# Effects of Magnesium Chloride on Smooth Muscle Actomyosin Adenosine-5′-triphosphatase Activity, Myosin Conformation, and Tension Development in Glycerinated Smooth Muscle Fibers†

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ABSTRACT: The contractile system of smooth muscle exhibits distinctive responses to varying $Mg^{2+}$ concentrations in that maximum adenosine-5′-triphosphatase (ATPase) activity of actomyosin requires relatively high concentrations of $Mg^{2+}$ and also that tension in skinned smooth muscle fibers can be induced in the absence of $Ca^{2+}$ by high $Mg^{2+}$ concentrations. We have examined the effects of $MgCl_2$ on actomyosin ATPase activity and on tension development in skinned gizzard fibers and suggest that the $MgCl_2$-induced changes may be correlated to shifts in myosin conformation. At low concentrations of free $Mg^{2+}$ ($\leq$1 mM) the actin-activated ATPase activity of phosphorylated turkey gizzard myosin is reduced and is increased as the $Mg^{2+}$ concentration is raised. The increase in $Mg^{2+}$ (over a range of 1–10 mM added $MgCl_2$) induces the conversion of 10S phosphorylated myosin to the 6S form, and it was found that the proportion of myosin as 10S is inversely related to the level of actin-activated ATPase activity. Activation of the actin-activated ATPase activity also occurs with dephosphorylated myosin but at higher $MgCl_2$ concentrations, between 10 and 40 mM added $MgCl_2$. Viscosity and fluorescence measurements indicate that increasing $Mg^{2+}$ levels over this concentration range favor the formation of the 6S conformation of dephosphorylated myosin, and it is proposed that the 10S to 6S transition is a prerequisite for the observed activation of ATPase activity. With glycerinated chicken gizzard fibers high $MgCl_2$ concentrations (6–20 mM) promote tension in the absence of $Ca^{2+}$. $MgCl_2$-induced tension is not associated with an increased extent of myosin phosphorylation, and this is supported by the observation that in the presence of ITP tension can also be developed by high concentrations of $MgCl_2$.

The regulation of smooth muscle contractile activity is thought to be effected via the phosphorylation–dephosphorylation of the two 20 000-dalton light chains of the myosin molecule [for reviews, see Adelstein & Eisenberg (1980) and Walsh & Hartshorne (1982)]. Phosphorylation is required for the activation by actin of myosin $Mg^{2+}$-ATPase activity and is correlated with isotonic shortening velocities in smooth muscle (Dillon et al., 1981). Relaxation and the loss of actin-activated ATPase activity occur when myosin is dephosphorylated. The $Ca^{2+}$ dependence of this process is attributable to calmodulin, which as the $Ca^{2+}$–calmodulin complex activates the myosin light chain kinase.

Smooth muscle myosin differs from striated muscle myosins in that under appropriate conditions it can form a looped or folded conformation (Onishi & Wakabayashi, 1982; Trybus et al., 1982; Craig et al., 1983). The extended and folded conformations are often referred to as 6S and 10S, respectively, since they sediment at different rates. A reduction in ionic strength promotes the formation of the 10S state, and in addition several other factors can influence the transition (Ikebe et al., 1983a). Of these the effect of phosphorylation is of particular interest. In general, phosphorylation favors the extended conformation (Trybus et al., 1983; Craig et al., 1983; Onishi et al., 1983), and under suitable solvent conditions the 10S to 6S transition requires the phosphorylation of both light chains (Ikebe et al., 1983a). Accompanying the conformational transition there is an alteration in the enzymatic activities of myosin and the 6S and 10S states also have distinct biological properties (Ikebe et al., 1983a). Thus, since phosphorylation can alter the conformation of myosin, it may also influence enzymatic activity. The point to emphasize, however, is that the shape of myosin, or some conformational change associated with the shape transition, is the determinant of enzymatic activity. Thus, phosphorylation influences enzymatic properties only as a result of a shift in the "shape–activity" relationship.

Smooth and skeletal muscles differ in regard to their requirements for myosin phosphorylation and they differ also with respect to the effects of $Mg^{2+}$ on their contractile systems. There are two areas in which differences have been noted. First, in assays with smooth muscle actomyosin the requirement for relatively high concentrations of $Mg^{2+}$ has been known for several years (Schirmer, 1965; Murphy et al., 1969; Russell, 1973; Moreland & Ford, 1981). It was shown that this unusual $Mg^{2+}$ dependence is not due to the myosin light chain kinase system (Hartshorne et al., 1980) but is more likely to be a feature of smooth muscle myosin. A second distinctive property has been observed frequently with skinned smooth muscle fibers, namely, that tension may be developed at relatively high concentrations of $Mg^{2+}$ in the absence of $Ca^{2+}$ (Gordon, 1978; Saida & Nonomura, 1978; Nakahata, 1979; Nakahata et al., 1981; Arner, 1983).

One of the variables that influence the conformational transition is the concentration of $Mg^{2+}$. In general, lower concentrations of $Mg^{2+}$ favor the formation of 10S and higher concentrations promote the reverse trend. Since the shape transitions are correlated to alterations in enzymatic activity, it seemed feasible that the unusual effects of $Mg^{2+}$ mentioned above might be attributable to changes in myosin conformation induced by varying the $Mg^{2+}$ concentrations. Some aspects of this hypothesis have been tested, and the results are presented here.

## Materials and Methods

Proteins were prepared from various tissues by the following procedures: myosin from frozen turkey gizzard (Persechini

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& Hartshorne, 1983); myosin light chain kinase from frozen turkey gizzards (Walsh et al., 1983); calmodulin from frozen bull testes (Walsh et al., 1983); actin from rabbit skeletal muscle and tropomyosin from frozen turkey gizzards (Driska & Hartshorne, 1975). [$\gamma$-$^{32}$P]ATP was obtained from New England Nuclear.

