The influence of Ca$^{2+}$ on the enzymatic and physical properties of smooth muscle myosin was studied. The actin-activated ATPase activity of phosphorylated gizzard myosin and heavy meromyosin is higher in the presence of Ca$^{2+}$ than in its absence, but this effect is found only at lower MgCl$_2$ concentrations. As the MgCl$_2$ concentration is increased, Ca$^{2+}$ sensitivity is decreased. The concentration of Ca$^{2+}$ necessary to activate ATPase activity is higher than that required to saturate calmodulin. The similarity of the pCa dependence of ATPase activity and of Ca$^{2+}$ binding to myosin and the competition by Mg$^{2+}$ indicate that these effects involved the Ca$^{2+}$-$\text{Mg}^{2+}$ binding sites of gizzard myosin. For the actin dependence of ATPase activity of phosphorylated myosin at low concentrations of MgCl$_2$, both $V_{\text{max}}$ and $K_v$ are influenced by Ca$^{2+}$. The formation of small polymers by phosphorylated myosin in the presence of Ca$^{2+}$ could account for the alteration in the affinity for actin. For the actin dependence of phosphorylated heavy meromyosin at low MgCl$_2$ concentrations, Ca$^{2+}$ induces only an increase in $V_{\text{max}}$. To detect alterations in physical properties, two techniques were used: viscosity and limited papain hydrolysis. For dephosphorylated myosin, 6 S or 10 S, Ca$^{2+}$-dependent effects are not detected using either technique. However, for phosphorylated myosin the decrease in viscosity corresponding to the 6 S to 10 S transition is shifted to lower KCl concentrations by the presence of Ca$^{2+}$. In addition, a Ca$^{2+}$ dependence of proteolysis rates is observed with phosphorylated myosin but only at low ionic strength, i.e. under conditions where myosin assumes the folded conformation.

It is generally accepted that the phosphorylation-dephosphorylation of the 20,000-dalton light chains of myosin form an important component of the regulatory mechanism in smooth muscle (1, 2). In its simplest interpretation the phosphorylation theory would predict that myosin phosphorylation allows the activation of Mg$^{2+}$-ATPase by actin and hence increases the cross-bridge cycling rate leading to tension development; dephosphorylation would reverse the process resulting in the formation of an "inactive" myosin and relaxation. It is apparent that this scenario is oversimplified and the physiological responses of smooth muscle require a more complex or versatile regulatory mechanism. For example, it was found that intact fibers from several types of smooth muscle exhibit a Ca$^{2+}$-dependent resistance to stretch (3, 4) that was thought to reflect attached noncycling cross-bridges. Subsequently, the attached noncycling bridges, termed latch-bridges, were shown to occur with dephosphorylated myosin (5) and possessed a Ca$^{2+}$ sensitivity distinct from that of the calmodulin-dependent myosin light chain kinase (6). Thus, it was proposed that light chain phosphorylation is correlated with the cross-bridge cycling rate (5) but that a second Ca$^{2+}$-dependent mechanism regulates stress maintenance (6, 7). The latter unidentified mechanism would presumably have a higher affinity for Ca$^{2+}$ than calmodulin since it operates when the calmodulin-myosin light chain kinase complex is inactive. This idea is consistent with recorded Ca$^{2+}$ transients in vascular smooth muscle cells that showed that tension can be maintained at a lower Ca$^{2+}$ level than that required for force development (8).

Another aspect that is not understood is the effect of Ca$^{2+}$ on shortening velocity. Although this is a controversial topic (see references in Ref. 9) there are several reports that claim that Ca$^{2+}$ influences shortening velocity of intact (10, 11) and skinned (9, 12, 13) smooth muscle fibers. This can occur at high levels of phosphorylation without any marked change in the extent of phosphorylation (9, 13) and is therefore different from the formation of latch-bridges. The requirement for Ca$^{2+}$ is not obligatory since contraction of skinned smooth muscle fibers can be induced in the absence of Ca$^{2+}$ by the addition of a Ca$^{2+}$-independent myosin light chain kinase (14). However, it is likely that under suitable conditions (the free Mg$^{2+}$ concentration in particular important), the cycling rate of a phosphorylated cross-bridge can be modified by varying the Ca$^{2+}$ concentration. The molecular basis for such an effect is unknown.

