Effect of Phosphorylation on the Binding of Smooth Muscle Heavy Meromyosin·ADP to Actin*

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Relaxation of both smooth and skeletal muscles appears to be caused primarily by inhibition of the step associated with Pi release in the actomyosin ATPase cycle, rather than by a block in the binding of the myosin·ATP and myosin·ADP·Pi complexes to actin. In skeletal muscle, troponin-tropomyosin not only causes marked inhibition of Pi release, but it also markedly inhibits the binding of myosin subfragment-1·ADP to actin, raising the possibility that the two phenomena are coupled in some way. In the present study we determined whether phosphorylation of smooth muscle heavy meromyosin (HMM) also affects both the binding of HMM·ADP to actin and the Pi release step. This was done by having phosphorylated and unphosphorylated HMM·ADP compete for sites on F·actin. At μ = 30 mM, phosphorylation increased the affinity of the HMM molecule for actin about 12-fold and at μ = 170 mM, there was less than a 3-fold increase in affinity of HMM. If phosphorylation affects the binding of each head of HMM to the same extent, then phosphorylation caused about a 4- and 2-fold increase in the affinity of each head of HMM for actin at μ = 30 and 170 mM, respectively. In contrast, at both ionic strengths, phosphorylation caused more than 100-fold increase in the affinity of each head of HMM for actin at μ = 30 and 170 mM, respectively. Therefore, the marked activation of P; release in the acto·HMM ATPase cycle upon phosphorylation of HMM is not accompanied by a comparable increase in the affinity of HMM·ADP for actin. We have also found that phosphorylation increases by only 4-fold the rate of P; release from HMM alone. These results suggest that in smooth muscle, phosphorylation accelerates the step associated with the release of P; both in the forward and the reverse direction without correspondingly affecting the binding of myosin·ADP to actin.

In both skeletal and smooth muscle, muscle contraction is regulated by the concentration of free calcium (1-3). In skeletal muscle, Ca²⁺ binds to the regulatory complex, troponin-tropomyosin, which then causes a shift in the position of the tropomyosin molecule on the thin filament, while in smooth muscle, Ca²⁺ binds to calmodulin which then activates the myosin light chain kinase. This results in the phosphorylation of the light chain of myosin, thereby causing contraction. Even though these muscles differ in the manner in which Ca²⁺ activates them, they appear to have a common mechanism of regulation, i.e. in both systems the primary step which is regulated appears to be the release of P; from actin·myosin·ADP·Pi (4-8). At the same time, the binding of myosin to actin in the presence of ATP does not appear to be significantly regulated in either system (4-8).

Not only does troponin-tropomyosin inhibit the release of P; in skeletal muscle, it also weakens the binding of myosin·ADP to actin by at least a factor of 20 (9). Therefore, troponin-tropomyosin appears to have a dual effect on the actomyosin ATPase cycle. Greene and Eisenberg (9) previously suggested that the weakening of the binding of myosin·ADP to actin may in fact be coupled to the inhibition of P; release. As shown in Scheme 1, which is a condensed actomyosin ATPase cycle, (A = actin and M = myosin), an increase in the free energy of the actin·myosin·ADP complex, i.e. a decrease in its stability, could be the origin of both of these effects.

An increase in the free energy of actin·myosin·ADP relative to acto·myosin·ADP·P; would occur if the rate constant for P; release decreased without a corresponding decrease in the reverse rate constant for P; binding. At the same time, an increase in the free energy of actin·myosin·ADP relative to myosin·ADP would decrease the strength of binding of myosin·ADP to actin. On this basis, there would be a coupling between inhibition of P; release and weakening of the binding of myosin·ADP to actin.

In this study, we examined whether such coupling occurs in smooth muscle. Sellers et al. (7) have previously shown that phosphorylation of smooth muscle HMM causes marked activation of the acto·HMM ATPase activity, while having only a small effect (about 4-fold) on the binding constant of HMM·ATP and HMM·ADP·P; to actin. Based on single turnover experiments, phosphorylation appears to activate P; release nearly 1000-fold (8). In the present study, we examined whether phosphorylation of smooth muscle HMM also strengthens the binding of HMM·ADP to actin. In contrast to skeletal muscle, we find that the binding of smooth muscle HMM to actin in the presence of ADP is not significantly affected as part of the regulatory process. This shows that, in contrast to skeletal muscle, inhibition of the actin-activated

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†The abbreviations used are: acto·HMM and acto·S-1, a complex between actin and either HMM or S-1; EGTA, [ethylene-bis(oxyethylenenitrilo)]tetraacetic acid; HMM, heavy meromyosin; S-1, myosin subfragment-one.
ATPase of myosin in relaxed smooth muscle is not linked to weak binding of myosin-ADP to actin.

