*Proc. Natl. Acad. Sci. USA*
Vol. 79, pp. 292–296, January 1982
Biochemistry

# Regulation of actin-activated ATP hydrolysis by arterial myosin

**(phosphorylation/myosin ATPase/$Ca^{2+}$ binding/myosin-mediated regulation)**

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*Communicated by Peter C. Nowell, September 17, 1981*

ABSTRACT **Myosin was isolated from the main pulmonary artery of swine and was phosphorylated or dephosphorylated by utilizing the endogenous kinase or phosphatase, respectively. The myosins, phosphorylated to various degrees, were purified free of kinase and phosphatase activities by gel filtration on Sepharose CL-4B agarose columns. The level of actin-activated ATPase activity was dependent upon the degree of myosin light chain phosphorylation. Fully phosphorylated myosin reconstituted with actin and tropomyosin (actin/tropomyosin = 6:1) had the highest ATPase activity (0.1 μmol of $P_i$/mg·min). The actin-activated ATPase activity showed maximal (60–65%) $Ca^{2+}$ sensitivity at 2 mol of $Ca^{2+}$ bound per mol of myosin. The actin-activated ATPase activity, $Ca^{2+}$ binding, and $Ca^{2+}$ sensitivity of arterial myosin were also dependent upon $Mg^{2+}$ concentration. The ATPase activity was maximal at 2–3 mM $Mg^{2+}$ and, at low (0.5 mM) $Mg^{2+}$ concentration, the activity was only one-third of the maximal activity. Increasing the $Mg^{2+}$ above 3 mM was not associated with a further increase in ATPase activity, but the $Ca^{2+}$ binding and $Ca^{2+}$ sensitivity decreased with increasing $Mg^{2+}$ concentration. The maximal $Ca^{2+}$ sensitivity was observed at 2–3 mM $Mg^{2+}$, a concentration at which the myosin bound 2 mol of $Ca^{2+}$/mol. Both the ATPase activity and the $Ca^{2+}$ sensitivity were more remarkable when actin that contained tropomyosin was used to activate the ATPase activity. The data indicate that calcium regulates the actin-activated ATP hydrolysis not only by its effects on the phosphorylation system but also by direct binding to the myosin.**

Phosphorylation of myosin is correlated with actomyosin ATPase in gizzard (1, 2), vas deferens (3), and in porcine and bovine stomach (4, 5). Using purified myosins in phosphorylated and unphosphorylated states, Chacko *et al.* (6) showed that the myosin ATPase measured at high ionic strength (0.5 M KCl) is the same for phosphorylated and unphosphorylated myosin. However, the actin-activated ATPase activity measured under physiological conditions is severalfold higher for phosphorylated myosin. This agrees with experiments in which platelet myosin was used (7). The degree of actin-activated ATP hydrolysis by myosin isolated from mammalian smooth muscle is linearly correlated with the amount of phosphate covalently bound to the 20,000-dalton light chain (5). Proponents of the phosphorylation mechanism agree that $Ca^{2+}$ activates actomyosin by its effect on $Ca^{2+}$-dependent kinase. Kerrick and co-workers (8), using skinned fiber preparations from avian gizzard and rabbit ileum to determine the relationship between phosphorylation and muscle tension, showed that the light chain became phosphorylated when tension was developed by the muscle strips. A relationship between phosphorylation and development of tension has also been reported when intact smooth muscle strips were used (9–11). The phosphorylation and tension development were correlated linearly by using hog carotid (11).

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In contrast to findings that the phosphorylation is correlated with actomyosin ATPase, Ebashi and co-workers (12) observed that the ATPase activity of myosin was activated by actin in the presence of $Ca^{2+}$ when the actomyosin was mixed with tropomyosin and leiotonins (leiotonin A, $M_r$ 100,000; leiotonin C, $M_r$ 18,000). Furthermore, relaxation of the muscle strip was not correlated with dephosphorylation (13). Alternatively, Butler and Siegman (14) observed correlation between dephosphorylation and cross-bridge cycling during relaxation.