Several other regulatory mechanisms, either as alternative or complementary to the phosphorylation theory, have been proposed. These include: the thin filament-linked leiotonin system (15); the possibility of thin filament phosphorylation (16); the actin- and calmodulin-binding protein, caldesmon (17, 18); and the presence of phosphorylation-independent activators of actomyosin ATPase activity (19, 20). In general, the mechanisms by which these systems achieve a regulatory or modulatory response have not been identified. However, there is one putative regulatory system that has a stronger experimental basis and that is the binding of Ca$^{2+}$ to myosin. It has been established that under appropriate conditions, myosin from each muscle type can bind Ca$^{2+}$ (vertebrate skeletal (21-31); cardiac (32-34); smooth (35-39); and invertebrate (25, 40-45)), but only in the case of molluscan and some other invertebrate muscles (41) is the binding of Ca$^{2+}$ to myosin an obligatory requirement for actin-activated ATPase activity. This reflects the presence of Ca$^{2+}$-specific binding sites that are present in addition to nonspecific Ca$^{2+}$-$\text{Mg}^{2+}$.

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The role of the Ca$^{2+}$-myosin interaction in the regulation of smooth muscle activity is controversial. In several reports, it is claimed that the actin-activated ATPase of phosphorylated, or, thiophosphorylated, myosin is not Ca$^{2+}$-dependent (43-53). Other investigators have observed that in the absence of Ca$^{2+}$ the actin-activated ATPase of phosphorylated myosin (39, 54-57) or heavy meromyosin (HMM) (68) is reduced. There is also a report (59) that the actin-activated ATPase of fully phosphorylated gizzard myosin is not Ca$^{2+}$-dependent, whereas the Mg$^{2+}$-ATPase of partially phosphorylated myosin is Ca$^{2+}$-dependent. Many of these apparently contradictory reports can be resolved if the ionic conditions, especially the Mg$^{2+}$ concentration, of the various ATPase assays are taken into consideration. In order to increase ATPase activity, many investigators use relatively high concentrations of Mg$^{2+}$ in their assays (for references see Ref. 60) and, as pointed out (39, 56), these conditions would reduce, or eliminate, a Ca$^{2+}$-sensitive response. If the binding of Ca$^{2+}$ to the Ca$^{2+}$-Mg$^{2+}$ sites of phosphorylated myosin is responsible for Ca$^{2+}$ sensitivity, as proposed by Chacko and Rosenfeld (39), such an effect would be expected.

From the evidence mentioned above, it is likely that Ca$^{2+}$ can influence the myosin-actin interaction in vitro and it is possible that the binding of Ca$^{2+}$ to myosin may play an important regulatory role in the intact muscle. To obtain more information on the effects of Ca$^{2+}$ on smooth muscle myosin we initiated this study. Of particular interest were the effects of Ca$^{2+}$ on actin-activated ATPase activity and on the properties and conformation of myosin. Previously it was suggested (61) that the folded (10 S) and extended (6 S) forms of monomeric myosin had distinct enzymatic properties and that some characteristic of the 6 S or 10 S conformation is a determinant of ATPase activity. With this in mind it was pertinent to determine if Ca$^{2+}$-dependent conformational changes of myosin could be detected that in turn might reflect the Ca$^{2+}$ dependence of ATPase activity. The results are presented below.

