

RESULTS

Dependence of the Actin-Activated ATPase Activity and Filament Stability of Thymus Myosin on $MgSO_4$ and KCl. The filament stability and actin-activated ATPase activity of thymus myosin were determined in 50 mM KCl, 2 mM ATP, and varying $MgSO_4$ (Table I). The fraction of filamentous myosin was estimated by sedimentation. The actin-activated ATPase assays shown in Table I were performed in the presence of 20 μM skeletal muscle actin and 5 μM thymus tropomyosin. In 10 mM $MgSO_4$ and 50 mM KCl, the K_{app} values of the actin-activated ATPase activities of unphosphorylated and phosphorylated thymus myosins in the presence of thymus tropomyosin are respectively about 20 and 4 μM, and the V_{max} of the unphosphorylated myosin is about 90% that of the phosphorylated myosin (Wagner & George, 1986). As expected from this difference in K_{app} values, the observed rate for the unphosphorylated myosin in 10 mM $MgSO_4$ was about half that of the phosphorylated myosin. Below 5 mM $MgSO_4$, both the fraction of the unphosphorylated myosin in filaments and the tropomyosin–actin-activated ATPase activity of the unphosphorylated myosin decreased. But even in 3 mM $MgSO_4$, almost 40% of the unphosphorylated myosin pelleted and its MgATPase activity was activated 8-fold by tropomyosin–actin. In 3–10 mM $MgSO_4$ and 50 mM KCl, the MgATPase activity of the phosphorylated myosin was stimulated about 10-fold by tropomyosin–actin, and most of the phosphorylated myosin was filamentous.

Table I: Effect of $MgSO_4$ on the ATPase Activity and Filament Assembly of Thymus Myosin[a]

	unphosphorylated myosin			phosphorylated myosin		
	ATPase activity (nmol min^{-1} mg^{-1})			ATPase activity (nmol min^{-1} mg^{-1})		
$[MgSO_4]$ (mM)	−actin	+actin[b]	filament stability, fraction pelleted[c]	−actin	+actin[b]	filament stability, fraction pelleted[c]
3	5	40	0.38 (0.04)	14	157	0.93 (0.04)
4		59	0.44 (0.04)		152	0.90 (0.05)
5		74	0.52 (0.05)		141	0.91 (0.04)
6		75	0.55 (0.03)		144	0.94 (0.06)
8		74	0.58 (0.07)		142	0.93 (0.03)
10	7	76	0.70 (0.05)	18	149	0.94 (0.02)

[a] The assays were performed at 37 °C in 50 mM KCl, 2 mM ATP, 1 mM EGTA, 0.5 mM DTT, 20 mM imidazole, pH 7.0, and $MgSO_4$ as indicated. Myosin was 0.3 mg/mL. [b] 20 μM skeletal muscle actin and 5 μM thymus tropomyosin. [c] Fraction myosin pelleted by a 15-min centrifugation at 125000*g*. The numbers in parentheses are standard deviations from three different determinations.

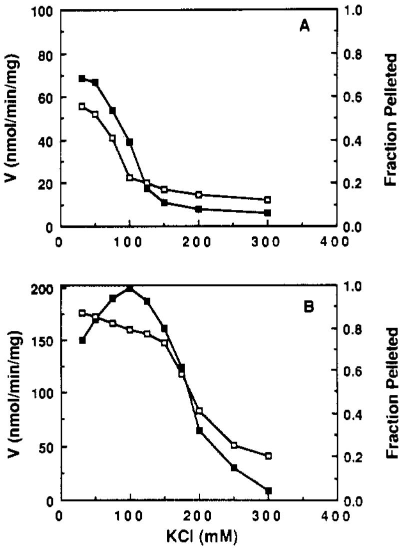

FIGURE 1: Effect of KCl on the actin-activated ATPase activity and filament stability of thymus myosin. The tropomyosin–actin-activated ATPase activity (■) and the fraction of myosin pelleted by a 15-min centrifugation at 125000*g* (□) were determined at 37 °C in 6 mM $MgSO_4$, 2 mM ATP, 1 mM EGTA, 0.5 mM DTT, 20 mM imidazole, pH 7.0, and varying KCl. The myosin was 0.3 mg/mL. The actin-activated ATPase activities were determined in 20 μM skeletal muscle actin and 5 μM skeletal muscle tropomyosin. Panel A shows the results with the unphosphorylated myosin, and panel B shows the results with the phosphorylated myosin. In the absence of actin, the MgATPase activity of the unphosphorylated myosin in 30, 150, and 300 mM KCl was respectively 3, 4, and 20 nmol min^{-1} mg^{-1}, and for the phosphorylated myosin it was 11, 11, and 20 nmol min^{-1} mg^{-1}. These activities have been subtracted from the activities observed in the presence of tropomyosin–actin.