Using competition experiments of Szent-Györgyi (15), Frederiksen (16) observed that the $Ca^{2+}$ regulation of actomyosin extracted from swine aorta was mediated by myosin. Similar observations were made by using actomyosin and acto-heavy meromyosin prepared from swine carotid artery (17). Neither the kinase activity nor the state of phosphorylation of the light chain was known in these studies. Hence, it is unclear whether the $Ca^{2+}$ sensitivity observed by Mrwa and Ruegg (17) is due to a direct effect of $Ca^{2+}$ on myosin—similar to that observed for invertebrate muscle (18)—or due to the activation of the phosphorylation system (2, 4, 5). It is also possible that these mechanisms may coexist in some smooth muscles. Using purified phosphorylated and unphosphorylated myosins from guinea pig vas deferens, Chacko *et al.* (6) showed that although phosphorylation is a prerequisite for activation of myosin ATPase by actin, $Ca^{2+}$ increases this activation of the ATPase even after phosphorylation. Rees and Frederiksen (19) recently reported that the actin-activation of phosphorylated aortic myosin was maximal in the presence of $Ca^{2+}$. Previous studies (6, 19) that reported $Ca^{2+}$ sensitivity of smooth muscle myosin do not show if the sensitivity is associated with $Ca^{2+}$ binding by myosin.

In this report we show that the actin-activated ATPase activity of myosin isolated from swine pulmonary artery is linearly correlated with the degree of myosin light chain phosphorylation. We also demonstrate that when myosin binds 2 mol of $Ca^{2+}$/mol, the actomyosin ATPase expresses the maximal $Ca^{2+}$ sensitivity. Furthermore, the actin-activated ATPase activity, $Ca^{2+}$ binding, and $Ca^{2+}$ sensitivity of arterial myosin depend on $Mg^{2+}$ concentration.

## EXPERIMENTAL PROCEDURE

**Preparation of Phosphorylated and Unphosphorylated Myosin.** Actomyosin that contained endogenous kinase and phosphatase was prepared from the main pulmonary arteries of swine by low-salt extraction followed by ammonium sulfate fractionation as described (5, 6). The times required for maximal phosphorylation and dephosphorylation were determined by using [$\gamma$-$^{32}$P]ATP (5). Myosins that were phosphorylated to various degrees were purified from other proteins by gel filtration on Sepharose CL-4B agarose columns equilibrated and eluted with 0.8 M KCl/1 mM EDTA/20 mM Tris·HCl, pH 7.5/2.5 mM dithiothreitol. The samples were made 10 mM with respect to MgATP prior to application to the columns. Dephosphoryl-

ated myosin was mixed with 1 mM EGTA before adding ATP. Fractions (5 ml) were collected at a flow rate of 30 ml/hr from 2.5 × 100 cm columns. The myosin peak was located by ATPase assay (6) and fractions from the peak were pooled. The myosin peak was routinely tested for the absence of kinase and phosphatase activities (5).

**Estimation of the Degree of Phosphorylation.** The amount of phosphate bound to the light chain of myosin, phosphorylated with [$\gamma$-$^{32}$P]ATP, was determined by elution of the radioactivity (6) from the 20,000-dalton light chain which was separated by $NaDodSO_4$/polyacrylamide gel electrophoresis (20). Myosin phosphorylated with nonradioactive ATP was subjected to electrophoresis on vertical slab gels of 10% polyacrylamide with 20 mM Tris/122 mM glycine buffer (pH 8.6) containing 8 M urea (21). A microgel electrophoresis unit (designed by Y. Nonomura and S. Ebashi) was utilized to minimize the amount of myosin required to observe the light chain. The phosphorylated light chain migrated faster than did the unphosphorylated light chain in this gel system. The gels were stained with Coomassie brilliant blue R and were scanned at 584 nm with a gel scanner-integrator (Transidyne, Ann Arbor, MI). The percent of light chain phosphorylation was obtained from areas under the fast migrating (phosphorylated) and slow migrating (unphosphorylated) bands on the urea gel.