**Materials and Methods**

Smooth muscle myosin was prepared from frozen turkey gizzards by a method modified from that of Perschini and Hartsoek (62) as follows. Turkey gizzards are trimmed of fat and connective tissue and minced. The mince (usually 250-500 g) is suspended in 3 volumes of buffer A, i.e. 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM EGTA, 25 mM MgCl$_2$, 0.2 mM dithiothreitol, 5% (v/v) Triton X-100, homogenized for 20 s in a Waring blender, and centrifuged at 1500 X g for 5 min. The supernatant is discarded and the pellet is subjected to 3 cycles of homogenization and centrifugation with buffer B, using a glass homogenizer. The procedures involved for the limited proteolysis of myosin by papain were as described previously (68). The extent of myosin degradation was estimated by integrating the area of intact myosin bands observed at different times of digestion using discontinuous SDS-polyacrylamide gels. Ca$^{2+}$-binding to gizzard myosin was estimated at pH 7.0 by equilibrium dialysis under the conditions given in the figure legend. The total concentration of EGTA (i.e. EGTA and Ca$^{2+}$-EGTA) was 0.1 mM. The apparent dissociation constant at pH 7.0 for Ca$^{2+}$-EGTA was assumed to be 1 X 10$^{-4}$ M. Myosin (4 mg/ml) was dialyzed overnight at 4 °C at different pH values using a EMD 101 equilibrium dialysis system (Hoefer Scientific Instruments). Ca$^{2+}$ (CaCl$_2$, New England Nuclear) was measured by scintillation counting (Beckman LS 8000 Scintillation System) in 100-μl aliquots from each side of the membrane bag. The amount of bound Ca$^{2+}$ was estimated. For practical reasons neither ATP nor actin were included in the samples for equilibrium dialysis. ATPase activities were measured at 25 °C, as described previously (69), under conditions given in the figure legends. Routine assays were carried out at pH 7.5, with the exception that pCa dependence of ATPase activity (Fig. 2) was determined at pH 7.0. The ATPase activity was expressed as described in the figure legends and determined by the procedure of Walsh et al. (65). Electrophoresis was carried out on 7.5-20% polyacrylamide gradient slab gels in the presence of 0.1% SDS using the discontinuous buffer system of Laemmli (70). The gels were stained with Coomassie Brilliant Blue R 250 (Sigma) and scanned by a GS 200 Scanning Densitometer (Hoefer Scientific Instruments) attached to a LCI-100 Laboratory Computing Integrator (Perkin Elmer). The measurements of viscosity and sedimentation velocity were carried out using a gravity column (67). Other procedures are given by Walsh et al. (71).

**Results**

The actin-activated ATPase of phosphorylated gizzard myosin (approximately 10:1 molar ratio of actin:myosin, respectively) at varying levels of MgCl$_2$ and in the presence and absence of Ca$^{2+}$ is shown in Fig. 1A. (The indicated concentration of MgCl$_2$ is the total concentration; in the presence of 1 mM ATP it is assumed that the free Mg$^{2+}$ concentration is approximately 1 mM less than the total concentration.) This figure illustrates an important point, namely that the level of actin-activated ATPase activity is markedly dependent on the MgCl$_2$ concentration, and that the Ca$^{2+}$ dependence of this

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1. The abbreviations used are: HMM, heavy meromyosin; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.
ATPase activity is more pronounced at lower MgCl₂ concentrations. It was suggested previously (60) that the low specific actomyosin ATPase activity, characteristic of lower MgCl₂ concentrations, reflects the presence of the 10 S conformation. Increasing the MgCl₂ concentration results in higher activities and the conversion of 10 S to 20 S myosin light chain kinase for 15 min at 25°C. The level of phosphorylation was 1.7-1.8 mol of P/mol of myosin under the conditions given in the legend to Fig. 1. precipitated by the addition of MgCl₂ to 10 mM, and then assayed at pH 7.0 at varying concentrations of MgCl₂, using a Ca²⁺-EGTA buffer system. The pCa of half-maximal activity (assuming the activity at pCa 4 to be the maximum) for the 0.1 mM MgCl₂ assays is approximately 10 μM, and for the 1 mM MgCl₂ assays is approximately 30 μM. Since the binding sites involved on the myosin are Ca²⁺-Mg²⁺ sites, a shift to lower affinity at higher MgCl₂ concentrations is expected. However, on this limited experimental basis and with the relatively slight shift of pCa values the significance of these results should not be overemphasized. Under identical conditions (i.e., pH, calmodulin concentration, etc.) activation of myosin light chain kinase occurs at lower Ca²⁺ concentrations, and half-maximal phosphorylation was obtained at approximately 1 μM Ca²⁺ (Fig. 2). Thus, it appears that under these assay conditions the Ca²⁺ sites on myosin activation of actin-activated ATPase activity in the presence of tropomyosin is not due to an effect of Ca²⁺ on binding of tropomyosin to actin.