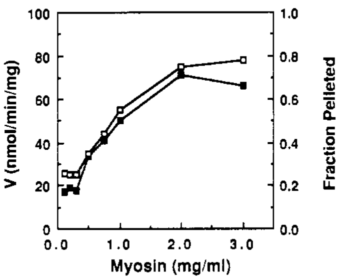

FIGURE 2: Dependence of the actin-activated ATPase activity and filament assembly of unphosphorylated thymus myosin on myosin concentration. The tropomyosin–actin-activated ATPase activity (■) and the fraction of myosin pelleted by a 15-min centrifugation at 125000*g* (□) were determined in 6 mM $MgSO_4$, 2 mM ATP, 125 mM KCl, 1 mM EGTA, 0.5 mM DTT, and 20 mM imidazole, pH 7.0 at 37 °C. The actin-activated ATPase activities were determined in the presence of 50 μM skeletal muscle actin and 12.5 μM skeletal muscle tropomyosin. The MgATPase activity in the absence of actin, 7 nmol min^{-1} mg^{-1}, has been subtracted from the activities observed in the presence of tropomyosin–actin.

The filament stability and actin-activated ATPase activities of thymus myosin were determined in 6 mM $MgSO_4$, 2 mM ATP, and varying KCl (Figure 1). The actin-activated ATPase assays were performed in the presence of 20 μM skeletal muscle actin and 5 μM skeletal muscle tropomyosin. In 30 mM KCl, the tropomyosin–actin-activated ATPase activity of the unphosphorylated myosin was 67 nmol min^{-1} mg^{-1}, 40% that of the phosphorylated myosin. As the KCl concentration was increased from 30 to 150 mM, the tropomyosin-activated ATPase activity of the unphosphorylated myosin and the fraction of unphosphorylated myosin pelleted decreased in parallel (Figure 1A).

As the KCl concentration was increased from 30 to 150 mM, there was a small decrease in the fraction of phosphorylated myosin pelleted, and the tropomyosin–actin-activated ATPase activity first increased and then decreased such that the tropomyosin–actin-activated ATPase activity in 150 mM KCl was about the same as that in 30 mM KCl (Figure 1B). As the KCl concentration was increased above 150 mM, both the fraction of phosphorylated myosin pelleted and the tropomyosin–actin-activated ATPase activity decreased rapidly. In 300 mM KCl, only 20% of the phosphorylated myosin pelleted and the tropomyosin–actin-activated ATPase activity was only 5% of that obtained in 150 mM KCl. At approximately physiological ionic strength, phosphorylation caused large increases in both filament formation and tropomyosin–actin-activated ATPase activity.

Filament formation was also checked by electron microscopy. Under conditions where about 20% of the myosin pelleted, e.g., unphosphorylated myosin in 6 mM $MgSO_4$, 2 mM ATP, and 125 mM KCl, very few myosin filament were observed in the electron microscope. On the other hand, unphosphorylated myosin in 6 mM $MgSO_4$, 2 mM ATP, and 30 mM KCl contained many filaments.

Effect of Myosin Concentration on the Actin-Activated ATPase Activity and Filament Stability of Thymus Myosin. The fraction of myosin that is filamentous depends upon the myosin concentration (Josephs & Harrington, 1966; Pollard, 1982; Kendrick-Jones et al., 1987). Below a critical concentration, all of the myosin is monomeric. Above this critical concentration, myosin polymerizes to form filaments that are in equilibrium with the critical concentration of myosin monomers. The critical concentration depends on the type of myosin and assay conditions. The myosin concentrations in the assays shown in Table I and Figure 1 were 0.3 mg/mL. Figure 2 shows the effect of myosin concentration on filament formation and tropomyosin–actin-activated ATPase activity of unphosphorylated thymus myosin in 6 mM $MgSO_4$, 2 mM ATP, and 125 mM KCl, conditions in which phosphorylation can cause a large increase in filament formation (Figure 1).