**MATERIALS AND METHODS**

**Preparation of Proteins**—Turkey gizzard heavy meromyosin was prepared as described previously (7), except that an Ultrogel AcA-34 column was used to purify HMM. Fully phosphorylated HMM (1.8–1.95 mol/mol) was prepared according to Sellers et al. (10). Rabbit skeletal HMM was prepared by a modification (11) of the method of Spudich and Watt (12).

**Actin-activated Assays**—The actin-activated Mg-ATPase activity was determined by using [γ-32P]ATP as described by Pollard and Korn (13) under the same conditions as the turbidity experiments.

**Reassociation of HMM with Actin**—The reassociation of HMM with actin was measured by turbidity at 340 nm in a Beckman DU-8 spectrophotometer in the kinetic mode at 25 °C as described by Sellers (8).

**Effect of Phosphorylation on the Binding of HMM-ADP to Actin**—Having measured the extent to which phosphorylation of smooth muscle HMM decreases the binding of HMM-ADP to actin to the same extent as it accelerates the Pi release at 25 °C, Sellers et al. (8) used turbidity to determine the rate of reassociation of HMM with actin; presumably the rate of HMM rebinding to actin at stoichiometric ATP is a measure of the rate-limiting step. It should be pointed out that even though ATP was added in a 2.5-fold mol ratio to myosin heads in this experiment, this is essentially a single turnover experiment because the small population of unregulated HMM molecules acts as scavengers, hydrolyzing the free ATP (8). In Fig. 1, the change in turbidity as unphosphorylated HMM reassociates with actin was measured at two different actin concentrations, 10 and 75 μM. We obtained the same low rate of 0.006 s⁻¹ at both actin concentrations. Therefore, the rate of Pᵢ release, which is 0.006 s⁻¹ under these conditions, is not activated by actin, just as was observed at low ionic strength.

We next measured the actin-activated ATPase activity of phosphorylated HMM at physiological ionic strength using steady-state measurements. Since we previously showed that increasing ionic strength does not weaken the binding of smooth muscle S-1 as much as it does skeletal muscle S-1 (16), we were also able to obtain a double reciprocal plot of ATPase activity versus actin concentration with phosphorylated HMM at μ = 0.17 M. As shown in Fig. 2, under this condition, the value of Kᵦ, the apparent binding constant of phosphorylated HMM for actin, is about 3 × 10⁻¹⁰ M⁻¹, while in the absence of actin, the maximum actin-activated ATPase activity, is 1.2 s⁻¹. Comparison of the rate of Vₘₐₓ obtained with phosphorylated HMM to the rate of Pᵢ release obtained with unphosphorylated HMM shows that phosphorylation causes a 200-fold increase in the Pᵢ release step in the acto-HMM ATPase cycle at physiological ionic strength.

**RESULTS**

**The Effect of Phosphorylation on the Acto-HMM ATPase Activity**—We wanted to determine whether phosphorylation of smooth muscle HMM strengthens the binding of HMM-ADP to actin to the same extent as it accelerates the Pᵢ release step in the actomyosin ATPase cycle. We made this comparison both at low ionic strength and at physiologic ionic strength.

At low ionic strength (μ = 0.012 M, 25 °C), Sellers et al. (7) originally found that phosphorylation caused a 25-fold activation of the maximal actin-activated smooth muscle HMM ATPase activity (Vₘₐₓ = 2 s⁻¹ for phosphorylated HMM). More recently, the rate of unphosphorylated HMM was determined from single turnover experiments (8) to be 0.002 s⁻¹. This rate, which is much less than that obtained by steady-state measurements (7), is not activated by the presence of actin. The much higher rate of the unphosphorylated HMM obtained in steady-state measurements is probably due to a small population of HMM molecules that are not properly regulated; even when dephosphorylated, these molecules hydrolyze ATP much faster than the majority of the population. Therefore, at low ionic strength, phosphorylation of HMM increases the Pᵢ release step in the acto-HMM ATPase cycle about 1000-fold.

