Effects of Phosphorylation, Magnesium, and Filament Assembly on Actin-Activated ATPase of Pig Urinary Bladder Myosin†

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ABSTRACT: The relationship between the light-chain phosphorylation and the actin-activated ATPase activity of pig urinary bladder myosin was either linear or nonlinear depending on the free Mg^{2+} concentration. Varying the free [Mg^{2+}] in the presence of 50 mM ionic strength (I) had a biphasic effect on the actin-activated ATPase. In 100 mM I, the activity increased on raising the free [Mg^{2+}]. The activity of the phosphorylated myosin was 3–23-fold higher than that of the unphosphorylated myosin at all concentrations of free Mg^{2+}, pH, and temperature used in this study. The increase in the turbidity and sedimentability of both phosphorylated and unphosphorylated myosins on raising the free [Mg^{2+}] was associated with a rise in the actin-activated ATPase activity. However, myosin light-chain phosphorylation still had a remarkable effect on the actin activation. The myosin polymers formed under these conditions were sedimented by centrifugation. Experiments performed with myosin polymers formed in mixtures of unphosphorylated and phosphorylated myosins showed that the presence of phosphorylated myosin in these mixtures had a slight effect on the sedimentation of the unphosphorylated myosin but it had no effect on the actin-activated ATP hydrolysis. Electron microscopy showed that the unphosphorylated myosin formed unorganized aggregates while phosphorylated myosin molecules assembled into bipolar filaments with tapered ends. These data show that although the unphosphorylated and phosphorylated myosins have the same level of sedimentability and turbidity, the filament assembly present only with the phosphorylated myosin can be associated with the maximal actin activation of Mg-ATPase.

Several reports demonstrate that the phosphorylation of smooth muscle myosin light chain augments the activation of myosin Mg-ATPase by actin [Chacko et al., 1977; Gorecka et al., 1976; Sobieszek & Small, 1977; Sellers et al., 1981; for a review, see Adelstein and Eisenberg (1980)]. Both linear (Sobieszek, 1977; Chacko, 1981; Chacko & Rosenfeld, 1982; Wagner et al., 1985) and nonlinear (Hartshorne & Persechini, 1980; Ikebe et al., 1982; Sellers et al., 1983) correlations between ATPase and light-chain phosphorylation have been reported. The linear relationship between light-chain phosphorylation and actin-activated ATP hydrolysis suggests that the two heads are regulated independently. On the other hand, the nonlinear relationship suggests that a 50% phosphorylation of the myosin is essential before it is activated by actin, indicating that both heads of the myosin are required to be phosphorylated for the activation of the Mg-ATPase of myosin by actin.

The actin-activated ATPase activity of smooth muscle myosin is also modulated by the conformation of the myosin molecule (Ikebe et al., 1984). Smooth muscle and nonmuscle myosins have been shown to exist either in a bent or in an extended conformation (Suzuki et al., 1978; Onishi & Wakabayashi, 1982; Trybus et al., 1982; Craig et al., 1983; Cross, 1988). Ionic conditions alter the equilibrium between the two forms of myosin molecules. Myosin light-chain phosphorylation favors the extended conformation in the presence of ATP at an ionic strength close to the physiological condition (Trybus et al., 1982; Trybus & Lowey, 1984). The folded form of gizzard myosin has a lower actin-activated ATPase activity than that of the extended form (Ikebe & Hartshorne, 1985). It has been suggested that the folded myosin molecules form antiparallel dimers (Trybus & Lowey, 1984). The antiparallel dimers assemble into side-polar filaments (Craig & Megerman, 1977). Myosin filaments which are stable in the presence of ATP are formed by the phosphorylated myosin, but not by the unphosphorylated myosin (Suzuki et al., 1978; Kendrick-Jones et al., 1982; Trybus et al., 1982). Under the ionic conditions that give the maximum actin-activated ATPase activity, the majority of the myosin molecules are assembled into filaments that can be sedimented by centrifugation, leaving behind the remaining myosin molecules either as a folded monomeric form or as an antiparallel dimer (Trybus & Lowey, 1984; Fillers & Chacko, 1987).

In order to study the effect of myosin filament assembly on actin-activated ATPase activity, Wagner and Vu (1987, 1988) allowed unphosphorylated myosins isolated from mammalian smooth and nonmuscle cells to assemble into filaments by increasing the Mg^{2+} concentration, and the actin-activated ATPase activities of these myosins were determined. They report that these myosins are active in the unphosphorylated form if they are assembled into filaments. On the basis of this finding, it has been suggested that the actin-activated ATP hydrolysis by smooth and nonmuscle myosins is regulated by the filament assembly. Hence, the increased actin activation of the phosphorylated myosin is attributed to be mediated through the effect of phosphorylation on the filament assembly (Wagner & Vu, 1988).

We demonstrate that the relationship between light-chain phosphorylation and actin activation of myosin ATPase is modulated by free Mg^{2+} concentrations. Raising the [Mg^{2+}] increases the turbidity and the sedimentability of myosin ir-

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respective of the state of phosphorylation; however, the actin-activated ATPase activity of the phosphorylated myosin is 3–23-fold higher than that of the unphosphorylated myosin under a variety of ionic conditions. Electron microscopy of the myosin kept under conditions of the ATPase assay reveals that the phosphorylated myosin is assembled into bipolar tapered filaments whereas the unphosphorylated myosin forms unorganized sedimentable aggregates. Although mammalian urinary bladder myosins in the unphosphorylated and the phosphorylated states have almost the same level of sedimentability and turbidity in high [Mg^{2+}], the maximal actin activation of the Mg-ATPase can be related only to the filament assembly present with the phosphorylated myosin.