**ATPase Assay.** The $K_2EDTA$ and $Ca^{2+}$-activated myosin ATPase activities were determined in 0.5 M KCl/20 mM Tris·HCl, pH 7.5/2 mM ATP and either 2 mM EDTA or 10 mM $CaCl_2$ with a myosin concentration of 15 $\mu$g/ml. Actin-activated ATPase activity was assayed in the presence of 10 mM imidazole·HCl, pH 7.1/2 mM ATP/15 mM KCl/6 mM $MgCl_2$ (except in experiments in which $Mg^{2+}$ concentration was varied) and either 0.1 mM $CaCl_2$ or 2 mM EGTA. A CaEGTA buffer (22) was used for experiments in which $Ca^{2+}$ concentration was varied. The protein concentrations used, unless indicated otherwise, were: myosin, 0.14 mg/ml; rabbit skeletal muscle actin, 0.25 mg/ml; tropomyosin, 0.07 mg/ml. Samples were incubated at 37°C and aliquots were removed at zero time and at two additional times to ascertain linearity of phosphate release. $P_i$ was measured according to Martin and Doty (23).

**Preparation of Other Proteins.** Rabbit skeletal muscle actin was prepared according to Spudich and Watt (24). Tropomyosin was extracted from the alcohol/ether powder (25) of the residue after myosin extraction by using 1 M KCl/20 mM imidazole·HCl, pH 7.0/5 mM dithiothreitol. Tropomyosin was purified by isoelectric precipitation at pH 4.3 and ammonium sulfate fractionation (40–65% saturation) (25). All proteins were assayed for biological activity by monitoring their effect on ATPase activity of purified skeletal muscle myosin. They were all free of kinase and phosphatase activity and other calcium regulatory proteins.

Protein concentrations were determined (26) with bovine serum albumin as a standard.

**Measurements of $Ca^{2+}$ Binding.** All solutions used for $Ca^{2+}$ binding were analyzed by atomic absorption spectrometry using internal standards. The CaEGTA buffer was made using a value for the dissociation constant of $1.9 \times 10^{-7}$ M at pH 7.0 (27). $Ca^{2+}$ binding by myosin was determined according to Chantler *et al.* (28). The pooled column fractions that contained pure myosin were dialyzed for 5 hr against 10 mM imidazole·HCl, pH 7.0/10 mM $MgCl_2$/2.5 mM dithiothreitol. The myosin, a fine silky precipitate after dialysis, was sedimented by centrifugation (48,000 × *g*, 15 min), resuspended in 0.5 M KCl/1 mM EDTA/1 mM EGTA/10 mM imidazole·HCl, pH 7.0/2.5 mM dithiothreitol, and dialyzed for 5 hr against the same buffer. The protein was then dialyzed for 5 hr against the above buffer without EGTA and EDTA. After dialysis the protein concentration was determined. Aliquots of protein were precipitated by dilution with buffer (10 mM imidazole·HCl, pH 7.0/2.5 mM dithiothreitol to obtain a KCl concentration of 40 mM. The precipitated protein was sedimented by centrifugation and then was resuspended in 40 mM KCl/10 mM imidazole·HCl, pH 7.0/2.5 mM dithiothreitol/0–8 mM $MgCl_2$ (based on the experiment) and CaEGTA buffer that contained $^{45}Ca$ and [$^3$H]glucose. The final count in each channel was 30,000 cpm/20 $\mu$l. The protein was then resedimented by centrifugation and the process was repeated; the final pellet was dissolved in 0.5 M KCl/10 mM imidazole·HCl, pH 7.0/2.5 mM dithiothreitol. Aliquots (0.05 ml) of the protein solution were transferred into scintillation vials, mixed with 10 ml of Ready-Solve HP (Beckman), and assayed in a liquid scintillation counter (Beckman 2000 LS).