Throughout the experiments illustrated in Fig. 1, the level of myosin phosphorylation remained constant, at 1.7 to 1.8 mol of P/mol of myosin. There was no detectable dephosphorylation for up to 30 min after the addition of EGTA. It is unlikely, therefore, that any of the observed differences in ATPase activity are due to altering levels of myosin phosphorylation.

The Ca²⁺ dependence of the actin-activated ATPase activity of phosphorylated gizzard myosin at two concentrations of MgCl₂, 0.1 and 1 mM (total), is shown in Fig. 2. As in the previous experiment the myosin was prephosphorylated (~1.8 mol of myosin) and then assayed at pH 7.0 at varying concentrations of Ca²⁺, using a Ca²⁺-EGTA buffer system. The pCa of half-maximal activity (assuming the activity at pCa 4 to be the maximum) for the 0.1 mM MgCl₂ assays is approximately 10 μM, and for the 1 mM MgCl₂ assays is approximately 30 μM. Since the binding sites involved on the myosin are Ca²⁺-Mg²⁺ sites, a shift to lower affinity at higher MgCl₂ concentrations is expected. However, on this limited experimental basis and with the relatively slight shift of pCa values the significance of these results should not be overemphasized. Under identical conditions (i.e., pH, calmodulin concentration, etc.) activation of myosin light chain kinase occurs at lower Ca²⁺ concentrations, and half-maximal phosphorylation was obtained at approximately 1 μM Ca²⁺ (Fig. 2). Thus, it appears that under these assay conditions the Ca²⁺ sites on myosin activation of actin-activated ATPase activity in the presence of tropomyosin is not due to an effect of Ca²⁺ on binding of tropomyosin to actin.
are occupied subsequent to the binding of Ca\textsuperscript{2+} to calmodulin.

The binding of Ca\textsuperscript{2+} to dephosphorylated myosin at 3 levels of MgCl\textsubscript{2} is shown in Fig. 3. For practical reasons these determinations differed from the ATPase assays in that ATP and actin were not included. The results show clearly that the binding of Ca\textsuperscript{2+} to myosin is dependent on the MgCl\textsubscript{2} concentration and is almost eliminated at 6 mM MgCl\textsubscript{2}. Half-saturation in 0.1 mM MgCl\textsubscript{2} occurred at approximately 35 \(\mu\)M Ca\textsuperscript{2+} and at approximately 50 \(\mu\)M Ca\textsuperscript{2+} in 1 mM MgCl\textsubscript{2}. The binding of Ca\textsuperscript{2+} to phosphorylated myosin in 0.1 mM MgCl\textsubscript{2} also is shown (Fig. 3) and the binding curve is the same as that obtained with dephosphorylated myosin.

In general the above results confirm those of others (39, 56) in that it is demonstrated that the Ca\textsuperscript{2+} sensitivity of actin-activated ATPase and binding of Ca\textsuperscript{2+} to myosin are markedly affected by the Mg\textsuperscript{2+} concentration. The following experiments explore in more detail the Ca\textsuperscript{2+} dependence of actin-activated ATPase activity using gizzard myosin and HMM.