Materials and Methods

Swine urinary bladders were collected from the slaughterhouse, stored in ice, and transported to the laboratory. The muscular layer was separated from the mucosa and the serosa, frozen in liquid nitrogen, and crushed into small pieces with a cold stainless-steel mortar. The muscle pieces were washed in wash buffer containing 60 mM KCl, 20 mM imidazole hydrochloride (pH 6.9), 2 mM EDTA, 2 mM DTT, 0.4 mM PMSF, 1 μM antipain, 1 μM pepstatin A, and 5 μM trypsin inhibitor, and homogenized in wash buffer (3 × volumes) using a Waring blender. The tissue homogenate was centrifuged for 15 min (30000g), and the supernatant was discarded. The tissue residue after the wash was mixed with 4 × volumes of an extraction buffer (pH 7.1) which had the same composition as the wash buffer except that it also contained 10 mM ATP. The muscle was homogenized for 5 min and centrifuged for 20 min at 30000g, and the tissue extract was collected. The extract was subjected to ammonium sulfate fractionation (0–35% and 35–70%). The major portion of the myosin was found in the 35–70% fraction. In addition, this fraction contained other proteins, including actin, tropomyosin, kinase, phosphatase, and calmodulin. Prior to purification, the myosin was phosphorylated to various levels using the endogenous kinase according to the method previously described by Chacko et al. (1977). The level of phosphorylation was controlled by varying the time of phosphorylation (15 s to 3 min). The phosphorylation reaction was stopped by adding EGTA (final concentration, 2 mM). After phosphorylation, the [KCl] was raised to 0.9 M, the sample was made 10 mM with respect to ATP, and it was loaded onto a 4B Sepharose column. To prepare the unphosphorylated myosin, the 35–70% ammonium sulfate fraction was incubated at 25 °C for 10 min in 2 mM EGTA in the absence of ATP in order to allow the endogenous phosphatase to remove the residual level of phosphorylation. The column was equilibrated with 0.8 M KCl, 15 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.4 mM PMSF, and 1 mM DTT. The myosin peak, as determined from the UV absorption, was pooled and dialyzed against 10 mM $MgCl_2$, 20 mM imidazole hydrochloride (pH 7.0), and 1 mM DTT until the myosin formed a white silky precipitate. The precipitated myosin was collected by centrifugation (48000g, 20 min), dissolved in 0.4 M KCl, 20 mM imidazole hydrochloride (pH 7.0), 2 mM EDTA, 1 mM DTT, 0.4 mM PMSF, 1 μM antipain, and 0.02% NaN_3, and dialyzed against the same buffer overnight. The myosin so obtained was further dialyzed for 5 h against the same buffer without EDTA and centrifuged at 48000g for 10 min, and the supernatant containing pure monomeric myosin was stored at 4 °C. The purified myosin was utilized for a maximum of 1 week.

Gizzard actin was prepared according to Heaslip and Chacko (1985). The tropomyosin was isolated and purified according to Eisenberg and Kielley (1974). All proteins were tested for the kinase and phosphatase activity as described (Chacko, 1981) and found to be negative.

Actin-activated Mg-ATPase was measured as described (Chacko, 1981). Unless otherwise specified, all assays were carried out at 37 °C in 10 mM imidazole hydrochloride (pH 7.0), 2 mM ATP, 0.1 mM $CaCl_2$, 1 mM DTT, either 50 or 100 mM ionic strength (adjusted with KCl), and at varying magnesium concentrations. After addition of the $MgCl_2$, the pH of the buffer was adjusted to 7.0. Other conditions are indicated in the figure legends. Myosin (0.09 mg/mL) was reconstituted with either gizzard actin or tropomyosin–actin (molar ratio of tropomyosin to actin, 1:5). The actin-activated ATPase assays were performed at an actin concentration of 13 μM. The assay was initiated by adding the ATP mixed with [γ-^{32}P]ATP to obtain around 50 000–70 000 cpm in 10 μL. Aliquots of 0.1 mL were removed from a 0.5-mL assay at zero time and two other intervals to check the linearity of the reaction. The inorganic phosphate was analyzed according to Martin and Doty (1949) with a slight modification (Pollard & Korn, 1973). The reaction was stopped by adding the sample into tubes containing a mixture of 6% silicotungstic acid and 5 M sulfuric acid (5:2) and isobutyl alcohol–toluene (1:1). After mixing with 1 mL of 1.6 M HCl containing 1 mM Na_2HPO_4, 0.5 mL of 5% ammonium molybdate was added, and again mixed vigorously. The [^{32}P]P_i liberated from [γ-^{32}P]ATP was determined by counting the upper layer in a scintillation counter.

The turbidity measurements were done by using a Varian DMS 90 UV–visible spectrometer at 340 nm. The monomeric myosin (in 0.4 M KCl) was diluted with the ATPase buffer containing magnesium at varying concentrations. Before the addition of myosin, the instrument was calibrated with the cuvette containing the buffer. The myosin was then added to the buffer, and when the absorption leveled off, 2 mM ATP was added. The change in the absorption was recorded until it leveled off again.

The amount of myosin assembled into filaments was estimated by centrifugation in a Beckman airfuge for 20 min at 110000g. The monomeric myosin was diluted with the ATPase buffer containing 0.1 mM $CaCl_2$, 20 mM imidazole hydrochloride (pH 7.0), 2 mM ATP, and varying concentrations of $MgCl_2$. The ionic strength was adjusted to 50 mM with KCl. The amount of nonsedimentable monomeric myosin in the supernatant was determined according to Bradford (1976). In order to estimate the amount of unphosphorylated myosin sedimented as polymers in a mixture of unphosphorylated and phosphorylated myosins, known amounts of unphosphorylated myosin and myosin phosphorylated with [γ-^{32}P]ATP were used. The percentages of each type of myosin sedimented were determined from the total count in the myosin mixture before centrifugation and the count in the supernatant.

Protein concentrations were estimated according to either Lowry et al. (1951) or Bradford (1976). Protein purity was ascertained by electrophoresis on 4–12% gradient SDS–PAGE (Laemmli, 1970). The extent of light-chain phosphorylation was determined by electrophoresis on urea gels under the alkaline condition (Perrie & Perry, 1970). The actin activation, sedimentation, and turbidity measurements were repeated using seven different preparations of myosin which had similar K^+/EDTA- and Ca^{2+}-activated ATPase in high salt (0.5 M KCl). The data shown are representatives of these experiments.