Calcium bound to the myosin was determined according to Kendrick-Jones *et al.* (18). Supernatant trapped in the pellets was determined from the [$^3$H]glucose content. The mol of $Ca^{2+}$ bound per mol of myosin was calculated (myosin $M_r$ 470,000).

## RESULTS

**Effect of Light Chain Phosphorylation on Actin-Activation of Myosin ATPase Activity.** Myosins that were phosphorylated to various levels were purified free of kinase and phosphatase. The phosphate bound to the myosin was stable and was in the 20,000-dalton light chain. The level of light chain phosphorylation was determined either by elution of the $^{32}$P radioactivity from myosin phosphorylated with [$\gamma$-$^{32}$P]ATP or by urea gel electrophoresis. Fig. 1 shows the migration patterns of unphosphorylated and phosphorylated 20,000-dalton light chain on urea gels. In some experiments the degree of phosphorylation was estimated by both procedures; this was necessary because the phosphatase failed to completely dephosphorylate the myosin prior to phosphorylation with [$\gamma$-$^{32}$P]ATP. Estimation of the degree of phosphorylation based only on the $^{32}$P that was eluted from light chain band in $NaDodSO_4$/poly-

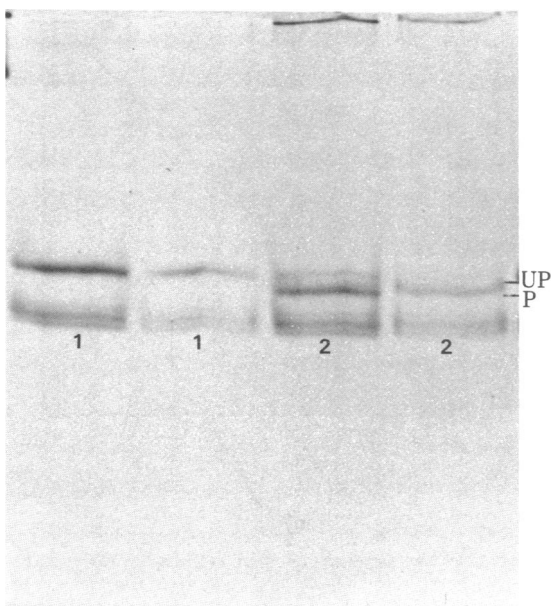


FIG. 1. Urea gels (different loadings) of unphosphorylated (lanes 1) and partially phosphorylated (lanes 2) 20,000-dalton light chain. The phosphorylated light chain (P) migrated faster than did the unphosphorylated light chain (UP). The myosin heavy chain did not enter the separating gel. The separation of 15,000-dalton light chain into multiple bands in this gel system was not related to phosphorylation (compare lanes 1 and 2). The nature of this phenomenon is unknown at present.

acrylamide gel gave a lower level of phosphorylation because it did not include the nonradioactive phosphate bound to the myosin prior to phosphorylation with [$\gamma$-$^{32}$P]ATP.

Fig. 2 shows the dependence of actin-activated ATPase activity on the percent of myosin phosphorylation. This activity was linearly correlated with the phosphate bound to the light chain. An ATPase activity of 0.1 $\mu$mol of $P_i$/mg of myosin per min was obtained when the myosin was fully phosphorylated.

The $K_2$EDTA-stimulated activities (in 0.5 M KCl) of myosins used (Fig. 2) were 1.2–1.5 $\mu$mol $P_i$/mg·min. The $Ca^{2+}$-activated ATPase activity was 0.5–0.65 $\mu$mol $P_i$/mg·min. A decrease in $K_2$EDTA-stimulated activity and an increase in $Ca^{2+}$-activated myosin ATPase activity was associated with a lower actin-activated ATPase activity even when the myosin was fully phosphorylated.