In Fig. 4 the actin dependence of phosphorylated (\(-1.9-2.0\) mol of P/mol of myosin) myosin ATPase is shown at two concentrations of MgCl\textsubscript{2} (2 and 10 mM, total) each in the presence and absence of Ca\textsuperscript{2+}. A Ca\textsuperscript{2+}-dependent response is seen only at the lower Mg\textsuperscript{2+} concentration. At 2 mM MgCl\textsubscript{2} in the presence of Ca\textsuperscript{2+} (\(-1 \times 10^{-4} M\)) \(V_{\text{max}}\) \(\sim 39\) nmol/min·mg (0.31 s\(^{-1}\)) and \(K_a\) (the apparent dissociation constant for actin as determined from ATPase measurements) is 2.3 \(\mu\)M. At 2 mM MgCl\textsubscript{2} in the absence of Ca\textsuperscript{2+} (i.e. 1 mM EGTA) \(V_{\text{max}}\) \(\sim 11.1\) nmol/min·mg (0.09 s\(^{-1}\)) and \(K_a\) \(\sim 40\) \(\mu\)M. Therefore, Ca\textsuperscript{2+} affects both \(V_{\text{max}}\) and \(K_a\) although the effect on the actin-myosin interaction appears more marked. In the inset of Fig. 4, are shown sedimentation velocity profiles of phosphorylated myosin in 2 mM MgCl\textsubscript{2}, 1 mM ATP, and the absence (upper) and presence (lower) of Ca\textsuperscript{2+} (other conditions given in the figure legend). Previously it has been shown (60, 61) that at low MgCl\textsubscript{2} concentrations and low ionic strength phosphorylated myosin sediments as a mixture of 10 S monomer and a small myosin polymer (in the order of 2 to 6 molecules). As shown in Fig. 4, the extent of polymer formation appears to be Ca\textsuperscript{2+}-dependent. In the presence of Ca\textsuperscript{2+} \((\sim 1 \times 10^{-4} M)\) the polymer boundary is considerably larger than in the absence of Ca\textsuperscript{2+}. The proportion of polymer-to-monomer could affect the ATPase properties, and it might be predicted that a myosin polymer would bind actin stronger than the myosin monomer. Therefore, this may be at least a partial explanation for the variations in \(K_a\) observed in these experiments.

In 10 mM MgCl\textsubscript{2} the actin-activated ATPase activity is not Ca\textsuperscript{2+}-sensitive; \(V_{\text{max}}\) \((\pm\text{Ca}^{2+})\) \(\sim 66\) nmol/min·mg (0.52 s\(^{-1}\)) and \(K_a\) \(\sim 3.3\) \(\mu\)M. Under these conditions monomeric myosin is not observed and the myosin aggregates are considerably larger than at lower MgCl\textsubscript{2} concentrations and are observed only at relatively low centrifugal forces.

To assist with an evaluation of whether the Ca\textsuperscript{2+} effects are primarily due to an alteration of \(V_{\text{max}}\) or \(K_a\), the ATPase properties of HMM were studied. HMM is soluble at low ionic strength and does not form aggregates.

The Mg\textsuperscript{2+} dependence of the actin-activated ATPase of phosphorylated HMM is distinct from that exhibited by myosin, as shown in Fig. 5. Increasing MgCl\textsubscript{2} concentrations tend to decrease the level of actin activation of HMM, (cf.}
Ref. 58) as opposed to an increase in the case of myosin. However, myosin and HMM are similar with respect to the effect of Mg$^{2+}$ on the Ca$^{2+}$ sensitivity of actin-activated ATPase. Ca$^{2+}$ sensitivity in both systems is observed only at lower MgCl$_2$ concentrations. In the case of HMM, Ca$^{2+}$ sensitivity is lost at about 3 mM MgCl$_2$ (total).

The actin dependence of the Mg$^{2+}$-ATPase of phosphorylated HMM is shown in Fig. 6 for two concentrations of MgCl$_2$ (1 and 4 mM, total) each in the presence and absence of Ca$^{2+}$. In 1 mM MgCl$_2$ and the presence of Ca$^{2+}$, $V_{\text{max}}$ and $K_a$ were calculated to be 833 nmol/min·mg (4.64 s$^{-1}$) assuming a molecular weight for HMM of 3.34 $\times$ 10$^5$ and 70 $\mu$M, respectively. In 1 mM MgCl$_2$ and the absence of Ca$^{2+}$, $V_{\text{max}}$ is unaltered and $K_a$ $\sim$ 555 nmol/min·mg (3.15 s$^{-1}$). At the higher MgCl$_2$ concentration, the ATPase activity was not sensitive to Ca$^{2+}$, and the values for $V_{\text{max}}$ and $K_a$ are 555 nmol/min·mg (3.15 s$^{-1}$) and 126 $\mu$M, respectively.