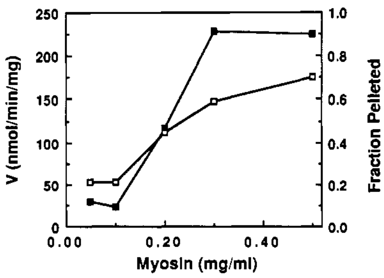

FIGURE 3: Dependence of the actin-activated ATPase activity and filament assembly of phosphorylated thymus myosin on myosin concentration. The tropomyosin-actin-activated ATPase activity (■) and the fraction of myosin pelleted by a 15-min centrifugation at 125000*g* (□) were determined in 3 mM $MgSO_4$, 2 mM ATP, 175 mM KCl, 1 mM EGTA, 0.5 mM DTT, and 20 mM imidazole, pH 7.0 at 37 °C. The actin-activated ATPase activities were determined in the presence of 50 μM skeletal muscle actin and 12.5 μM skeletal muscle tropomyosin. The MgATPase activity in the absence of actin, 14 nmol min^{-1} mg^{-1}, has been subtracted from the activities observed in the presence of tropomyosin–actin.

Below 0.3 mg/mL, most of the unphosphorylated myosin was monomeric, and its MgATPase activity was only slightly stimulated by 50 μM tropomyosin–actin. As the myosin concentration was increased above 0.3 mg/mL, the fraction of myosin pelleted and the tropomyosin–actin-activated ATPase activity increased in parallel. The rate obtained for unphosphorylated myosin at 2.0 mg/mL was 71 nmol min^{-1} mg^{-1}, 40% that of the phosphorylated myosin. Similar results were obtained with the unphosphorylated myosin in 3 mM $MgSO_4$, 2 mM ATP, and 75 mM KCl; when the myosin was 0.15 mg/mL, 24% was pelleted and the tropomyosin–actin-activated ATPase activity was 7 nmol min^{-1} mg^{-1}, and when the myosin was 3 mg/mL, 68% was pelleted and the tropomyosin–actin-activated ATPase activity was 38 nmol min^{-1} mg^{-1}. These results show that under the same assay conditions, monomeric and filamentous unphosphorylated thymus myosins have very different actin-activated ATPase activities.

In both of the above buffers, 0.1 mg/mL phosphorylated myosin was mostly filamentous, and its tropomyosin–actin-activated ATPase activity did not increase when the myosin concentration was increased from 0.1 to 0.5 mg/mL. However, in 3 mM $MgSO_4$, 2 mM ATP, and 175 mM KCl, the fraction of myosin pelleted and the tropomyosin–actin-activated ATPase activity did depend on the concentration of phosphorylated myosin (Figure 3). Below 0.1 mg/mL, most of the phosphorylated myosin was monomeric and its MgATPase activity was only slightly stimulated by tropomyosin–actin. Above 0.1 mg/mL, both the fraction pelleted and the tropomyosin–actin-activated ATPase activity increased. The tropomyosin–actin-activated ATPase activity at 0.3 mg/mL phosphorylated myosin was about 10 times that at 0.1 mg/mL. Thus, as observed with unphosphorylated myosin, monomeric and filamentous phosphorylated thymus myosins have very different actin-activated ATPase activities.