ATPase activities were determined at 25 °C as described by Ferenczi et al. (1978). Reactions were started by the addition of [$\gamma$-$^{32}$P]ATP (1 mM). Solvent conditions are given in the figure legends. Phosphorylation of isolated myosin was assayed as outlined previously (Walsh et al., 1983). Viscosities were measured at 25 °C in Cannon-Ubbelohde viscometers with water flow times of approximately 25 s. The myosin concentration was 2 mg/mL; other conditions are given in figure legends. The viscosity data are expressed as $\eta_{rel}$ (viscosity of protein solution/viscosity of solvent). Sedimentation velocity experiments were carried out as described earlier (Ikebe et al., 1983a). The intrinsic tryptophan fluorescence of myosin at 25 °C was measured by using a Ferrand spectrofluorometer, Mark I. Excitation and emission wavelengths were 295 and 340 nm, respectively. Conditions are given in the figure legends. Protein determinations and electrophoresis on 7.5–20% polyacrylamide gradient slab gels in the presence of 0.1% NaDodSO$_4$[1] were as described earlier (Walsh et al., 1982).

Skinned muscle fibers were prepared from fresh chicken gizzards by using a glycerol procedure similar to that described by Peterson (1980) with modifications kindly suggested by Dr. U. Mrwa (University of Heidelberg). Small strips of tissue (approximately 10 × 2 mm) were dissected from lateral muscle blocks in which the fibers were oriented and placed for 10 min in 50% glycerol, 20 mM imidazole hydrochloride (pH 7.0), 10 mM $MgCl_2$, 7.5 mM ATP, 4 mM EGTA, 0.2 mM dithiothreitol, and 5 $\mu$g/mL leupeptin at 23 °C. The muscle strips were then transferred to the same solution at 5 °C for 1 h and subsequently stored at −20 °C. Bundles of fibers (approximately 3–6 mm long × 0.2–0.4 mm in diameter) were teased out (with the aid of a dissecting microscope) and attached horizontally, with a cellulose nitrate–acetone glue, between a fixed glass rod and a glass rod extending from a Statham UC2, or Grass FT03, force transducer, mounted on a micrometer drive. The absolute force obtained with the fiber bundles used in this study was in the range 0.5–4 mN. The compliance of the apparatus amounted to 0.4% shortening at maximum isometric force. Fibers initially were immersed at 23 °C in relaxing solution, i.e., 50 mM KCl, 20 mM imidazole hydrochloride (pH 7.0), 2 mM EGTA, 2 mM ATP, 2 mM $MgCl_2$, 1 $\mu$M calmodulin, 10 mM phosphoenolpyruvate, and 10 units/mL pyruvate kinase (Type II, Sigma Chemical Co.). The ATP regenerating system was not used when ITP (2 mM) was substituted for ATP. In the relaxing solution fibers were stretched to a preload of approximately 0.05 mN, which was found to be optimal for isometric force. Normal contractions were induced by immersing the fibers into a solution where the EGTA of the relaxing solution was replaced by $Ca^{2+}$-EGTA. Contractions were induced also by increasing the $Mg^{2+}$ concentration of the relaxing solution (see the legend to Figure 6).

The level of myosin phosphorylation in the skinned fiber preparations was determined as follows: The fiber at the required physiological state was immersed for about 2 min in 0.5 N $HClO_4$ at 0 °C and then homogenized in 50 $\mu$L of 9.5 M urea, 30 mM Tris, 140 mM glycine, 0.2 mM EDTA, and 0.5 mM dithiothreitol (pH 8.8) and applied to polyacrylamide slab gel electrophoresis (9.6% acrylamide) at pH 8.6 under the conditions given by Perrie & Perry (1970). The gels were fixed overnight in 25% methanol and 7% formaldehyde and subjected to the silver stain procedure of Oakley et al. (1980) as modified by Eschenbruch & Burk (1982). Following the citric acid–methylamine wash (step 3 of the latter procedure), the gels frequently became opaque and were clarified by washing for about 30 s in Kodak fixer. The percentage of phosphorylated and dephosphorylated 20000-dalton light chain was determined by densitometry using a Zeiss PM6 spectrophotometer attached to a Spectra-Physics SP 4050 printer/plotter, an SP 4020 data interface, and an SP 4000 central processor.