For electron microscopy, a drop of myosin in the actin activation buffer was placed on a carbon-coated grid for about 30 s. The grid was then washed with the same buffer followed

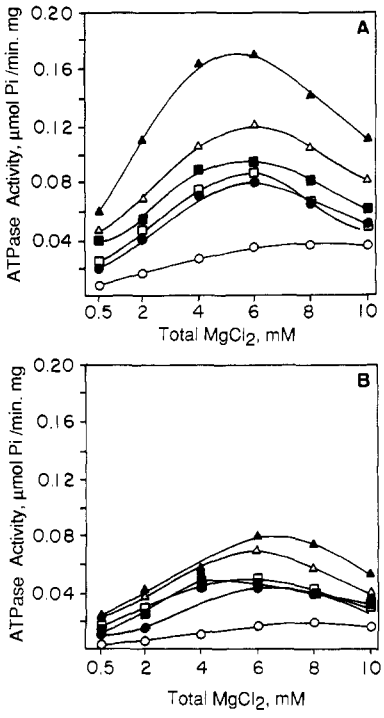

FIGURE 1: Magnesium dependence on the actin-activated ATPase activity of swine urinary bladder myosin phosphorylated at various levels before purification. Assays were carried out in 10 mM imidazole hydrochloride (pH 7.0), 0.1 mM $CaCl_2$, 2 mM DTT, 50 mM ionic strength (adjusted with KCl), 2 mM ATP, and $MgCl_2$ (total) as indicated. Myosin (0.09 mg/mL) was mixed with 13 μM of actin (B) or actin containing tropomyosin (A). Tropomyosin was mixed with actin at a tropomyosin:actin molar ratio of 1:5 and dialyzed overnight. Assays were conducted at 37 °C using unphosphorylated myosin (○) and myosin phosphorylated at various levels [20% (●); 33% (□); 44% (■); 68% (△); 100% (▲)].

by 5 drops of 1% uranyl acetate. The excess stain was removed, and the grids were air-dried at room temperature (Pepe et al., 1986). Grids containing negatively stained filaments were examined under a Phillips 201 electron microscope.

Results

Correlation between the Degree of Phosphorylation and the Actin-Activated ATPase at Varying Mg^{2+} Concentrations. In view of the previous finding that the actin-activated ATPase of smooth muscle myosin is dependent on the free Mg^{2+} concentration (Chacko & Rosenfeld, 1982; Nag & Seidel, 1983; Ikebe & Hartshorne, 1985), experiments were carried out to determine if the Mg^{2+} dependence for the actin activation of the Mg-ATPase is altered by the degree of myosin phosphorylation. The effect of Mg^{2+} on the actin-activated ATPase of myosin at various levels of phosphorylation (<5%, 20%, 33%, 44%, 68%, and 100%) is shown in Figure 1. The actin-activated ATPase activities of myosins at all levels of phosphorylation increased on raising the [Mg^{2+}] from 0.5 to 6 mM. Raising the [Mg^{2+}] higher than 6 mM is associated with a diminution of the ATPase activity. This biphasic effect of Mg^{2+} on actin activation was evident at all levels of myosin phosphorylation. Tropomyosin potentiated the actin-activated ATP hydrolysis by myosin at all levels of phosphorylation.

In order to determine if the effect of Mg^{2+} on the actin-activated ATPase is modulated by the ionic strength, the ATPase activity at 100 mM ionic strength was measured as a function of the free Mg^{2+} concentrations (Figure 2). The ATPase activities of both phosphorylated and unphosphorylated myosins increased on raising the free [Mg^{2+}]. The actin-activated ATPase activity of unphosphorylated

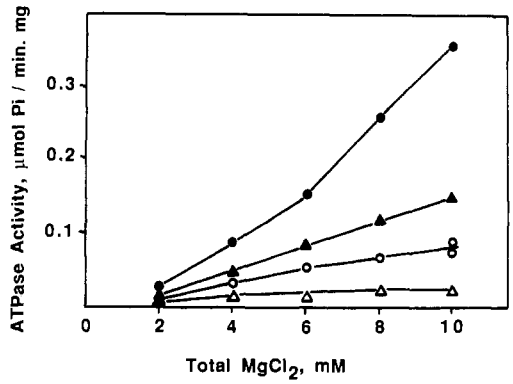

FIGURE 2: Magnesium dependence on the actin-activated ATPase at 100 mM ionic strength. Actin-activated ATPase activities of the phosphorylated (●, ▲) and unphosphorylated (○, △) bladder myosin are measured at 37 °C. Conditions are the same as in Figure 1 except that for the ionic strength which is 100 mM. Triangles represent the activity in the presence of actin, and circles indicate the ATPase activity in the presence of actin mixed with tropomyosin in a molar ratio of 5:1.

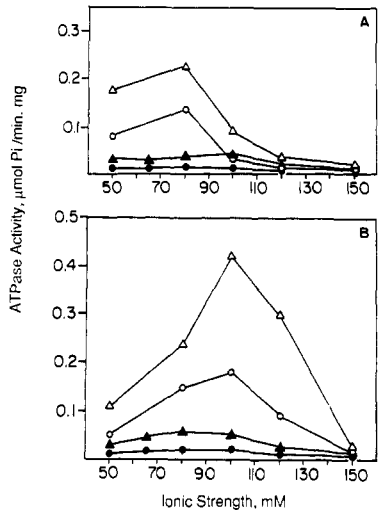

FIGURE 3: Effect of ionic strength on the actin-activated ATPase. The effect of ionic strength on the activation of Mg-ATPase by actin (○, ●) and tropomyosin–actin (△, ▲) at 6 mM (A) and 10 mM (B) $MgCl_2$ is shown for phosphorylated (○, △) and unphosphorylated (●, ▲) myosins. Conditions are same as in Figure 1 except for the ionic strength which varied as indicated.

myosin was lower than that of the phosphorylated myosin at all Mg^{2+} concentrations tested. At a free [Mg^{2+}] of around 8 mM (total 10 mM in the presence of 2 mM ATP), the actin-activated ATPase activity of phosphorylated myosin is around 4–10-fold higher than that of the unphosphorylated myosin both in the presence and in the absence of tropomyosin. The biphasic effect on the ATPase observed on varying the free [Mg^{2+}] in 50 mM ionic strength is not evident in 100 mM ionic strength.