Effect of Increasing Actin Concentration on the MgATPase Activity of Monomeric Thymus Myosin. The MgATPase activities shown in Figure 1 were determined in the presence of 20 μM tropomyosin–actin, and those shown in Figures 2 and 3 were determined in the presence of 50 μM tropomyosin–actin. The low stimulations of the MgATPase activity of the myosin monomers under these conditions could result from their actin-activated ATPase activities having either low V_{max} or high K_{app}s. To try to determine which, the MgATPase activities of thymus myosin were measured at varying concentrations of tropomyosin–actin. In 3 mM $MgSO_4$, 2 mM ATP, and 175 mM KCl, 0.5 mg/mL phosphorylated thymus myosin was mostly filamentous (Figure 3), and its tropomyosin–actin-activated ATPase activity had a V_{max} of 220 nmol min^{-1} mg^{-1} and a K_{app} of 6 μM. Under these same conditions, 0.1 mg/mL phosphorylated myosin was mostly monomeric, and its MgATPase activity in 20, 50, and 120 μM tropomyosin–actin was only about 20 nmol min^{-1} mg^{-1}. Thus, either the V_{max} of the monomeric myosin was less than 10% that of the filamentous myosin or its K_{app} was much greater than 120 μM. It was not practical to use more than about 120 μM tropomyosin–actin, as the solutions became too viscous to pipet reproducibly. If the monomer had the same V_{max} as the filament and if its MgATPase activity had a hyperbolic dependence on actin concentration, the K_{app} of the monomer would be about 1000 μM. As the highest tropomyosin–actin concentration used was 120 μM, this is only a rough estimate. If the low ATPase activity found for the mostly monomeric myosin was due to a small fraction that was filamentous, then the differences in the actin-activated ATPase activities of the monomer and filament would be even greater. In 6 mM $MgSO_4$, 2 mM ATP, and 125 mM KCl, 0.3 mg/mL unphosphorylated thymus myosin was mostly monomeric (Figure 2), and its MgATPase activity in the presence of 120 μM tropomyosin–actin was only 17 nmol min^{-1} mg^{-1}; 0.3 mg/mL phosphorylated myosin was mostly filamentous, and its tropomyosin–actin-activated ATPase activity had a V_{max} of 180 nmol min^{-1} mg^{-1} and a K_{app} of about 2 μM. Under these conditions, 2.0 mg/mL unphosphorylated myosin was mostly filamentous, and its MgATPase activity in 50 μM tropomyosin–actin was 71 nmol min^{-1} mg^{-1}; V_{max} was not determined because the rate of ATP hydrolysis at this high myosin concentration was too fast.

In 6 mM $MgSO_4$, 2 mM ATP, and 50 mM KCl, 52% of 0.3 mg/mL unphosphorylated myosin and 85% of 0.3 mg/mL phosphorylated myosin were pelleted (Figure 1). Under these conditions the tropomyosin–actin-activated ATPase activity of the unphosphorylated myosin had a V_{max} of 101 nmol min^{-1} mg^{-1} and a K_{app} of 13 μM, and the phosphorylated myosin had a V_{max} of 190 nmol min^{-1} mg^{-1} and a K_{app} of 2 μM. The low K_{app} of the unphosphorylated myosin under this condition is similar to that we previously obtained for filamentous, unphosphorylated myosin (Wagner & George, 1986). This result is consistent with the fraction of unphosphorylated myosin that was filamentous having a V_{max} comparable to that of filamentous, phosphorylated myosin and with the monomeric, unphosphorylated myosin not being stimulated by actin.

Conformation of Thymus Myosin Monomers. Vertebrate smooth muscle and nonmuscle myosins monomers can exist in two different conformations. On the basis of their sedimentation coefficients they are referred to as the 6S and 11S monomers (Suzuki et al., 1982; Trybus et al., 1982; Scholey et al., 1982). In the 6S conformation the myosin tail has an extended conformation, and in the 11S conformation, the tail bends back on itself and interacts with the myosin heads (Onishi & Wakabayashi, 1982; Trybus et al., 1982; Craig et al., 1983). Thymus myosin in 0.6 M KCl, 10 mM $MgCl_2$, 0.2 mM DTT, and 10 mM imidazole, pH 7.0, has a sedimentation coefficient of 5.9 S, and unphosphorylated thymus myosin in 150 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 0.2 mM DTT, and 10 mM imidazole, pH 7.0, has a sedimentation coefficient of 11.2 S. Similar values have been reported by Scholey et al. (1982). Thymus myosin in these two buffers was used to standardize a Pharmacia Superose 6 HR10/30 gel filtration column. As has been reported for gizzard myosin (Trybus et al., 1982), 6S thymus myosin monomer eluted faster than did the 11S thymus monomer. The 6S monomer eluted after 9