To check if Ca$^{2+}$-induced conformational changes in myosin could be detected, two approaches were taken. The first was to determine if the viscosity of myosin is altered in the presence and absence of Ca$^{2+}$. As can be seen (Fig. 7) the presence of Ca$^{2+}$ shifts the viscosity transition to lower KC1 concentrations. This could be due either to a relative resistance of the 6 S myosin plus Ca$^{2+}$ to form the folded conformation, or to an increased formation of small polymers with 10 S myosin plus Ca$^{2+}$. As shown above (Fig. 4), the sedimentation velocity profiles of phosphorylated myosin indicated an enhanced tendency for polymer formation in the presence of Ca$^{2+}$. For dephosphorylated myosin an effect of Ca$^{2+}$ is not detected and the viscosity transitions in the presence and absence of Ca$^{2+}$ are identical (Fig. 7).

It has been demonstrated that the rate of limited papain hydrolysis of myosin is sensitive to conformation (68, 73) in that 10 S myosin is more resistant to proteolysis than 6 S myosin. This approach was used to screen for possible Ca$^{2+}$-dependent conformational changes in gizzard myosin. The extent of proteolysis was estimated from the area of the myosin heavy chain observed on SDS-polyacrylamide gels. Time courses of heavy chain disappearance for myosin exposed to papain under various conditions are shown in Fig. 8. For 6 S myosin, both phosphorylated and dephosphorylated, and in the presence and absence of Ca$^{2+}$, the rates of hydrolysis are approximately the same. (The 6 S conformation was achieved by using a KC1 concentration of 0.4 M. It was shown in control experiments on casein digestion that variations in KC1 concentrations from 50 mM to 0.4 M had negligible effects on the rate of papain hydrolysis.) For 10 S dephosphorylated myosin the proteolysis rate is considerably slower than that for 6 S myosin and there is no effect of Ca$^{2+}$ (Fig. 8). (The concentration of papain used with dephosphorylated 10 S myosin was 3-fold higher than that used in the other experiments.) The only conditions under which an effect of Ca$^{2+}$ could be detected was for phosphorylated 10 S myosin. For phosphorylated 10 S myosin in the absence of Ca$^{2+}$, the proteolysis rate was faster than for dephosphorylated 10 S myosin, and this indicates an alteration in the molecule induced by phosphorylation (see "Discussion") that is not evident from sedimentation velocity experiments. In the presence of Ca$^{2+}$ the proteolysis rate was reduced to a rate similar to that for 6 S myosin.
The data presented above indicates that the actin-activated ATPase of phosphorylated gizzard myosin can be modified by a Ca\(^{2+}\)-dependent process. The Ca\(^{2+}\)-dependent response is seen both in the presence and absence of tropomyosin and is seen also for the ATPase activity of acto-HMM. In all instances the Ca\(^{2+}\) effect is influenced by the prevalent Mg\(^{2+}\) concentration and in general is favored by lower concentrations of Mg\(^{2+}\). Although these results are qualitatively similar to previous studies (39, 56, 58) there are some distinctions. For example, in the presence of tropomyosin and using skeletal muscle actin we find both a Mg\(^{2+}\) and Ca\(^{2+}\) dependence of actin-activated ATPase activity. Nag and Seidel (56) found little Ca\(^{2+}\) dependence using skeletal actin plus tropomyosin, whereas with gizzard actin plus tropomyosin they observed a marked Ca\(^{2+}\) dependence at lower MgCl\(_2\) concentrations. Ca\(^{2+}\) dependence in our system is seen even at low Mg\(^{2+}\) levels and does not show an optimum at approximately 2 mM free Mg\(^{2+}\) as found with arterial myosin (39). Rees and Fredericksen (55) with porcine aorta myosin found a stimulation by Ca\(^{2+}\) of the actin-activated ATPase of dephosphorylated myosin and this is not found with our system. Previously it was reported that the ATPase activity of acto-arterial HMM showed a marked Ca\(^{2+}\) dependence only in the presence of tropomyosin (58), whereas in our system tropomyosin is not essential.