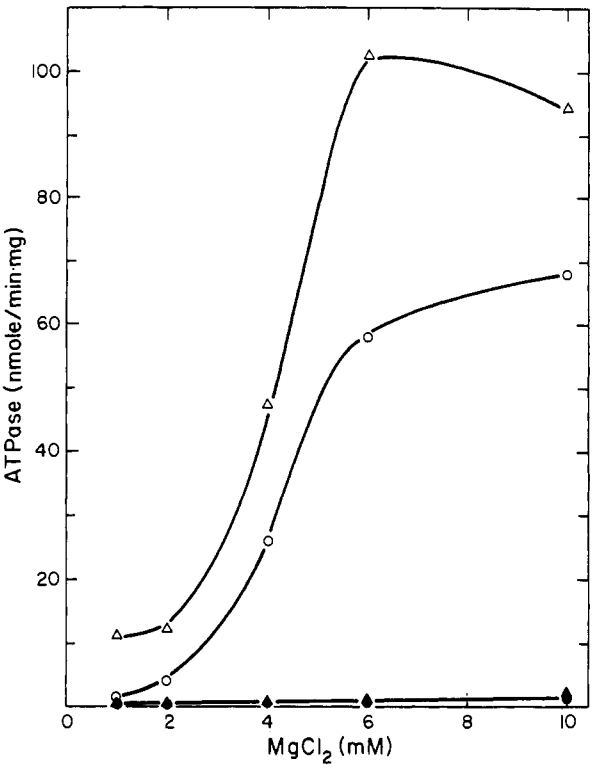


FIGURE 1: $MgCl_2$ dependence of actin-activated ATPase activity of gizzard myosin in the presence and absence of tropomyosin. Myosin (0.5 mg/mL) was preincubated at each $MgCl_2$ concentration (1–10 mM) for 15 min at 25 °C in 85 mM KCl, 30 mM Tris-HCl (pH 7.5), 1 mM [$\gamma$-$^{32}$P]ATP, 7.5 $\mu$g/mL myosin light chain kinase, 10 $\mu$g/mL calmodulin, and either 0.1 mM $CaCl_2$ (○, △) or 1 mM EGTA (●, ▲) and in the presence [0.2 mg/mL (△, ▲)] or absence (○, ●) of turkey gizzard tropomyosin. In the presence of $Ca^{2+}$ myosin was phosphorylated to approximately 1.8 mol of P/mol of myosin; in the absence of $Ca^{2+}$ phosphorylation was not detected. Actin-activated ATPase activity was initiated by the addition of skeletal muscle F-actin (to 1 mg/mL) to dephosphorylated myosin (●, ▲) and F-actin and EGTA (1 mM) to phosphorylated myosin (○, △).

## Results

*Effect of Low Concentrations of $MgCl_2$ (Up to 10 mM).* The $MgCl_2$ dependence of the actin-activated ATPase activity of turkey gizzard myosin is shown in Figure 1. Myosin was incubated with myosin light chain kinase, calmodulin, and ATP, in the presence and absence of $Ca^{2+}$, and following this period the ATPase reaction was started by the addition of actin or actin plus tropomyosin. The ATPase assays were carried out in the absence of $Ca^{2+}$, i.e., in the presence of EGTA (1 mM). Preincubation in the presence of $Ca^{2+}$ and at all concentrations of $MgCl_2$ resulted in the incorporation of approximately 1.8 mol of phosphate/mol of myosin, and this was

[1] Abbreviations: NaDodSO$_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HMM, heavy meromyosin.

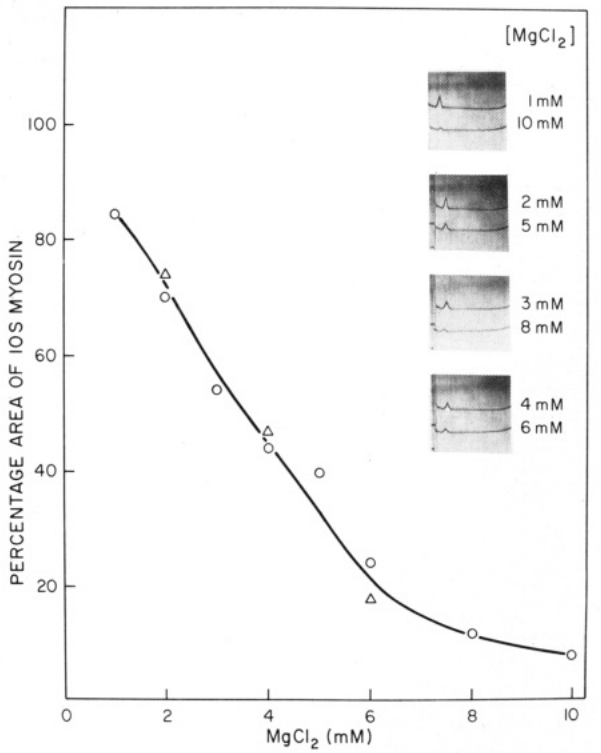


FIGURE 2: $MgCl_2$ dependence of 10S formation from phosphorylated myosin. Phosphorylation of myosin (1.5 mg/mL) was carried out in the absence of tropomyosin as described in Figure 1. Aliquots in 85 mM KCl, 1 mM ATP, and various concentrations of $MgCl_2$ (1–10 mM) were examined by analytical ultracentrifugation, as described under Materials and Methods. Schlieren patterns recorded 5 min after reaching a speed of 60 000 rpm are shown in the inset. At this time myosin polymers were sedimented and do not appear in the sedimentation patterns. The area of the sedimenting boundary for 100% 10S was measured with dephosphorylated myosin in 1 mM $MgCl_2$, 85 mM KCl, 1 mM ATP, 1 mM EGTA, and 30 mM Tris-HCl (pH 7.5). The area of the 10S peak for phosphorylated myosin at each $MgCl_2$ concentration was measured and expressed as a percentage of the 10S dephosphorylated myosin boundary. The two symbols represent data from two experiments.