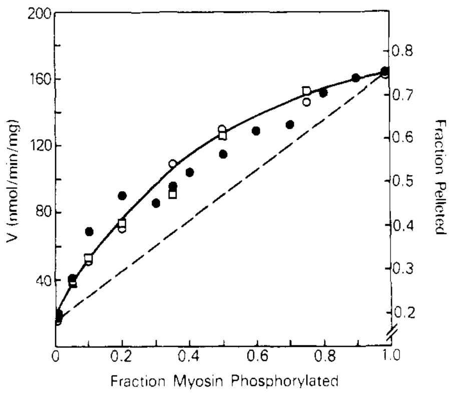

FIGURE 4: Effect of phosphorylated thymus myosin on the actin-activated ATPase activity and filament assembly of unphosphorylated thymus myosin. Varying ratios of unphosphorylated myosin and phosphorylated myosin were mixed either as monomers in 0.3 M KCl and 2 mM EGTA (○, ●) or as preformed filaments in 125 mM KCl, 5 mM $MgSO_4$, and 2 mM EGTA (□). The tropomyosin–actin-activated ATPase activities of these mixtures (○, □) and the fraction of myosin in these mixtures pelleted by a 15-min centrifugation at 125000*g* (●) were determined in 6 mM $MgSO_4$, 2 mM ATP, 125 mM KCl, 1 mM EGTA, 0.5 mM DTT, and 20 mM imidazole, pH 7.0 at 37 °C. The actin-activated ATPase activities were determined in the presence of 50 μM skeletal muscle actin and 12.5 μM skeletal muscle tropomyosin. The total myosin concentration in the ATPase and sedimentation assays was 0.3 mg/mL. The MgATPase activity in the absence of actin, 7 nmol min^{-1} mg^{-1}, has been subtracted from the activities.

mL, and the 11S monomer eluted after 11 mL. This column was used to determine the conformation of the myosin monomers under the conditions used for the ATPase assays; 100-μL samples of 0.5–3.0 mg/mL myosin were loaded, and the myosin eluted over about 1 mL.

Unphosphorylated myosin in 6 mM $MgSO_4$, 2 mM ATP, and 125 mM KCl and in 3 mM $MgSO_4$, 2 mM ATP, and 75 mM KCl eluted from the Superose column at the position of the 11S myosin monomer. Phosphorylated myosin in 6 mM $MgSO_4$, 2 mM ATP, and 200 mM KCl and in 3 mM $MgSO_4$, 2 mM ATP, and 175 mM KCl eluted from the Superose column in two peaks. About 70–90% eluted in the same position as the 11S monomer, and the rest eluted in the void volume. Myosin filaments also eluted in the void volume. Unphosphorylated and phosphorylated myosins in 6 mM $MgSO_4$, 2 mM ATP, and 300 mM KCl eluted in the same position as the 6S monomer.

Interactions between Unphosphorylated and Phosphorylated Thymus Myosins. The interaction of phosphorylated myosin with unphosphorylated myosin was examined under conditions in which the unphosphorylated myosin was mostly monomeric and the phosphorylated myosin was mostly filamentous. Varying ratios of unphosphorylated and phosphorylated myosin were mixed, and their tropomyosin–actin-activated ATPase activities and filament stabilities were determined in 6 mM $MgSO_4$, 2 mM ATP, and 125 mM KCl (Figure 4). The total myosin concentration used in these assays was 0.3 mg/mL. The ATPase assays were performed in the presence of 50 μM tropomyosin–actin, so that if the unphosphorylated myosin was filamentous, its MgATPase activity would be a significant fraction of that of the phosphorylated myosin. Both the fraction of myosin pelleted and the tropomyosin–actin-activated ATPase activity of these mixtures were not simply additive but were greater than if there was no interaction between the unphosphorylated and phosphorylated myosins. The same result was obtained when the myosins were mixed as monomers in 300 mM KCl and 2 mM EGTA (open circles) or as preformed filaments in 125 mM KCl, 5 mM $MgSO_4$, and 2 mM EGTA (open squares). Thus, phosphorylated myosin appeared to promote the assembly of the unphosphorylated into filaments, most likely by forming filaments that contained both types of myosin. The MgATPase activity of the unphosphorylated myosin in these mixed filaments was activated by tropomyosin–actin. Low levels of phosphorylated myosin did not cause all of the unphosphorylated myosin to assemble into filaments. If it had, the fraction of myosin pelleted would have been the same at all ratios of phosphorylated to unphosphorylated myosin.