This large effect of phosphorylation on Pᵢ release also occurs at physiological ionic strength (μ = 0.17 M). The ATPase activity of unphosphorylated HMM was measured from single turnover experiments to avoid the problems encountered from steady-state measurements due to the small population of unregulated HMM molecules. As was previously done by Sellers (8), we used turbidity to determine the rate of reassociation of HMM with actin; presumably the rate of HMM rebinding to actin at stoichiometric ATP is a measure of the rate-limiting step. It should be pointed out that even though ATP was added in a 2.5-fold mol ratio to myosin heads in this experiment, this is essentially a single turnover experiment because the small population of unregulated HMM molecules acts as scavengers, hydrolyzing the free ATP (8). In Fig. 1, the change in turbidity as unphosphorylated HMM reassociates with actin was measured at two different actin concentrations, 10 and 75 μM. We obtained the same low rate of 0.006 s⁻¹ at both actin concentrations. Therefore, the rate of Pᵢ release, which is 0.006 s⁻¹ under these conditions, is not activated by actin, just as was observed at low ionic strength.

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**Effect of Phosphorylation on the Binding of HMM-ADP to Actin**—Having measured the extent to which phosphorylation of smooth muscle HMM increases the rate of Pᵢ release at both low and high ionic strength, we next wanted to determine the effect of phosphorylation on the binding of smooth muscle HMM-ADP to actin under these conditions. This made it necessary for us to determine the concentration of ADP...
concentration was 0.6 mM dithiothreitol, both with phosphorylated and unphosphorylated HMM.

The competition method was used because HMM-ADP binds too tightly to measure the binding directly. Because of this tight binding, it was also necessary to check that the system had reached equilibrium before we made our measurement. This was accomplished by verifying that the order of addition of phosphorylated HMM and unphosphorylated HMM to actin did not significantly affect the results. However, this control experiment also showed that it was necessary to incubate the solutions for many hours to achieve equilibrium, probably because of the slow rate of dissociation of HMM from actin. We routinely incubated the solutions at 4 °C for 16 h, followed by 1.5 h at 25 °C, which provided sufficient time to reach equilibrium (see open and closed symbols in Fig. 1). Extending the time at 25 °C from 1.5 to 8 h did not significantly alter the results.

The data from the competition experiments were plotted according to the following equation:

$$\frac{\text{[HMM-P]}_{\text{bound}}}{\text{[HMM]}_{\text{bound}}} = \frac{K^*}{K} \frac{\text{[HMM-P]}_{\text{free}}}{\text{[HMM]}_{\text{free}}}$$

where HMM-P and HMM are phosphorylated and unphosphorylated HMM, respectively, and $K^*$ and $K$ are the association constants for the binding of actin to HMM-P-ADP and HMM-ADP, respectively. In this plot the slope is equal to $K^*/K$. Competition experiments were carried out at low ($\mu = 0.03$ M) and physiologic ($\mu = 0.17$ M) ionic strength (Fig. 4). It is seen that $K^*/K$ is affected by ionic strength with values of 11.5 ± 0.6 and 2.6 ± 0.1 obtained at low and high ionic strength, respectively. This shows that the phosphorylated HMM-ADP complex binds about 12-fold stronger to actin than does the unphosphorylated HMM-ADP complex at low ionic strength but only about 3-fold stronger at high ionic strength.