**Effect of $Mg^{2+}$ on Actin-Activation.** Fig. 3 shows the dependence of arterial myosin on $Mg^{2+}$ concentration for actin-activation. The ATPase activity was increased 4-fold by increasing the free $Mg^{2+}$ concentration from 0.5 mM to 3 mM. At higher $Mg^{2+}$ concentration (8 mM), the ATPase activity was only 3-fold higher than that observed at 0.5 mM. The state of phosphorylation was not altered during the ATPase assay, as determined from urea gel electrophorograms of aliquots of myosin removed from the enzyme assay mixture at the same time that samples were removed for $P_i$ determinations.

In another experiment ATPase assay was begun at 0.5 mM $Mg^{2+}$ and additional $Mg^{2+}$ was added every 5 min for 30 min (final $Mg^{2+}$ concentration, 10 mM). Aliquots for $P_i$ determinations were taken at each time before the $MgCl_2$ addition. The $P_i$ liberated increased as $Mg^{2+}$ was added to the incubation until the final $Mg^{2+}$ concentration reached 4 mM. The $Mg^{2+}$ effect is seen over a range (13–80 mM) of KCl concentrations.

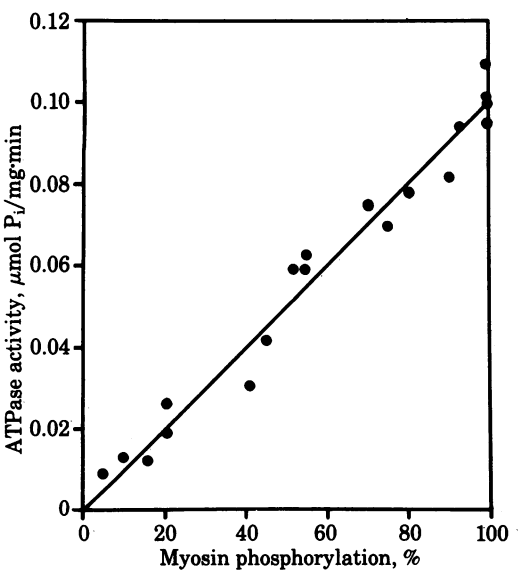


FIG. 2. Relationship between myosin light chain (20,000 daltons) and actin-activated ATPase activity. Myosins were phosphorylated or dephosphorylated by using the endogenous kinase or phosphatase, respectively, prior to separation of the myosin from these enzymes. The degree of phosphorylation was determined as described. Myosins that were phosphorylated to various degrees were reconstituted with rabbit skeletal muscle actin (myosin/actin molar ratio = 1:20). Actin was complexed with smooth muscle tropomyosin (tropomyosin/actin molar ratio = 1:6). Assay conditions were as described in text [myosin, 0.14 mg/ml; $CaCl_2$, 0.1 mM; $MgCl_2$, 4 mM (total); ATP, 2 mM]. The ATPase activity was correlated linearly with the degree of myosin phosphorylation. Fully phosphorylated (2 mol of $P_i$/mol of myosin) had the highest (0.1 $\mu$mol of $P_i$/mg·min) ATPase activity under the assay conditions.

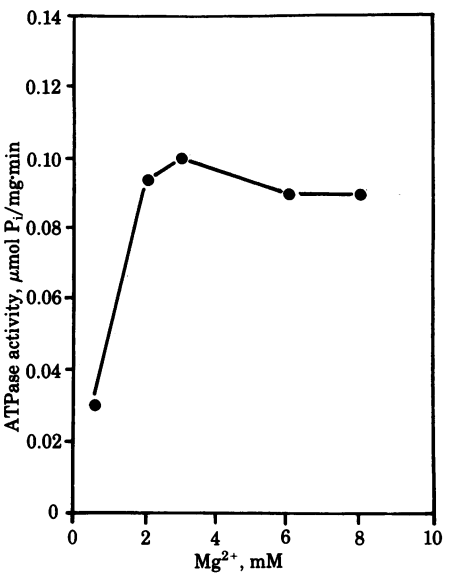


FIG. 3. Dependence of actin-activated ATP hydrolysis on $Mg^{2+}$. Assay conditions were as in Fig. 2. The $Ca^{2+}$ concentration was 0.01 mM. Fully phosphorylated myosin was reconstituted with actin complexed with tropomyosin. Molar ratios are myosin/actin = 1:20; tropomyosin/actin = 1:6. Free $Mg^{2+}$ concentration ranged from 0.5 mM to 8 mM. Notice that the ATPase activity is maximal at 3 mM $Mg^{2+}$.