DISCUSSION

Thymus myosin filament assembly and actin-activated ATPase activity were affected by ionic strength (Figure 1), Mg^{2+} concentration (Table I), myosin concentration (Figures 2 and 3), and LC20 phosphorylation. Phosphorylated thymus myosin remained filamentous at higher KCl and lower $MgSO_4$ concentrations than did the unphosphorylated myosin. When unphosphorylated and phosphorylated myosin were filamentous, their MgATPase activities were stimulated 10–20-fold by actin. The K_{app} of the tropomyosin–actin-activated ATPase activity of filamentous, unphosphorylated thymus myosin is greater than that of the filamentous, phosphorylated myosin (Wagner & George, 1986). Thus, even under conditions where both myosins were mostly filamentous, the observed tropomyosin–actin-activated ATPase activity of the phosphorylated myosin was usually greater than that of the unphosphorylated myosin. When unphosphorylated and phosphorylated thymus myosins were monomeric, their MgATPase activities were not activated or only very slightly activated by actin. Under the conditions used for the ATPase assays described in this paper, thymus myosin monomers were mostly in the folded 11S conformation. Increasing the tropomyosin–actin concentration from 20 to 120 μM did not cause any significant increase in the MgATPase activities of the myosin monomers. Thus, either the V_{max} values of the actin-activated ATPase activities of the monomers were only about 10% of those of the filamentous myosins or the monomers have very high K_{app} values, as much as 100 times greater than that of the filamentous myosin.

By varying the myosin concentration, it was possible to have under the same assay conditions mostly monomeric, unphosphorylated myosin or mostly filamentous, unphosphorylated myosin (Figure 2); the actin-activated ATPase activity of the filamentous myosin was much greater than that of the monomeric myosin. Similar results were obtained with phosphorylated thymus myosin except, because of the greater stability of the phosphorylated myosin filaments, the assays were performed at lower $MgSO_4$ and higher KCl. Thus, the state of myosin assembly appears to be of primary importance in determining the actin-activated ATPase activity of thymus myosin. This conclusion is different from that obtained with gizzard smooth muscle heavy meromyosin (Sellers et al., 1981). Since gizzard heavy meromyosin cannot form filaments, regulation of its actin-activated ATPase activity by light chain phosphorylation must be independent of filament assembly. This result with gizzard heavy meromyosin has been used to infer that the regulation of the actin-activated ATPase activities of nonmuscle myosins by light chain phosphorylation is also independent of filament assembly (Sellers et al., 1981; Citi & Kendrick-Jones, 1986); i.e., the V_{max} values of the actin-activated ATPase activities of filamentous, unphosphorylated nonmuscle myosins would be much less than those of the filamentous, phosphorylated myosins. However, these authors did not determine the actin-activated ATPase activities of unphosphorylated and phosphorylated nonmuscle myosins under conditions in which both types of myosin are filamentous. On the other hand, we have shown that at high actin concentrations filamentous, unphosphorylated and phosphorylated thymus myosins have comparable ATPase activities (Wagner & George, 1986).

While there was a reasonable correlation between actin activation and the fraction of myosin pelleted, it was not possible to calculate specific activities in terms of filament concentration. One difficulty is that while an increase in the fraction of myosin pelleted appears to be a good indication of increased filament formation, the fraction of myosin pelleted is not the same as the fraction of myosin in filaments. The fraction of monomeric myosin pelleted depended on whether it was 6S or 11S. Under conditions where the myosin was in the 6S conformation (0.6 M KCl), 5–10% of the myosin pelleted, and under conditions where the myosin was in the 11S conformation, 10–20% of the myosin pelleted. Under both of these conditions, almost no myosin filaments could be observed by electron microscopy. Another difficulty is that actin may affect myosin filament assembly. In 10 mM $MgSO_4$ and 150 mM KCl, conditions in which unphosphorylated thymus myosin should be mostly monomeric (Scholey et al., 1980), the MgATPase activity of the unphosphorylated myosin in the presence of high concentrations of tropomyosin–actin is not linear; the rate of ATP hydrolysis increases with time (Wagner & George, 1986). This time-dependent increase in actin-activated ATPase activity is consistent with actin promoting myosin filament assembly. However, in 6 mM $MgSO_4$ and 50 mM KCl, conditions in which the unphosphorylated myosin was a mixture of monomers and filaments, the tropomyosin–actin-activated ATPase assays were linear. Also under conditions in which the unphosphorylated myosin was mostly monomeric, 6 mM $MgSO_4$, 2 mM ATP, and 125 mM KCl, the addition of 55 and 110 μM tropomyosin–actin did not increase the amount of unphosphorylated myosin pelleted (Wagner and Vu, unpublished observations). Thus, if actin affects myosin filament formation, the effect is small and only detectable at high actin concentrations.