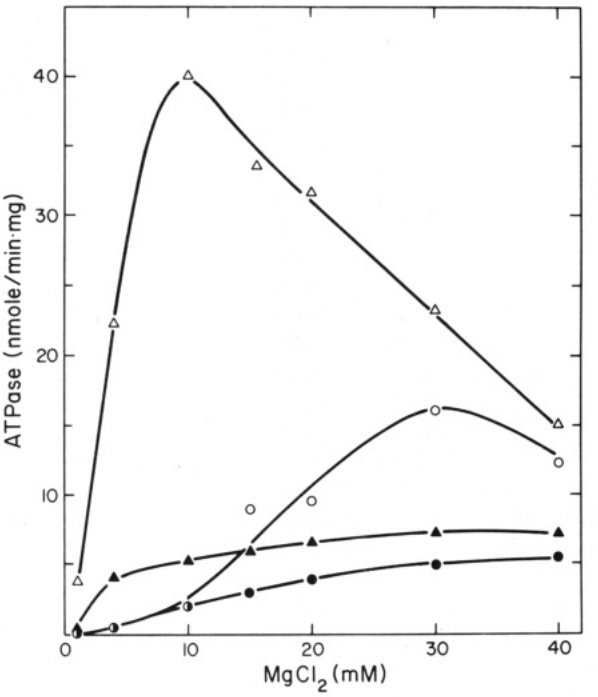


FIGURE 3: $MgCl_2$ dependence of actin-activated ATPase activity of gizzard myosin. Preincubation of myosin (0.5 mg/mL) at different $MgCl_2$ concentrations (1–40 mM) in the absence of tropomyosin was as described in Figure 1. $Mg^{2+}$ dependence of ATP hydrolysis is shown for phosphorylated (△, ▲) and dephosphorylated (○, ●) myosins in the absence (●, ▲) and presence of skeletal muscle F-actin [1 mg/mL (○, △)].

constant during the ATPase assay. In the absence of $Ca^{2+}$ during the preincubation period and at all $Mg^{2+}$ concentrations, myosin phosphorylation was not detected. The actin-activated ATPase activity of phosphorylated myosin shows a marked dependence on the added $MgCl_2$ concentration, and concentrations of free $Mg^{2+}$ in the millimolar range (~5 mM) are required for maximum activity. The same trend is observed in the presence of tropomyosin although the ATPase activities are increased (Figure 1). These results are similar to those reported earlier (see the introduction) and confirm that relatively high concentrations of free $Mg^{2+}$ are required for maximum actomyosin ATPase activity.

The increase in ATPase activity is not due merely to an increase in ionic strength since the actin-activated ATPase activity of phosphorylated myosin (1.8 mol of P/mol of myosin) in 2 mM $MgCl_2$ (other conditions identical with those used for Figure 1) is not activated over a range of KCl solutions from 85 to 135 mM.

To test whether the variations in ATPase activity might be related to the conformation of myosin, sedimentation patterns of phosphorylated myosin were determined under solvent conditions identical with those used for the enzyme assays. As shown in Figure 2, the major component at low concentrations of $MgCl_2$ is the 10S or folded myosin species. As the $MgCl_2$ concentration is increased, the area of the 10S boundary decreases with a corresponding increase in a more rapidly sedimenting species characteristic of myosin aggregates. At 1 and 2 mM added $MgCl_2$ a relatively small myosin polymer is formed [see Ikebe et al. (1983a)] and at higher $MgCl_2$ levels only large aggregates are observed. From a comparison of Figures 1 and 2 it is apparent that the proportion of 10S myosin is inversely related to the level of actin-activated ATPase activity. It should also be emphasized that despite the tendency of phosphorylation to stabilize the 6S conformation the 10S species can be formed with phosphorylated myosin.

*Effects of Higher Concentrations of $MgCl_2$ (Up to 40 mM).* The effect of increasing $MgCl_2$ concentrations up to 40 mM on the $Mg^{2+}$-ATPase activity of myosin and actomyosin is shown in Figure 3. The experimental design was similar to that used in Figure 1, in that myosin was preincubated in the presence or absence of $Ca^{2+}$ with myosin light chain kinase and calmodulin and the ATPase activities were monitored following the addition of actin or in the absence of actin over a fixed time period. For the actin-activated ATPase of phosphorylated myosin, there is an initial increase (as shown also in Figure 1) followed by a gradual decrease in activity. The inhibition of activity over the range 10–40 mM $MgCl_2$ is not due entirely to increased ionic strength, since equivalent concentrations of KCl caused a much smaller decrease. The tendency of actomyosin to form large aggregates at higher $MgCl_2$ concentrations may be partly responsible for the loss of activity. The interesting point of Figure 3 is illustrated by the actin-activated ATPase activity of dephosphorylated myosin. At concentrations of $MgCl_2$ below 10 mM there is virtually no actin-activated activity and the level of ATP hydrolysis is equivalent to that of dephosphorylated myosin in the absence of actin. At higher concentrations of $MgCl_2$ (10–30 mM) there is an increase in the level of actin-activated hydrolysis, followed by a gradual decline (30–40 mM) that parallels that observed with phosphorylated myosin. Myosin light chain kinase is not activated by these concentrations of