The most likely site for Ca\(^{2+}\) binding, and therefore the site responsible for these Ca\(^{2+}\) effects, is the myosin molecule. The evidence for this is as follows. 1) Myosin from each muscle type is known to bind Ca\(^{2+}\). With the exception of the mussels-type myosin that possesses Ca\(^{2+}\)-specific sites, other myosins bind Ca\(^{2+}\) at Ca\(^{2+}\)-Mg\(^{2+}\) sites (25). The reported dissociation constants in the presence of low concentrations of Mg\(^{2+}\) are variable but are in the range of 5 to 30 \(\mu\)M (see Refs. 22, 32, and 33). These values are consistent with the Ca\(^{2+}\) levels necessary to activate ATPase activity (from Fig. 2, 10 \(\mu\)M in 0.1 mM MgCl\(_2\), and 30 \(\mu\)M in 1 mM MgCl\(_2\)) and with rough estimates of half-maximal Ca\(^{2+}\) binding (from Fig. 3, 35 \(\mu\)M in 0.1 mM MgCl\(_2\), and 50 \(\mu\)M in 1 mM MgCl\(_2\)) of the concentrations of Ca\(^{2+}\) that elicit the in vitro effects are therefore in the range expected from the affinity of Ca\(^{2+}\) binding to myosin. These Ca\(^{2+}\) concentrations are higher than those reported by Chacko and Rosenfeld (39) who found activation of ATPase activity at \(10^{-5}\) M Ca\(^{2+}\). Activation in our system occurred only at greater than \(10^{-6}\) M Ca\(^{2+}\), and this is similar to the findings of Nag and Seidel (56). 2) The cation binding sites on gizzard myosin are Ca\(^{2+}\)-Mg\(^{2+}\) sites (25) and the relationship between increasing MgCl\(_2\) concentrations and Ca\(^{2+}\) binding or Ca\(^{2+}\) sensitivity of ATPase activity is consistent with competition by Ca\(^{2+}\) or Mg\(^{2+}\) for
these sites. 3) A Ca\textsuperscript{2+} dependence of ATPase activity is seen in the presence of only myosin and actin; and in the absence of other proteins actin is not credited as conferring Ca\textsuperscript{2+} sensitivity. 4) Ca\textsuperscript{2+}-dependent conformational changes are found with myosin alone.

The effect of Ca\textsuperscript{2+} on the actin-activated myosin ATPase was to alter both the apparent dissociation constant for actin (K\textsubscript{a}) and V\textsubscript{max}. It is suggested that the alteration of K\textsubscript{a} could be associated with the polymer formation which was shown to occur in the presence of Ca\textsuperscript{2+} (see Fig. 4). To evaluate the relative contributions of K\textsubscript{a} and V\textsubscript{max} to the Ca\textsuperscript{2+} dependence of ATPase and to eliminate complications due to polymer formation, we studied the actin-activated ATPase of gizzard HMM. For the ATPase activity of acto-HMM a Ca\textsuperscript{2+} dependence is observed at lower MgCl\textsubscript{2} concentrations but not at higher MgCl\textsubscript{2} concentrations (see Figs. 5 and 6). The Ca\textsuperscript{2+} effect is due to a shift in V\textsubscript{max}, K\textsubscript{a} in the presence and absence of Ca\textsuperscript{2+} remains constant. In the intact muscle one would not expect to see effects due to myosin polymerization since it has been reported that thick filaments exist both in resting and contracting muscle (74). Thus, it might be predicted that the increase in V\textsubscript{max} observed with phosphorylated myosin would be reflected in vivo by an increase in shortening velocity. In support of this, Siegman et al. (13) found that an increase in external Ca\textsuperscript{2+} caused approximately a 2-fold increase in the unloaded shortening velocity of intact taenia coli fibers. With skinned gizzard fibers at constant levels of ionic strength a fraction of the phosphorylated myosin forms polymers. Whether or not polymers form at levels lower than that necessary to "activate" calmodulin. Murphy and colleagues (6, 7) report that latch-bridges possess a greater sensitivity to Ca\textsuperscript{2+} than calmodulin. In other words the latch process would be subject to regulation at Ca\textsuperscript{2+} levels lower than that necessary to bind to calmodulin. The Ca\textsuperscript{2+} sensitivities of the two systems relative to the calmodulin threshold therefore are different. In addition, one would predict that the formation of attached cross-bridges is associated with an increased affinity of myosin and actin. We think that the changes observed for K\textsubscript{a} can be explained by polymer formation and we do not find an increased affinity for actin in the absence of Ca\textsuperscript{2+}, but rather a reduced affinity. It is possible that the effect of Ca\textsuperscript{2+} on myosin filaments is different from that shown in our in vitro experiments. With this as a reservation, it can be proposed that our current data is not consistent with the involvement of Ca\textsuperscript{2+} binding to myosin in the formation of slowly cycling cross-bridges.