In another experiment, the actin-activated ATPase activities at 6 and 10 mM $MgCl_2$ were measured as a function of the ionic strength (Figure 3). The ATPase activity of the unphosphorylated myosin remained low at all ionic strengths in both 6 and 10 mM $MgCl_2$ (free [Mg^{2+}], 4 mM and 8 mM respectively), but the maximum activity was obtained at an ionic strength of around 80–90 mM. The effect of Mg^{2+} on the actin-activated ATPase of the phosphorylated myosin was also dependent on the ionic strength. At a free Mg^{2+} concentration of 4 mM, the maximum activity was observed at an ionic strength of around 80 mM. Further increase in the ionic strength caused a decrease in the ATPase activity. In 50 mM ionic strength, the ATPase activity at 4 mM Mg^{2+}

Table I: Effect of [$MgCl_2$], Temperature, and pH on the Actin-Activated ATPase Activity of Urinary Bladder Smooth Muscle Myosin[a]

	ATPase activity (nmol of P_i min^{-1} mg^{-1})											
	unphosphorylated myosin						phosphorylated myosin					
	25 °C at pH			37 °C at pH			25 °C at pH			37 °C at pH		
$MgCl_2$ (mM)	6.8	7.0	7.5	6.8	7.0	7.5	6.8	7.0	7.5	6.8	7.0	7.5
6	5	6	6	16	11	10	61	45	21	243	219	124
8	7	6	5	39	30	22	93	95	58	324	271	200
10	8	8	7	80	70	48	90	88	97	350	291	246

[a] Actin-activated ATPase activities were determined in 50 mM KCl, 20 mM imidazole hydrochloride, 2 mM ATP, 0.5 mM EGTA, and 0.5 mM DTT; temperature, [$MgCl_2$], and pH as indicated. 40 μM smooth muscle actin mixed with tropomyosin and 0.25 mg/mL myosin were used.

was higher than that at 8 mM Mg^{2+}. The activity at 8 mM Mg^{2+} increased 4-fold on raising the ionic strength from 50 to 100 mM. In 100 mm ionic strength and 8 mM free Mg^{2+}, the actin-activated ATPase of phosphorylated myosin was 8-fold higher than that of the unphosphorylated myosin. The difference between activities of the phosphorylated and unphosphorylated myosins was around 10-fold in 110 mM ionic strength and 8 mM free Mg^{2+}.

The effect of pH and temperature on the Mg^{2+} dependence of actin-activated ATPase activity is shown in Table I. The effect of pH on the Mg^{2+} dependence for the ATPase activity is minimal at 25 °C for the unphosphorylated myosin. The ATPase activity of the phosphorylated myosin in 6 mM Mg^{2+} decreases on raising the pH. At all pHs used, there is a 3.5–13-fold difference in the ATPase activity between unphosphorylated and phosphorylated myosins at 25 °C. The activity of both myosins increases on raising the [Mg^{2+}] at all pHs irrespective of the temperature of the assay. At 37 °C, the difference in activity between unphosphorylated and phosphorylated myosin decreases from 15–20-fold to 3–4-fold on raising the [Mg^{2+}] from 6 to 10 mM irrespective of the pH of the ATPase assay. At 25 °C, the difference in the activity between unphosphorylated and phosphorylated myosins, in response to a rise in the [Mg^{2+}], is around 8–15-fold at pH 6.8 and 7.0. At pH 7.5 and 25 °C, the activity of phosphorylated myosin is over 3-fold higher than that of the unphosphorylated myosin in 6 mM Mg^{2+}, and this difference is increased to 13-fold on raising the [Mg^{2+}] to 10 mM.

The ATPase activity as a function of the degree of phosphorylation at varying Mg^{2+} concentrations (0.5, 2, 4, 6, 8, and 10 mM total $MgCl_2$) is depicted in Figure 4. At all Mg^{2+} concentrations, the actin-activated ATPase activity rose on increasing the levels of phosphorylation. The relationship between phosphorylation and actin-activated ATPase was linear below 6 mM $MgCl_2$ (free [Mg^{2+}], around 4 mM). On raising the free Mg^{2+} concentration above 4 mM, the correlation curves became flat until the level of phosphorylation reached 50%; the activity increased linearly on raising the degree of phosphorylation from 50 to 100%. As shown in Figures 2 and 3, tropomyosin caused a 3–4-fold increase in the actin-activated ATPase at all levels of phosphorylation, but it did not alter the relationship between the ATPase and the degree of phosphorylation.

The relationship between the actin-activated ATPase and the degree of phosphorylation was also determined by using a myosin polymer made by mixing fully phosphorylated myosin with varying proportions (20, 40, 60, and 80%) of unphosphorylated myosin. The actin-activated ATPase activity of the myosin in 8 mM free Mg^{2+} increased on raising the percent of the phosphorylated myosin in the polymer population in the mixture of unphosphorylated and phosphorylated myosins (Figure 5).

The actin-activated ATPase activities of unphosphorylated and phosphorylated myosins in experiments described above

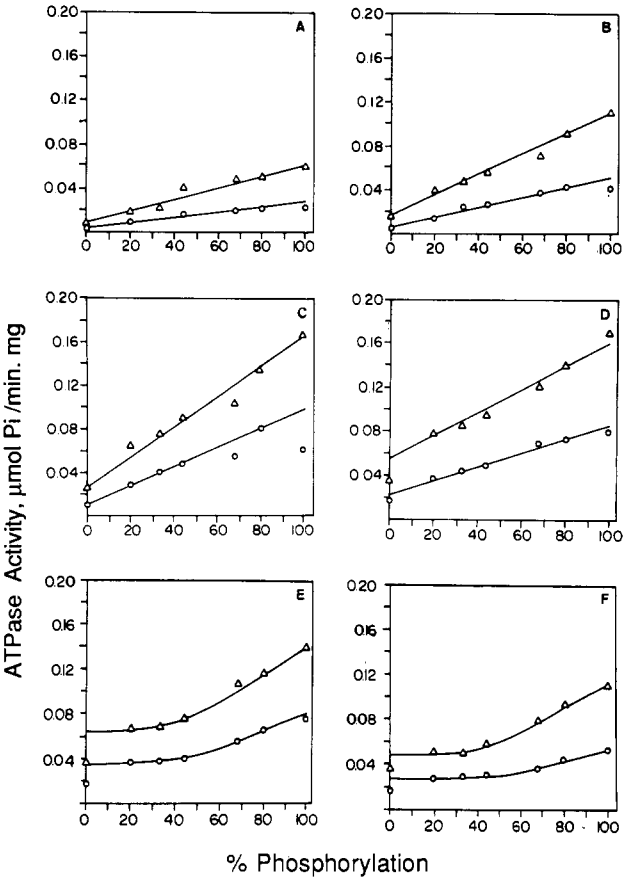

FIGURE 4: Effect of Mg^{2+} on the relationship between actin-activated ATPase activity and the degree of phosphorylation. The ATPase activities of myosins at varying levels of phosphorylation shown in Figure 1 are depicted to show the relationship between the degree of phosphorylation and actin-activated ATP hydrolysis. The ATPase activities in the presence of actin (○) and actin mixed with tropomyosin (△) are plotted as a function of the percentage of phosphorylation. Concentrations of the $MgCl_2$ are (A) 0.5 mM, (B) 2 mM, (C) 4 mM, (D) 6 mM, (E) 8 mM, and (F) 10 mM.