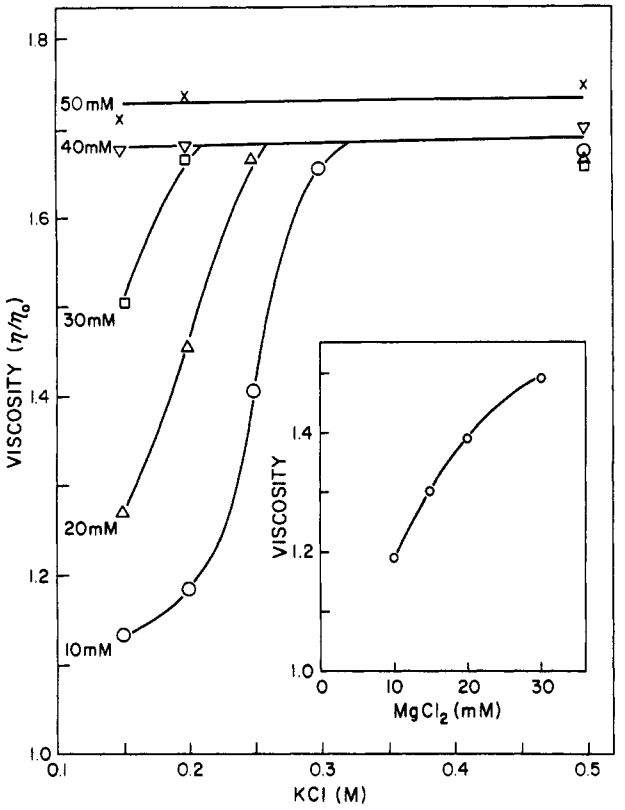


FIGURE 4: Relative viscosity of dephosphorylated myosin at various $MgCl_2$ concentrations. Viscosity was measured as outlined under Materials and Methods. Conditions: 2 mg/mL myosin, 1 mM ATP, 30 mM Tris-HCl (pH 7.5), varying concentrations of KCl, and the following $MgCl_2$ concentrations, 10 (○), 20 (△), 30 (□), 40 (▽), and 50 mM (×). In the inset the same levels of $MgCl_2$ were used but the ionic strength was maintained at ~0.23 by adjusting the KCl concentrations.

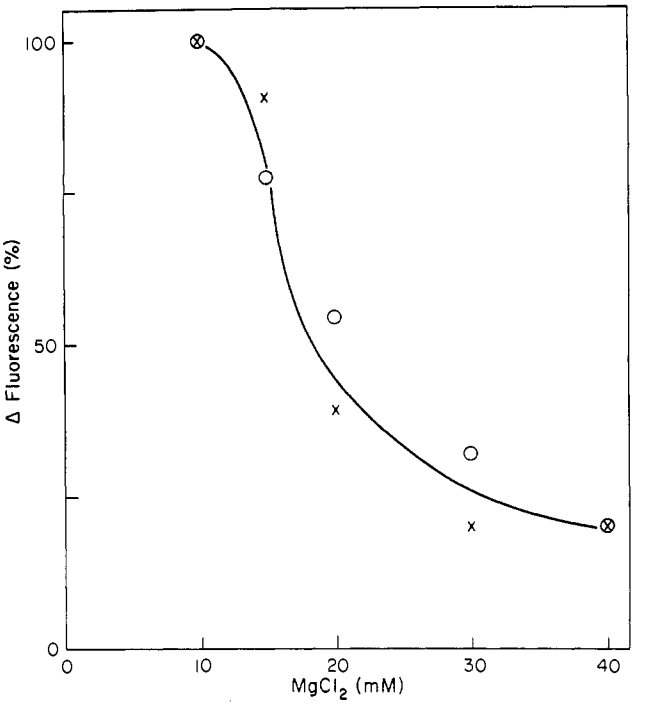


FIGURE 5: Effect of $MgCl_2$ on the ATP-induced intrinsic tryptophan fluorescence of myosin. Fluorescence was monitored by two procedures: (1) Increasing $MgCl_2$ concentrations (10–40 mM) were added to dephosphorylated myosin (0.5 mg/mL) in 0.15 M KCl, 0.1 mM ATP, 1 mM EGTA, and 30 mM Tris-HCl (pH 7.5). The fluorescence change (○) is recorded as a percentage of the change associated with the complete 10S to 6S transition (achieved by the addition of KCl to 0.3 M with dephosphorylated myosin in 10 mM $MgCl_2$ and other solvent conditions as given above). (2) The fluorescence change between dephosphorylated and phosphorylated myosin is recorded at different $MgCl_2$ concentrations (×). Initially the ATP-induced fluorescence of dephosphorylated myosin was measured in 0.15 M KCl, 1 mM ATP, 30 mM Tris-HCl (pH 8.5), and various levels of $MgCl_2$. (At 10 mM $MgCl_2$ dephosphorylated myosin is in the 10S conformation.) The myosin was then phosphorylated to form the 6S species by using the conditions given in Figure 2 (except that a pH of 8.5 was used to prevent aggregation) and the fluorescence level (now of the 6S conformation) again measured. The change in fluorescence intensity induced by $MgCl_2$ relative to the fluorescence change associated with the 10S to 6S transition is plotted for various concentrations of $MgCl_2$.

$MgCl_2$ in the absence of $Ca^{2+}$, and therefore the increase in ATPase activity is not due to myosin phosphorylation. The $Mg^{2+}$-ATPase activity of myosin alone, both phosphorylated and dephosphorylated (Figure 3), shows a gradual increase but not as marked as the actin-activated activity. Thus, it can be concluded that the level of actin-activated ATPase activity of dephosphorylated myosin can be modified by alterations in the $Mg^{2+}$ concentration.

An increase in KCl concentration to 200 mM, at fixed $MgCl_2$ concentration (10 mM), does not activate ATPase activity. However, higher concentrations of KCl up to 300 mM do induce a slight (approximately 40%) increase in the level of actin-activated ATPase activity.

Some of the data shown in Figure 3 can be explained from previous results (Ikebe et al., 1983a); i.e., the difference in the $Mg^{2+}$-ATPase activities (in the absence of actin) of phosphorylated and dephosphorylated myosin at $MgCl_2$ concentrations of 1–10 mM reflects predominantly the distribution of the 6S and 10S conformations (see also Figure 1). Whether or not the activation occurring at higher $MgCl_2$ concentrations is due also to a conformational transition is addressed in the following experiments. Initially we investigated the effect of increasing $MgCl_2$ concentrations on the KCl dependence of the viscosity of dephosphorylated myosin (Figure 4). As shown previously (Ikebe et al., 1983a), the relative viscosity of dephosphorylated myosin at 10 mM $MgCl_2$ is reduced markedly at KCl concentrations below 0.3 M, and this is due to the transition of the 6S to the 10S species. Increasing $MgCl_2$ concentrations increased $\eta_{rel}$ and favor the formation of 6S. The sedimentation pattern of dephosphorylated myosin in 40 mM $MgCl_2$ showed only one boundary characteristic of 6S myosin. For the data shown in Figure 4 polymeric myosin was not observed in sedimentation velocity experiments. However, for $MgCl_2$ concentrations above 20 mM and at KCl concentrations below 0.15 M, visible aggregates were formed.