**Effect of Phosphorylation on the Rate of P$_i$ Release from HMM Alone**—This study has shown that phosphorylation of smooth muscle HMM accelerates the P$_i$ release step in the actomyosin ATPase cycle much more than it strengthens the binding of HMM-ADP to actin. Previously, Sellers et al. (7) found that phosphorylation causes only a 4-fold increase in the binding constant of HMM-ADP-P$_i$ (and HMM-ATP) to actin at low ionic strength. Since it is necessary to maintain detailed balance in Scheme 1, there must be some step that is altered by phosphorylation other than the release of P$_i$ from actin-myosin-ADP-P$_i$. One likely possibility is that both the release and binding of P$_i$ to the actin-myosin-ADP-P$_i$ complex are equally affected by phosphorylation. However, another possibility is that phosphorylation increases the rate of P$_i$ release to the same extent whether or not HMM is bound to actin. This latter possibility was tested by measuring the rate of P$_i$ release from the smooth muscle HMM-ADP-P$_i$ complex with phosphorylated and unphosphorylated HMM at $\mu = 0.032$ M with a rapid gel-filtration technique (8). As shown in Fig. 5, phosphorylation caused only a 4-fold increase in the rate of P$_i$ release from HMM-ADP-P$_i$. Fitting the data to a single exponential, the rates of P$_i$ release from the unphosphorylated and phosphorylated HMM-ADP-P$_i$ complex were 0.002 s$^{-1}$ and 0.007 s$^{-1}$, respectively.

Therefore, it is necessary to saturate the acto-HMM complex with nucleotide, both with phosphorylated and unphosphorylated HMM. This was done by directly measuring the binding of [H]ADP to acto-HMM at both low and high ionic strength ($\mu = 0.03$ M and $\mu = 0.17$ M), the conditions we planned to use in our studies on the binding of HMM-ADP to actin. Fig. 3 shows the Scatchard plot obtained for the binding of ADP to acto-HMM. This binding was not significantly affected by either phosphorylation (circles versus triangles) or ionic strength (open versus closed symbols). A linear fit to the data gives a binding constant of $2 \times 10^5$ M$^{-1}$ with 0.8 mol of ADP binding per head of HMM. There is some nonlinearity in the plot at both low and high values of $\theta$, but since these points tend to be relatively inaccurate (17), it is not clear whether there is cooperativity or heterogeneity in the binding of ADP to acto-HMM. In any case, these results clearly show that at 3 mM ADP, the concentration used in the actin-binding experiments, the acto-HMM complex was completely saturated with nucleotide whether phosphorylated or unphosphorylated HMM was used.

We then determined the effect of phosphorylation on the binding of HMM-ADP to actin by having phosphorylated and unphosphorylated HMM compete for sites on F-actin. The competition method was used because HMM-ADP binds too tightly to measure the binding directly. Because of this tight binding, it was also necessary to check that the system had reached equilibrium before we made our measurement. This was accomplished by verifying that the order of addition of phosphorylated HMM and unphosphorylated HMM to actin did not significantly affect the results. However, this control experiment also showed that it was necessary to incubate the solutions for many hours to achieve equilibrium, probably because of the slow rate of dissociation of HMM from actin. We routinely incubated the solutions at 4 °C for 16 h, followed by 1.5 h at 25 °C, which provided sufficient time to reach equilibrium (see open and closed symbols in Fig. 1). Extending the time at 25 °C from 1.5 to 8 h did not significantly alter the results.

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where HMM-P and HMM are phosphorylated and unphosphorylated HMM, respectively, and $K^*$ and $K$ are the association constants for the binding of actin to HMM-P-ADP and HMM-ADP, respectively. In this plot the slope is equal to $K^*/K$. Competition experiments were carried out at low ($\mu = 0.03$ M) and physiologic ($\mu = 0.17$ M) ionic strength (Fig. 4). It is seen that $K^*/K$ is affected by ionic strength with values of 11.5 ± 0.6 and 2.6 ± 0.1 obtained at low and high ionic strength, respectively. This shows that the phosphorylated HMM-ADP complex binds about 12-fold stronger to actin than does the unphosphorylated HMM-ADP complex at low ionic strength but only about 3-fold stronger at high ionic strength.
phosphorylation of smooth muscle HMM causes only a slight increase in the rate of Pi release from HMM-ADP-P_i complex in the absence of actin, unlike the profound acceleration obtained in the presence of actin.