were measured at 13 μM actin. In order to ensure that the actin concentration used is close to the actin concentration needed to maximally activate the Mg-ATPase of both phosphorylated and unphosphorylated myosins, ATP hydrolysis by both myosins was measured as a function of actin concentration. As shown in Figure 6, the ATPase curve reached a plateau at an actin concentration of around 30 μM (myosin to actin molar ratio around 1:150). At 13 μM actin, the molar ratio of myosin to actin is around 1:70, and the ATPase activity is over 60% of the V_{max} for both phosphorylated and unphosphorylated myosins.

Modulation of the Actin-Activated ATPase by Myosin Filament Assembly. Smooth muscle myosin in monomeric form was made by dissolving the myosin in 0.4 M KCl (Trybus & Lowey, 1984). The monomeric myosin was then allowed

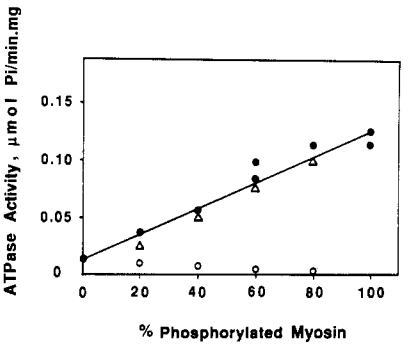

FIGURE 5: Effect of phosphorylation on the actin-activated ATPase of myosin polymers made with mixtures of unphosphorylated and phosphorylation myosins. The various levels of phosphorylation in the polymer population were achieved by mixing fully phosphorylated and unphosphorylated myosins at varying proportions as indicated. The assay was conducted at 37 °C in 10 mM imidazole hydrochloride (pH 7.0), 50 mM ionic strength, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, and 2 mM ATP. The actin–tropomyosin (molar ratio of actin to tropomyosin 5:1) used for the assay contains 13 μM actin. Closed circles (●) represent the experimental results from the polymer population in the mixture. Contributions by the various proportions of the phosphorylated myosin in the ATPase of the polymers in the mixture were calculated from the activity at 100% phosphorylation. These calculated values are represented by the triangles (△). Similarly, the contribution of the unphosphorylated myosin on the ATPase of the polymers in the mixture, calculated from the activity of the 100% unphosphorylated myosin, is shown by the open circle (○).

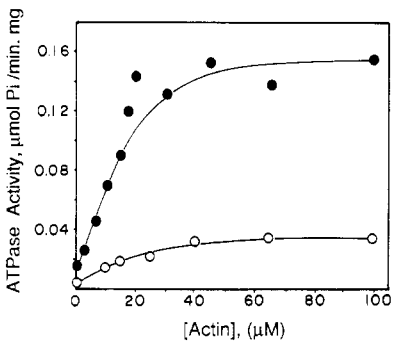

FIGURE 6: Effect of increasing amounts of tropomyosin–actin on actin-activated ATP hydrolysis. Assay conditions: [myosin], 0.09 mg/mL; actin concentrations as indicated; [$MgCl_2$], 6 mM; [EGTA], 0.5 mM; ionic strength, 50 mM (adjusted with KCl); [imidazole hydrochloride] (pH 7.0), 10 mM; temperature, 37 °C. Open circles represent unphosphorylated myosin, and closed circles represent phosphorylated myosin.

to assemble into filaments by diluting the myosin solution in low-salt buffers (pH 7.0) made with varying Mg^{2+} concentrations (final ionic strength, 50 mM). Assembly of myosin into filaments was associated with an increase in the turbidity of the myosin solution. The turbidity exhibited by the unphosphorylated myosin upon raising the Mg^{2+} concentration was slightly lower than that of the phosphorylated myosin. The turbidity diminished upon the addition of ATP at all Mg^{2+} concentrations, but the percentage of the decrease was more remarkable when the free Mg^{2+} concentration was below 4 mM (data not shown).

The percent of myosin filament assembled under conditions of the ATPase assays at varying concentrations of Mg^{2+} was also determined by estimating the amount of myosin polymer sedimented by rapid centrifugation in an airfuge (Pollard, 1982). Raising the [Mg^{2+}] above 4 mM (free around 2 mM) caused a linear increase in the percent of myosin sedimented. At Mg^{2+} concentrations between 2 and 8 mM (total between 4 and 10 mM), phosphorylated myosin sedimented around 20% more than did the unphosphorylated myosin. The actin-activated ATPase activities of unphosphorylated and fully

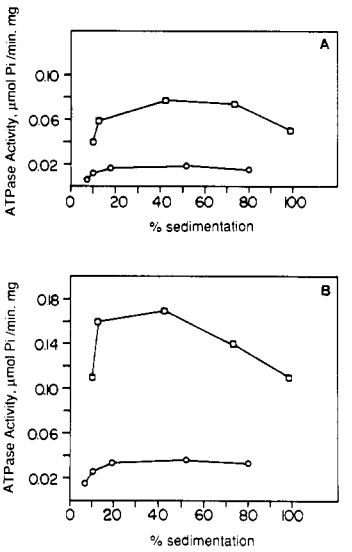

FIGURE 7: Relationship between myosin filament assembly and actin-activated ATP hydrolysis. Myosins were diluted in the ATPase buffer. The Mg^{2+} concentrations (2–10 mM) of the buffers were adjusted to obtain the same level of sedimentability for phosphorylated and unphosphorylated myosin. Myosin samples were reconstituted with actin (A) or tropomyosin–actin (B), and the ATPase activity was determined. The actin-activated ATPase activities of phosphorylated (□) and unphosphorylated (○) myosin were plotted as a function of the myosin sedimented. The assay was conducted at 50 mM ionic strength in the presence of 0.1 mM $CaCl_2$, 1 mM DTT, 2 mM ATP, and at varying Mg^{2+} concentrations.