Variations in ionic strength associated with the different $MgCl_2$ levels obviously influence the viscosity data, but the increased viscosity observed in Figure 4 cannot be explained solely on the basis of increasing ionic strength. If the data are considered as a function of total ionic strength, the tendency to form 6S myosin at higher $MgCl_2$ concentrations is still apparent. This is illustrated in the inset of Figure 4. Viscosity was determined at increasing $MgCl_2$ concentrations but at constant ionic strength (~0.23), and again the $Mg^{2+}$ dependence of viscosity is apparent.

The ATP-induced fluorescence of smooth muscle myosin also is sensitive to conformation (Ikebe et al., 1983b), and the $MgCl_2$ dependence of the change in fluorescence is shown in Figure 5. Data are plotted as a percentage of the 10S to 6S fluorescence change. Two experimental procedures were followed: (1) The ATP-induced fluorescence of dephosphorylated myosin in the 10S state (see figure legend for conditions) was monitored at increasing $MgCl_2$ concentrations. As the $MgCl_2$ level increased, the ATP-induced fluorescence approached that characterizing the 6S state. (2) The

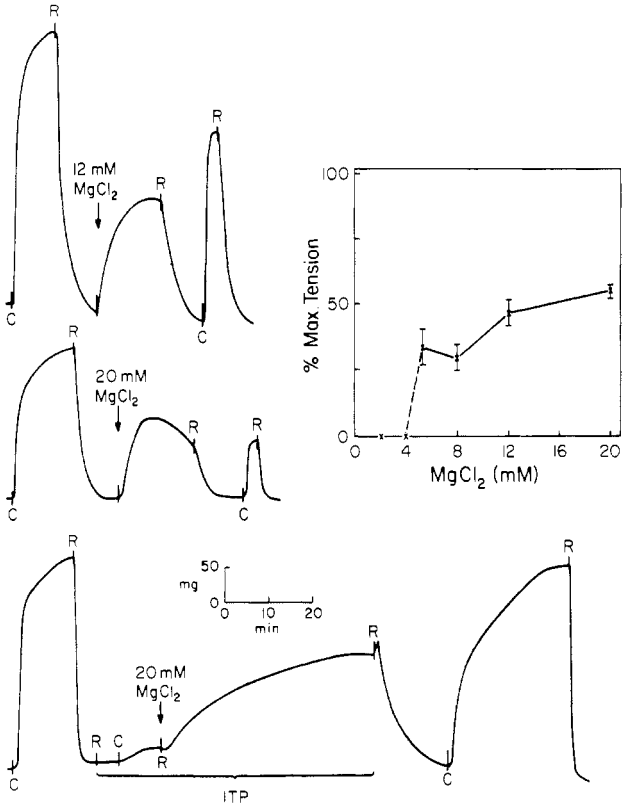


FIGURE 6: Effects of $MgCl_2$ on tension development in glycerinated chicken gizzard fibers. Details of the fiber preparation and the composition of various solutions are given under Materials and Methods. The upper and middle traces show tension induced by ATP and $Ca^{2+}$ (solution C) followed by relaxation in the absence of $Ca^{2+}$ (solution R). In the relaxing solution, i.e., the presence of EGTA, the total $MgCl_2$ concentration is increased to 12 mM (upper) and 20 mM (middle) and the subsequent development of tension recorded. Reduction of the $MgCl_2$ concentration to that normally used in the relaxation solution (at R) causes relaxation. A second control contraction–relaxation cycle is shown to assess the damage incurred by exposure to high $MgCl_2$ levels. In the inset the relationship between tension and total $MgCl_2$ over a range of concentrations is shown. The degree of $MgCl_2$-induced tension is given as a fraction of the $Ca^{2+}$-induced tension in the first contraction. The symbols represent the means of at least six different fibers ± SEM. The lower trace shows a control contraction–relaxation cycle and the substitution of ITP for ATP (2 mM) in solutions R and C. In the relaxing solution plus ITP, the concentration of total $MgCl_2$ is increased to 20 mM and tension is developed. Substitution by normal relaxing solution causes relaxation. A second control cycle of contraction and relaxation is also shown.

fluorescence of dephosphorylated myosin at various concentrations of $MgCl_2$ was recorded, and myosin light chain kinase, calmodulin, and $Ca^{2+}$ were then added to achieve complete phosphorylation. This resulted in the formation of 6S myosin. The difference between the initial level of fluorescence for the dephosphorylated myosin and the final level of fluorescence for the phosphorylated myosin is shown in Figure 5. The values obtained by this procedure are identical with those obtained by the more direct initial approach and indicate that increasing concentrations of $MgCl_2$ favor the formation of 6S, as judged by intrinsic tryptophan fluorescence.