Under the one set of conditions examined, phosphorylated thymus myosin appeared to promote the assembly of the unphosphorylated myosin into filaments. Similar results have recently been reported by Citi et al. (1987) for both thymus and brush border myosins. The ability of phosphorylated myosin to cause unphosphorylated myosin to form filaments could allow for small changes in phosphorylation to cause large changes myosin filament assembly (Citi et al., 1987). Under very different assay conditions, Trybus and Lowey (1985) found that unphosphorylated gizzard myosin did not assemble into minifilaments and phosphorylated gizzard myosin had no effect on the assembly of the unphosphorylated myosin. At this time it is not known whether this difference between gizzard myosin and thymus and brush border myosins is due to an inherent difference in these myosins or if it is due to the different assay conditions used.

The observation that actin activates the MgATPase activity of filamentous, unphosphorylated thymus myosin may be applicable to other vertebrate cytoplasmic myosins. Citi and Kendrick-Jones (1986) have examined the effect of LC20 phosphorylation on chicken brush border myosin filament assembly and actin-activated ATPase activity. They used the myosin as isolated that contained 10–20% phosphorylated LC20, phosphatase-treated myosin that contained less than 10% phosphorylated LC20, and myosin that was fully phosphorylated. Under conditions in which the dephosphorylated myosin was mostly monomeric and fully phosphorylated myosin was mostly filamentous, about 80% of the myosin with 10–20% phosphorylated LC20 appeared to be filamentous. Citi and Kendrick-Jones concluded that the presence of phosphorylated myosin stabilizes filaments of the unphosphorylated myosin. They also found that while the actin-activated ATPase activity of dephosphorylated brush border myosin was less than 20% that of the fully phosphorylated myosin, the actin-activated ATPase activity of the myosin with 10–20% phosphorylated LC20 was about 60% that of the fully phosphorylated myosin. While the authors gave no explanation for this observation, a likely explanation is that the unphosphorylated myosin in the sample with 10–20% phosphorylated LC20 was activated by actin. The large increase in ATPase activity caused by a small change in LC20 phosphorylation may have resulted from a large increase in myosin filament formation. However, they did not determine what fraction of the myosin was filamentous under the conditions in which the ATPase assay was performed.

Light chain phosphorylation has been shown to increase the actin-activated ATPase activities of cytoplasmic myosins isolated from macrophages (Trotter & Adelstein, 1979), platelets (Sellers et al., 1981), lymphocytes (Fechheimer & Cebra, 1982), brain (Barylko & Sobieszek, 1983), and erythrocytes (Wong et al., 1985). However, these assays were usually performed at relatively low actin concentrations, and the states of myosin assembly were not determined.

Under approximately physiological conditions, light chain phosphorylation can cause large increases in thymus myosin filament formation (Figure 1). However, the applicability of this observation to the state of myosin assembly in the cell is uncertain. Under approximately physiological conditions, light chain phosphorylation also regulates smooth muscle myosin filament assembly in vitro (Suzuki et al., 1978; Scholey et al., 1980), but in intact relaxed smooth muscles, the unphosphorylated myosin is filamentous (Somlyo et al., 1981). A possible explanation for this difference is that the concentration of myosin in the smooth muscle is greater than the critical concentration for filament assembly. The state of myosin filament assembly in vertebrate nonmuscle cells is unknown. The myosin concentration in thymocytes is about 0.4 mg/mL (Scholey et al., 1982), but the local concentration may be higher. Also, other proteins in the cell may influence myosin filament formation.

If phosphorylation regulates filament assembly in vivo, it could be an effective system for regulating actomyosin-based motile activities in nonmuscle cells. In the unstimulated cell, the unphosphorylated myosin monomer would be in the 11S conformation. In this folded conformation, it may be able to diffuse more readily through the cell than if it were in the extended 6S conformation. Upon stimulation, a rise in internal Ca^{2+} concentration activates myosin light chain kinase, which then phosphorylates myosin. The phosphorylated myosin forms filaments that interact with actin to cause movement. However, if the unphosphorylated myosin is filamentous in vivo, the difference in the tropomyosin–actin-activated ATPase activities of unphosphorylated and phosphorylated thymus myosins would be too small for this phosphorylation to be an effective on–off switch, and the cell must have some other mechanism to regulate its interaction with actin. One potential thin filament regulatory protein is caldesmon (Owada et al., 1984; Bretscher & Lynch, 1985; Ngai & Walsh, 1985; Sobue et al., 1985).

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