**DISCUSSION**

Greene and Eisenberg (9) previously suggested that the inhibition of the P_i release step in the actomyosin ATPase cycle of relaxed muscle may be coupled to a weakening of the binding of myosin-ADP to actin. Their proposal was based on results obtained with skeletal muscle acto-S-1 where troponin-tropomyosin was found to inhibit both the release of P_i from actin-myosin-ADP-P_i, and the binding of S-1-ADP to actin. These effects of troponin-tropomyosin have also been observed using skeletal muscle HMM (18). However, the present study has shown that at both low and high ionic strength, the increase in the rate of release of P_i from actin-myosin-ADP-P_i upon phosphorylation of smooth muscle HMM is not coupled to an increase in the binding constant of HMM-ADP to actin. At low ionic strength, phosphorylation increases P_i release about 1000-fold, whereas there is only about a 12-fold increase in the binding constant of HMM-ADP to actin. Furthermore, results obtained with skeletal muscle HMM indicate that various agents (ionic strength, temperature, and nucleotide) which affect the binding of HMM to actin appear to affect each of the two HMM heads to the same extent (19). If this also applies to the strengthening effect of phosphorylation on the binding of smooth muscle HMM to F-actin, then the 12-fold increase in the binding constant of HMM-ADP to actin due to phosphorylation may actually be less than a 4-fold increase in the binding constant of each head of HMM for actin.

**FIG. 5.** Rate of phosphate release from phosphorylated and dephosphorylated HMM in the absence of actin. The conditions were: 20 mM KCl, 10 mM imidazole (pH 7.0), 1.8 mM MgCl_2, 0.1 mM EGTA, 1 mM dithiothreitol, 15 μM HMM, 25°C, using either phosphorylated HMM (●) or dephosphorylated HMM (□). The reaction was initiated by the addition of 30 μM [γ-32P]ATP, followed by the addition of 1 mM nonradioactive ATP at 5 s. There is an undetermined error in the time dimension due to the fact that centrifugation is used to separate bound and unbound products. The data provided a reasonable fit to a single exponential equation with rate constants of 0.007 and 0.0017 s⁻¹ obtained with phosphorylated and dephosphorylated HMM, respectively. The data with unphosphorylated HMM is taken from Sellers (8). The ordinate intercept is less than one, probably due to a population of HMM molecules which do not irreversibly bind ATP in cold chase studies.
troponin-tropomyosin not only markedly affects the equilibrium constant of myosin-ADP binding to actin, but also appears to decrease the rate constant of P_i release from actin-myosin-ADP-P_i more than it does the rate constant of P_i binding.

The observation that blocking P_i release, by itself, can cause muscle relaxation leads to the question of why troponin-tropomyosin also weakens the binding of S-1 and S-1-ADP to actin. The weakening by troponin-tropomyosin of the binding of S-1 ADP (or S-1-PP_i) to actin occurs at low levels of occupancy of the actin with S-1 (9, 10). In the absence of Ca^{2+}, S-1-ADP binds to regulated actin about 20-fold weaker than when most of the actin sites are occupied with S-1, i.e. the binding is positively cooperative. Similar cooperativity also appears to occur in vivo (21). This is based on stiffness measurements and x-ray diffraction patterns of skeletal muscle fibers in PP_i which show that, over a narrow range of ionic strength, the myosin cross-bridges go from being essentially all attached to all detached from actin.

One possible function for this phenomena is that it may allow the binding of force producing cross-bridges, as well as Ca^{2+}, itself, to modulate the number of turned-on actin units in vivo. In this regard, recent studies of Williams and Eisenberg (22) examining the effect of tropomyosin on the acto-S-1 ATPase activity indicate that when the regulated actin filament is activated by Ca^{2+}, the number of S-1 heads bound to actin may greatly affect the ATPase rate. The ability of force-producing cross-bridges to modulate the number of turned-on actin units could make the response of the muscle to Ca^{2+} highly cooperative. It could also allow the velocity of muscle contraction to affect the ATP turnover rate by changing the number of force-producing cross-bridges bound to actin. More work is required to determine whether this is indeed the role of the weak binding of S-1-ADP to regulated actin in skeletal muscle.

The observation made in this study that phosphorylation does not significantly affect the binding of smooth muscle myosin ADP complexes to actin may be related to the physiology of the latch state (23, 24) which has been shown to occur in smooth muscle after the initial rise in Ca^{2+} concentration and is marked by high force and low shortening velocity. During latch, myosin phosphorylation levels sometimes decline to near resting levels and presumably tension is being maintained largely by dephosphorylated myosin-ADP cross-bridges. Our in vitro studies suggest that the affinity of these myosin-ADP cross-bridges for actin should not be greatly affected by phosphorylation.

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REFERENCES