*Effect of $MgCl_2$ on Skinned Smooth Muscle Fibers.* Figure 6 shows the influence of different concentrations of $MgCl_2$ on the tension developed with glycerol-treated smooth muscle fibers. In each of the tension traces an initial control contraction–relaxation cycle is shown. The symbols C and R refer to "contracting" and "relaxing" solutions containing $Ca^{2+}$ and EGTA, respectively (see Materials and Methods). In the upper trace the fiber was immersed in relaxing solution containing 12 mM $MgCl_2$ (added concentration) and tension is developed, although at a slower rate than in $Ca^{2+}$-induced contractions. Reduction of the $MgCl_2$ level to that used in the relaxing solution, i.e., 2 mM, results in relaxation. A similar series of experiments at 20 mM added $MgCl_2$ is shown in the middle trace. This tension trace illustrates another point that was consistently observed in these experiments; i.e., at higher levels of $MgCl_2$ the developed tension is not stable and shows a measurable decay. The decay is irreversible, and subsequent contractions in control media attain only the reduced level of tension and not the original tension (Figure 6). In general, the response of different fiber preparations was variable at lower $MgCl_2$ concentrations ($\leq$ 8 mM) and fibers stored in glycerol for less than 3 weeks were more reproducible. Tension is not developed at high $MgCl_2$ concentrations in the absence of ATP. The contractions induced by $MgCl_2$ are not merely a consequence of increasing ionic strength since the addition of KCl to 300 mM in relaxing solution does not cause tension development. (In the presence of $Ca^{2+}$ the level and rate of tension development are not influenced over the range of KCl concentrations from 0 to 150 mM.) When the contraction induced by $MgCl_2$ is maximum (e.g., 12–20 mM $MgCl_2$), the addition of KCl to 125 mM at peak isometric tension causes an irreversible loss in tension. However, the addition of KCl to 125 mM to submaximum $Mg^{2+}$-induced contractions (e.g., at 6–8 mM $MgCl_2$) causes an increase in the extent of isometric tension.

The level of myosin phosphorylation was determined at different phases of the control and $Mg^{2+}$-induced contractions. For the control contraction at peak tension, the extent of myosin light chain phosphorylation was 50 ± 9% ($n$ = 8); at rest the phosphorylation level was 9 ± 3% ($n$ = 5), and for the $Mg^{2+}$ contraction determined at peak tension for 20 mM $MgCl_2$ was 9 ± 4% ($n$ = 6). Thus, the $Mg^{2+}$ contractions occur in the absence of myosin phosphorylation, which is consistent with the observation that myosin light chain kinase is not activated by elevated $MgCl_2$ levels in the absence of $Ca^{2+}$. The lower tension trace in Figure 6 also provides evidence that the $Mg^{2+}$ contractions occur without myosin phosphorylation. After the control, contraction–relaxation cycle the fiber was washed sequentially in relaxing and contracting solutions in which ITP was substituted for ATP. It is known that in the absence of myosin phosphorylation ITP will not support tension in skinned smooth muscle fibers (Cassidy & Kerrick, 1982) and that ITP is not a substrate of myosin light chain kinase (Pires & Perry, 1977). Thus, with the usual contraction solution only slight tension is developed with ITP, and this probably reflects a slight but significant level of myosin phosphorylation (presumably close to 9% as quoted above). However, when the $MgCl_2$ concentration of the relaxing solution is raised to 20 mM, a considerably larger and relatively slow increase in tension occurs similar to that shown previously by Watanabe (1980). Relaxation of the fiber follows when the $MgCl_2$ level is reduced. Some damage to the contractile apparatus is incurred by the treatment with 20 mM $MgCl_2$ since the subsequent contraction in ATP-containing solution shows an unusual slow phase of tension development (Figure 6).

## Discussion

The previous results are best considered under two categories, i.e., the effects of lower concentrations ($\leq$10 mM) and the effects of higher concentrations of $MgCl_2$ (10–40 mM). In the former category the unusual requirement (see the introduction) of smooth muscle actomyosin for excess $Mg^{2+}$ levels (i.e., in excess of the ATP concentration) is addressed.

This effect is not associated with the phosphorylating system since myosin light chain kinase does not require high $Mg^{2+}$ levels (Hartshorne et al., 1980) and also the effect can be demonstrated with fully phosphorylated myosin (Figure 1). It is therefore restricted to either myosin or actin, and since skeletal muscle actin was used and the effect is not observed with skeletal muscle actomyosin, the former is the more likely candidate. Previously it was shown (Ikebe et al., 1983a) that at free $Mg^{2+}$ concentrations in the range of approximately 0.1–1 mM phosphorylated myosin can form the 10S conformation under appropriate solvent conditions. This observation is confirmed in the present paper, and further it is shown (Figure 2) that as the $MgCl_2$ concentration is increased the proportion of 10S decreases. A more interesting correlation, however, is that the proportion of 10S has an inverse relationship to the level of actin-activated ATPase activity, suggesting that 10S phosphorylated myosin is not activated appreciably by actin. The distribution of 10S myosin as a function of the $MgCl_2$ concentration offers a reasonable explanation for the requirement of smooth muscle actomyosin for relatively high levels of $Mg^{2+}$. Further, it is apparent that if any of the other assay conditions (e.g., solvent or added proteins) favor the 10S state, then these may yield a reduced actomyosin ATPase activity that need not be proportional to the degree of myosin phosphorylation. Suzuki et al. (1981) found that superprecipitation of acto-phosphorylated myosin required relatively high concentrations of $MgCl_2$, which was related to thick filament formation, but that the actin-activated ATPase activity of phosphorylated myosin was optimal at equimolar $MgCl_2$ and ATP. With regard to the latter point, it should be noted that the $Mg^{2+}$ sensitivity (i.e., the dependence of ATPase activity of acto-phosphorylated myosin on relatively high concentrations of $MgCl_2$; see Figure 1) of actomyosin is very susceptible to proteolysis and is not apparent with degraded myosin or with HMM (M. Ikebe and D. J. Hartshorne, unpublished observations).