phosphorylated myosins are presented as functions of percentages of myosin sedimented in Figure 7. Irrespective of the amounts of myosin sedimented by centrifugation, the Mg-ATPase activity of phosphorylated myosin reconstituted with either actin or tropomyosin–actin was 4–6-fold higher than that of the unphosphorylated myosin. A 20% increase in the amount of myosin sedimented was associated with a rise in the actin-activated Mg-ATPase activities of both phosphorylated and unphosphorylated myosins. Further increase in the amount of the sedimented myosin up to 80% is not associated with a rise in the ATPase activity of the unphosphorylated myosin. The activity of the phosphorylated myosin is slightly diminished when the myosin sedimented was very high.

In order to determine if the amount of unphosphorylated myosin in the polymer population formed in the mixtures of unphosphorylated and phosphorylated myosins increased on raising the proportion of phosphorylated myosin, myosin phosphorylated with [γ-^{32}P]ATP was mixed in 0.4 M KCl buffer with varying proportions of unphosphorylated myosin. The mixtures of known amounts of phosphorylated and unphosphorylated myosins was then diluted with the low-salt ATPase buffer (0.05 M) to form filaments. Filaments formed were sedimented by centrifugation. Amounts of myosin in the pellet and the supernatant (both ^{32}P phosphorylated and unphosphorylated myosins) were quantified. The amount of the unphosphorylated myosin in the polymer population that sedimented increased slightly on raising the proportion of the phosphorylated myosin in the mixture (Table II).

Myosins in the phosphorylated and unphosphorylated states kept under the condition of the ATPase as in Figure 7, a condition in which both myosins sedimented to the same level, were negatively stained to determine if there was any difference in the filament assembled. The electron microscopic appearance of myosin filaments under these conditions is depicted in Figure 8A,B. The phosphorylated myosin (Figure 8A) formed bipolar filaments with a tapered end. The size of the

Table II: Effect of Phosphorylated Myosin on the Formation of Polymer by Unphosphorylated Myosin[a]

% myosin in the assay		% protein in the sedimented polymer		
P	UP	total	P[b]	UP[b]
100	0	78	78	0
80	20	69	74	48
60	40	55	64	43
40	60	48	62	36
20	80	34	53	31
0	100	24	0	24

[a] The unphosphorylated (UP) and phosphorylated (P) (with [γ-^{32}P]ATP) myosins in 0.4 M KCl were mixed in various proportions (total 0.4 mg/mL) and diluted with the ATPase buffer containing 6 mM $MgCl_2$, 2 mM ATP, and 0.1 mM $CaCl_2$. After incubation for 20 min, the myosin polymers were sedimented by centrifugation. [b] The percentage of the proportions of each type of myosin in the original assay mixture sedimented upon centrifugation.

filaments was around 0.6–0.8 μm. On the other hand, the unphosphorylated myosin molecules (Figure 8B) assembled into very small filament structures or structureless aggregates.

Discussion

The force development by chemically skinned and intact smooth muscle strips (Saida & Nonomura, 1978; Altura & Altura, 1981; Ruegg & Paul, 1982) and the activation of smooth muscle actomyosin ATPase (Chacko & Rosenfeld, 1982; Ikebe et al., 1984; Gagelmann et al., 1986) are modulated by the free Mg^{2+}. Utilization of smooth muscle myosin at various levels of phosphorylation in the present study enables us to determine if the degree of myosin phosphorylation influences the response of actomyosin ATPase to free Mg^{2+}. The biphasic effect of free Mg^{2+} on the actin-activated ATPase activity, exhibited by bladder myosin phosphorylated at varying degrees in the present study, has been observed previously in experiments which utilized fully phosphorylated gizzard myosin and heavy meromyosin (Chacko & Rosenfeld, 1982; Nag & Seidel, 1983; Ikebe et al., 1984; Kaminiski & Chacko, 1984). The Mg^{2+}-induced effect on the ATPase is unlikely to be caused by a direct effect of Mg^{2+} on the active sites, since the decrease in the actin activation of myosin in high Mg^{2+} is abolished when the ionic strength is raised from 50 to 100 mM (Figures 2 and 3). The increase in the ATPase activity in the presence of high Mg^{2+} and 100 mM ionic strength may be associated with modulations in the myosin conformation and filament assembly.

Tropomyosin potentiates the activity at all levels of Mg^{2+} concentrations, although it is less remarkable at very low concentrations of Mg^{2+}, presumably due to the decreased binding of tropomyosin to actin in low Mg^{2+} (Miyata & Chacko, 1986). It has also been suggested that the effect of Mg^{2+} on the actin-activated ATPase is due to the effect of this divalent cation on the activity of the myosin light-chain kinase (Moreland & Ford, 1981). The effect of Mg^{2+} on the actin-activated ATPase observed in this study is independent of any effect the free Mg^{2+} may have on the myosin phosphorylation (Moreland & Ford, 1981) since the myosin is stably phosphorylated prior to the ATPase assay and proteins used for the ATPase assay are free of kinase, phosphatase, or calmodulin.

The level of activation of the Mg-ATPase of the myosin depends on phosphorylation at all pHs. Raising the Mg^{2+} concentration decreases the difference in the activity between phosphorylated and unphosphorylated myosins depending on the temperature and the pH. At pH 7.5, the myosin appeared to be less turbid (data not presented), and under this condition,