REFERENCES

7. Barsotti, M. Kobe, and D. J. Hartshorne, unpublished observation.
8. Hartshorne, R. Barsotti, M., Ikebe, and D. J. Hartshorne, unpublished observation.

myosin. Thus, the use of the term 10 S to describe both the phosphorylated and dephosphorylated forms implies an identity in conformation that may not be justified. It should be emphasized, however, that although the gross hydrodynamic properties of the two myosins are similar, the sedimentation coefficient of the phosphorylated myosin (at low ionic strength, etc.) was not calculated, and more exacting measurements might reveal a difference in sedimentation rates. Additional evidence to indicate a difference between 10 S phosphorylated myosin and 10 S dephosphorylated myosin is apparent from the actin-activated ATPase activities. Although the level of actin activation for phosphorylated myosin at low MgCl\textsubscript{2} concentrations (i.e. operationally defined as 10 S) is low, it is considerably higher than the actin-activated ATPase activity of dephosphorylated myosin. Therefore, it is suggested that phosphorylated 10 S myosin in the absence of Ca\textsuperscript{2+} is distinct in some way from dephosphorylated myosin in the presence or absence of Ca\textsuperscript{2+}. This difference becomes more evident when Ca\textsuperscript{2+} is bound to the phosphorylated 10 S myosin.

We suggested earlier (68, 69), based on limited proteolysis studies, that the interaction of subfragment 1 and subfragment 2 may be affected by the 6 S-10 S transition. In our opinion this interaction is probably more important in determining enzymatic activity than the folding and/or interactions involving the tail portion of the molecule. This could be the region that is altered by phosphorylation at low ionic strength and, as such, may not be easily detected by sedimentation velocity measurements.

It is important to establish if Ca\textsuperscript{2+} influences the binding affinity of myosin to actin, as this may be pertinent to the attached or slowly cycling cross-bridges observed in fiber measurements (4, 5). Although we cannot be conclusive at this time, we feel it is unlikely that Ca\textsuperscript{2+} binding to myosin is important in this process. Our results indicate that the range of Ca\textsuperscript{2+} concentrations over which the Ca\textsuperscript{2+} effects are observed are higher than that necessary to "activate" calmodulin. Murphy and colleagues (6, 7) report that latch-bridges possess a greater sensitivity to Ca\textsuperscript{2+} than calmodulin. In other words the latch process would be subject to regulation at Ca\textsuperscript{2+} levels lower than that necessary to bind to calmodulin. The Ca\textsuperscript{2+} sensitivities of the two systems relative to the calmodulin threshold therefore are different. In addition, one would predict that the formation of attached cross-bridges is associated with an increased affinity of myosin and actin. We think that the changes observed for K\textsubscript{a} can be explained by polymer formation and we do not find an increased affinity for actin in the absence of Ca\textsuperscript{2+}, but rather a reduced affinity. It is possible that the effect of Ca\textsuperscript{2+} on myosin filaments is different from that shown in our in vitro experiments. With this as a reservation, it can be proposed that our current data is not consistent with the involvement of Ca\textsuperscript{2+} binding to myosin in the formation of slowly cycling cross-bridges.