If the 10S myosin conformation represents an "inhibited" form, then activation of ATPase activity must be accompanied by the formation of an "active" conformation. The simplest explanation is that the inactive–active transformation reflects the 10S to 6S conformational change. However, this relationship is not established since 6S myosin easily aggregates (possibly forming filaments). The increase of actin-activated activity therefore could be a consequence of either the 6S formation or subsequent myosin aggregation. The importance of filament formation for superprecipitation and also as a prerequisite for smooth muscle contraction is emphasized by Suzuki et al. (1982b). Dephosphorylated 10S myosin will aggregate at low ionic strength without an increase in actin-activated ATPase activity, and it is unlikely that aggregation alone is the explanation. In addition, filamentous myosin exists in an inactive state in relaxed smooth muscle (Somlyo et al., 1981). Thus, it is reasonable to propose that the conversion from an inactive to an active form follows some conformational change in myosin, although the assembly into myosin filaments may impose further modifications on enzymatic properties.

In the preceding discussion the effects of $MgCl_2$ were focused on the 10S to 6S transition of phosphorylated myosin. In the secondary category the effects of higher levels of $MgCl_2$ are concerned with the 10S to 6S transition of dephosphorylated myosin. Increasing concentrations of $MgCl_2$ will induce an activation of the actin-activated ATPase of dephosphorylated myosin (Figure 3). This effect is not due to increasing ionic strength since ionic equivalents of KCl do not activate enzymatic activity. A possible explanation for the activation is that the increased levels of $MgCl_2$ achieve a conversion of the 10S form of dephosphorylated myosin to the 6S species, and this is supported by the viscosity (Figure 4) and fluorescence (Figure 5) data. Myosin aggregation is promoted at these high levels of $MgCl_2$, and again the previous uncertainty is raised, namely, whether 6S formation per se or its subsequent aggregation is responsible for activation of ATPase activity.

The experiments with the skinned smooth muscle fibers suggest that even in filamentous myosin the conformation of dephosphorylated myosin may be altered by changing the $MgCl_2$ concentrations. The activation of the intact contractile system occurs at lower $MgCl_2$ levels than the activation of the actomyosin, and this difference may reflect an altered sensitivity to $MgCl_2$ for the two systems. In support of this it is known that smooth muscle skinned fibers differ from actomyosin in that maximum tension development does not require the high levels of free $Mg^{2+}$ that characterize the in vitro enzymatic properties (Endo et al., 1977; Gordon, 1978). Thus, allowing for a shift in the $MgCl_2$ sensitivities, it is possible that both the activation of ATPase activity and the tension increase reflect the same conformational transition of myosin.

It was shown previously that the $Mg^{2+}$-, $Ca^{2+}$- and $K^+$-EDTA-ATPase activities for 10S and 6S myosins were distinct (Ikebe et al., 1983a), and it was suggested that the 10S to 6S transition, or its equivalent in filamentous myosin, could be involved in the regulation of the contractile apparatus in smooth muscle. This hypothesis was based on indirect evidence since no data were available for the relevant enzymatic activity, i.e., the actin-activated ATPase activity of myosin. In this paper some direct evidence is presented that supports the general hypothesis. Most of the evidence used to develop the relationship between myosin shape and enzymatic activity was obtained with monomeric myosin, because of technical reasons. Thus, it should be emphasized that although the enzymatic properties of myosin correlated closely with the gross conformational transition, the determinant of ATPase activity could be an associated but more subtle conformational change. It is unlikely that the 10S to 6S transition occurs in filamentous myosin although some component(s) of this transition may be allowed in the more restricted environment of the myosin filament.

The situation with smooth muscle HMM poses an interesting problem. It is known that the actin-activated ATPase activity of HMM is regulated by phosphorylation (Ikebe et al., 1982; Sellers et al., 1981), and it is obvious that HMM cannot form a looped structure since the tail portion of the myosin molecule is missing. Thus, it appears that the properties of HMM do not fit a simple 6S (active) to 10S (inactive) scheme. It has been shown, however, that the sedimentation velocity and $Mg^{2+}$-ATPase activity of HMM vary with KCl concentration (at levels similar to those used to demonstrate the 6S to 10S transition with myosin), and these variations were attributed to a conformational change (Suzuki et al., 1982a). It is possible, therefore, that some component of the 6S to 10S transition is preserved in HMM. If this supposition is correct, then it would indicate that in myosin the formation and release of the looped structure is not the critical event but rather that an associated and more subtle conformational change is responsible for the alteration of enzymatic activity.

With the above qualifications in mind, the effects of $MgCl_2$ on the gross conformation of myosin may be discussed. The correlation of reduced ATPase activity with the 10S conformation suggests that the folded form of myosin, or some conformational equivalent, represents the "inhibited" state. In

the in vivo state activation of the contractile apparatus is achieved by phosphorylation of myosin, and it has been shown that under defined solvent conditions phosphorylation favors the 6S conformation (Trybus et al., 1982; Craig et al., 1983; Ikebe et al., 1983a; Onishi et al., 1983). High $MgCl_2$ concentrations will also activate the contractile system in the absence of phosphorylation and will promote the formation of 6S myosin. Thus, elevated $MgCl_2$ levels can mimic to some extent the effects of phosphorylation, and the common effect is the formation of 6S myosin. The simplest relationship, therefore, is that 10S represents an inactive form of myosin, presumably present in relaxed muscle, and that the formation of 6S, or equivalent, results in the activation of the contractile apparatus. This is undoubtedly an oversimplification of the real situation, and the extent to which this hypothesis is valid remains to be established.

**Registry No.** Mg, 7439-95-4; ATPase, 9000-83-3.

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