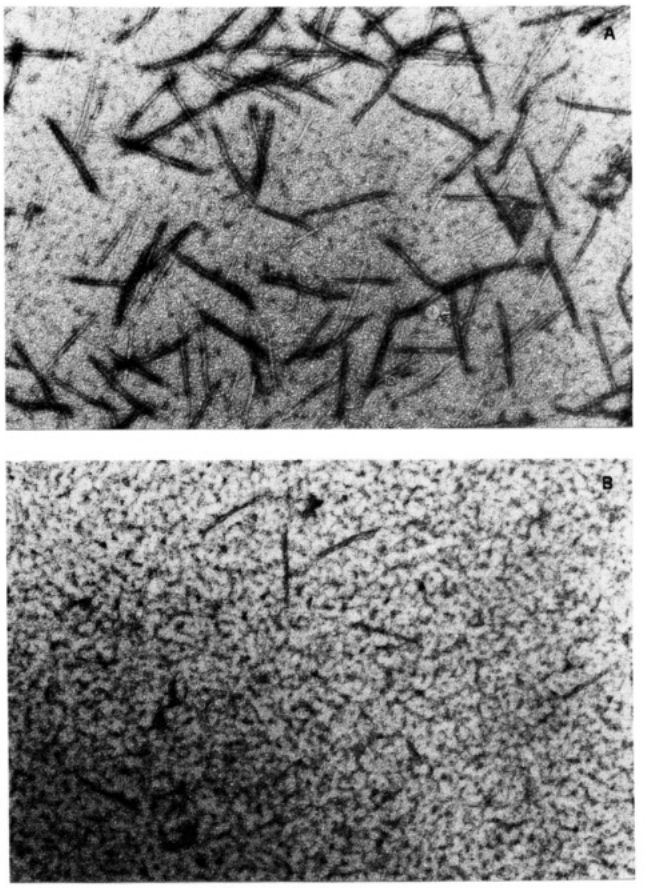

FIGURE 8: Electron micrographs of myosin filaments formed by phosphorylated (A) and unphosphorylated (B) swine urinary bladder myosins. Myosin monomers in 0.4 M KCl were slowly diluted with 10 mM imidazole hydrochloride (pH 7.0) containing 2 mM ATP, 1 mM DTT, and 0.1 mM $CaCl_2$. The concentration of $MgCl_2$ in the buffers was adjusted (6 mM for phosphorylated and 7.5 mM for unphosphorylated) to obtain the same amount of sedimentable myosins. The ionic strength was 50 mM for both myosins. Magnification (A and B) 26400×.

the increase in activity on raising the [Mg^{2+}] at both 25° and 37 °C (Table I) is presumably due to the assembly of filament. It is also possible that the increased activity observed on raising the [Mg^{2+}] and the ionic strength is caused by an increased affinity of myosin to actin. However, the binding of heavy meromyosin to smooth muscle actin is slightly decreased on raising the ionic strength at 3 mM free Mg^{2+} (Chacko & Eisenberg, 1990). However, the ionic strength effect on the binding of smooth muscle myosin subfragment 1 to skeletal muscle actin is not remarkable (Greene et al., 1983). Further studies are needed to determine if the high ATPase activity observed in high [Mg^{2+}] and 100 mM ionic strength (Figure 2) is associated with an increase in the affinity of myosin heads to actin.

The relationship between phosphorylation and actin activation of the bladder myosin is linear at free Mg^{2+} below 4 mM. This is in agreement with previous reports on mammalian arterial (Chacko & Rosenfeld, 1982; Wagner & Vu, 1986) and bovine stomach muscle myosin (Chacko, 1981). Raising the free [Mg^{2+}] above 4 mM causes a change in the relationship between phosphorylation and actin activation; the ATPase begins to rise only when the myosin is 50% phosphorylated. These data are similar to previous reports on gizzard myosin which show that both heads of the myosin have to be phosphorylated before it is activated by actin (Ikebe et al., 1982; Sellers et al., 1983). Results presented in Figure 4 demonstrate that the same preparation of myosin shows

either a linear or a nonlinear correlation depending on the free Mg^{2+} concentration. Hence, the relationship between myosin light-chain phosphorylation and actin-activated ATPase activity is dependent on the free Mg^{2+} concentration. In experiments utilizing myosin polymers formed by myosin molecules, which have both heads either in phosphorylated or in unphosphorylated states (Figure 5), the relationship between myosin phosphorylation and actin-activated ATPase activity is linear even though the ATPase assay is carried out at 10 mM $MgCl_2$. This implies that myosins with low levels of phosphorylation used in Figure 4, which at high [Mg^{2+}] shows a nonlinear correlation between myosin phosphorylation and ATPase, are not a mixture of myosin molecules phosphorylated at both heads and molecules with heads that are not phosphorylated. It is likely that myosins at low levels of phosphorylation used for experiments in Figure 4 are randomly phosphorylated, and single head phosphorylated molecules are more susceptible to modulation by Mg^{2+}.

It has been suggested that the tropomyosin modulates the myosin ATPase and the relationship between myosin phosphorylation and actin-activated ATPase (Merkel et al., 1984, 1989). It is unlikely that the tropomyosin played a role in the relationship between myosin light-chain phosphorylation and actomyosin ATPase in the present study since both the nonlinear and linear relationships are observed either with actin or with tropomyosin–actin depending on the [Mg^{2+}].

The actin concentration used for most of the experiments in this study is 13 μM. This gives a myosin (0.19 μM) to actin molar ratio of 1:70, and at this concentration, the actin-activated ATPase activity is over 60% of the maximal activity. An effect on the K_{app} for actin caused by phosphorylation is not evident in this study which utilized intact myosin molecules. Using heavy meromyosin, Sellers et al. (1987) show that compared to the effect of phosphorylation on the V_{max}, the difference between phosphorylated and unphosphorylated myosin in their K_{app} for actin is less significant.

Results from experiments with polymers in a mixture of phosphorylated and unphosphorylated myosins indicate that the presence of increasing ratios of phosphorylated myosin causes a slight increase in the unphosphorylated myosin in the sediment (see Table II). The presence of unphosphorylated myosin in the sediment obtained from mixtures of unphosphorylated and phosphorylated myosins is not definite proof for the formation of copolymers. If copolymers are formed and the ATPase activity of unphosphorylated myosin in the copolymer is modulated by the phosphorylated myosin, the ATPase activity of the copolymer is expected to be higher than the combined activities of both types of myosin. The ATPase activity of the myosin polymers in mixtures of phosphorylated and unphosphorylated myosins under this condition is additive (Figure 5), indicating that if copolymers are formed, the phosphorylated myosin in the copolymer has no effect on the ATP hydrolysis by the unphosphorylated myosin. These data are different from those observed by Wagner and Vu (1988), who carried out similar experiments with phosphorylated and unphosphorylated thymus myosin. They found that the interaction between the phosphorylated myosin and the unphosphorylated myosin increased the ATPase activity of the copolymer. The ionic strength utilized for the ATPase assay for Wagner and Vu (1988) was higher than that used for the present study (around 150 mM compared to the 50 mM). The actin-activated ATPase activity of bladder myosin either phosphorylated or unphosphorylated is very low under the ionic conditions utilized by Wagner and Vu (1988). It is not clear if the difference between myosins isolated from thymus and bladder is due to an inherent difference in these myosins.

Data presented in Figures 2 and 3 and Table I clearly demonstrate that the actin-activated Mg-ATPase of phosphorylated myosin is severalfold higher than that of the unphosphorylated myosin; the difference in activity between unphosphorylated and phosphorylated myosins is amplified when actin containing bound tropomyosin is used to activate the myosin ATPase. The actin-activated ATPase activities of both phosphorylated and unphosphorylated myosins are modulated by temperature, pH, ionic strength, and Mg^{2+} concentration; however, the steady-state ATPase activity of phosphorylated myosin is severalfold higher than that of the unphosphorylated myosin.

Phosphorylation of the myosin increases the population of the extended myosin monomer that assembles into stable filaments in the presence of ATP (Trybus et al., 1982; Kendrick-Jones et al., 1983). Hence, it has been suggested that the sedimentability of myosin, presumably determined by the state of filament assembly, modulates the actin-activated ATPase of the smooth muscle myosin (Wagner & Vu, 1987). The data presented in Figure 7 show that the increase in the actin-activated ATPase activity on increasing the amount of sedimentable myosin is not evident once 20% of the myosin is sedimented. This suggests that the increase in sedimentable myosin polymer, either phosphorylated or unphosphorylated, is not proportionally associated with a rise in the actin-activated ATPase activity. Moreover, the activity of the unphosphorylated myosin never reaches the level of activity exhibited by phosphorylated myosin in spite of the similarity in their sedimentability at high concentrations of Mg^{2+}.

The increase in turbidity of both phosphorylated and unphosphorylated myosin on raising the Mg^{2+} concentration indicates that both types of myosin assemble into filaments. The decrease in turbidity upon the addition of ATP is more pronounced in the case of the unphosphorylated myosin (data not shown). This observation is compatible with previous reports (Kendrick-Jones et al., 1982; Trybus et al., 1982). The electron microscopic appearance of filaments formed by phosphorylated myosin under this condition is similar to that observed in previous studies which utilized smooth muscle myosins from several sources (Wachsberger & Pepe, 1974; Hinssen et al., 1978; Chowrashi et al., 1987). Despite the fact that the unphosphorylated myosin shows high turbidity and it sediments at the same level as the phosphorylated myosin (Figure 7), recognizable filaments are rarely formed (Figure 8A,B). The very small filament and the structureless aggregates formed by the unphosphorylated myosin are similar to those observed by Suzuki et al. (1978). Clearly, there is a remarkable difference between the phosphorylated and unphosphorylated myosin in their abilities to assemble into filaments.

Effects of Mg^{2+} and phosphorylation on the monomer/polymer equilibrium of myosin have been studied using smooth muscle (Watanabe, 1985; Trybus & Lowey, 1985; Fillers & Chacko, 1987) and nonmuscle myosin (Citi & Kendrick-Jones, 1986). The increase in the population of myosin polymer has been associated with increased actin-activated ATPase activity. The nonmuscle myosin polymers, formed under conditions that give high ATPase activity, consist of both bipolar and side-polar filaments (Citi & Kendrick-Jones, 1986). Myosin filaments formed by phosphorylated myosin in the present study are bipolar. The small filament structure formed by unphosphorylated myosin may contain side-polar filaments; however, this requires further study.

The requirement for phosphorylation for actin activation of the Mg-ATPase of myosin, independent of the effect of phosphorylation on the filament assembly, has been studied using heavy meromyosin, a proteolytic fragment of myosin (Sellers et al., 1982). However, it may be argued that the proteolysis may affect the regulation of the enzymatic activity of the myosin. Data from experiments carried out in this study demonstrate that the actin-activated ATPase of phosphorylated myosin is severalfold higher than that of the myosin in the unphosphorylated state even when their turbidity and sedimentability are the same. The difference in the activity between phosphorylated and unphosphorylated myosin observed under some conditions used in the present study is not so different from that observed by Wagner and Vu (1987). Electron microscopy of the myosin polymer clearly demonstrates that the filament assembly for unphosphorylated and phosphorylated polymers is different. While this study emphasizes the requirement for phosphorylation for actin activation, it does not rule out the need for filament assembly for the maximal activation of the Mg-ATPase of myosin. However, our data indicate that the filament assembly associated with maximal activation of myosin is present only with phosphorylated myosin.

There are other contractile systems in which myosin filament assembly is important for the regulation of actomyosin ATPase. For example, myosin filament assembly, mediated by dephosphorylation of the myosin heavy chain, regulates the actin-activated ATPase activity of myosin II from *Acanthamoeba castelleni* (Collins & Korn, 1981; Kuznicki et al., 1984). The myosin concentration and the ionic conditions in the intact smooth muscle cell are different from those used for the in vitro studies. Hence, it is difficult to apply the information obtained from the in vitro experiments directly to the intact cell. The observation that the well-organized myosin filaments, formed by the phosphorylated myosin, are compatible with maximal enzymatic activity of the myosin is significant. The finding that the relaxed muscle with very low level of phosphorylation contains thick filaments (Somlyo et al., 1981) suggests that once the myosin is organized into filaments, dephosphorylation may not dismantle the filaments to a degree that can be appreciated during electron microscopic observation. Quantifying the number and the size of filaments at various levels of phosphorylation will be helpful to confirm if the assembly of myosin into filaments in the intact cell is modulated by phosphorylation. It is also possible that the packing of the entire myosin molecule in the filament or the position of myosin heads and the S-2 region on the filament structure may be modulated by phosphorylation. Alteration of the arrangement of myosin heads on the filament back bone is likely to have an effect on the cross-bridge attachment. Interestingly, the high economy of ATP utilization in the maintenance of force in smooth muscle is achieved with slowly cycling or noncycling cross-bridges (Dillon et al., 1981; Butler & Siegman, 1982) which are not phosphorylated (Hai & Murphy, 1988).

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