**$Ca^{2+}$ Dependence of Actin-Activated ATP Hydrolysis.** Fig. 4 shows the effect of varying $Ca^{2+}$ on the actin-activated ATP hydrolysis. The ATPase activity was maximal at pCa 4, and a 60% inhibition was observed by lowering the $Ca^{2+}$ concentration (pCa 8). The $Ca^{2+}$ sensitivity of the ATPase activity was noticed both for myosin that was reconstituted with pure actin and for myosin that was reconstituted with actin complexed with tropomyosin. Both the actin-activated ATPase activity and the $Ca^{2+}$ sensitivity were more pronounced when actin was complexed with tropomyosin at an actin/tropomyosin molar ratio of 6:1.

Because the actomyosin ATPase of arterial myosin was dependent on $Mg^{2+}$ concentration, the $Ca^{2+}$ sensitivity was determined at various $Mg^{2+}$ concentrations. Fig. 5 shows the ef-

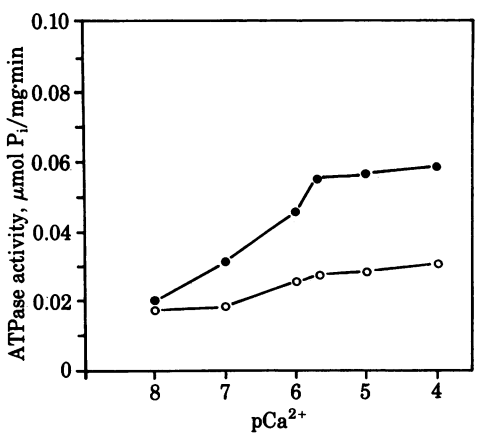


FIG. 4. Effect of varying $Ca^{2+}$ on actin-activated ATPase activity. Assay conditions were as in Fig. 2 except for $Ca^{2+}$ concentration which varied as indicated. The actin-activated ATPase activities of phosphorylated myosin (65% phosphorylated) reconstituted with pure actin (○) and actin complexed with turkey gizzard tropomyosin (●) are shown at various $Ca^{2+}$ concentrations. $Ca^{2+}$ sensitivity was observed in both instances; however, the ATPase activity and the $Ca^{2+}$ sensitivity were more remarkable in the presence of tropomyosin.

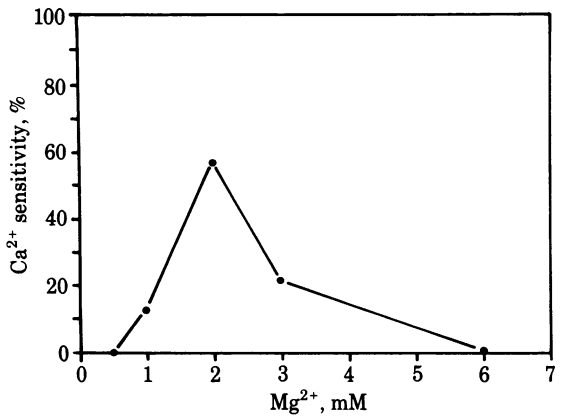


FIG. 5. Effect of $Mg^{2+}$ concentration on $Ca^{2+}$ sensitivity. Assay conditions were as in Fig. 2. The ATPase assay was done at pCa 8 or pCa 5 at various $Mg^{2+}$ concentrations. $Ca^{2+}$ sensitivity = [(ATPase activity at pCa 5 − ATPase activity at pCa 8)/ATPase activity at pCa 5] × 100. The $Ca^{2+}$ sensitivity increased until the free $Mg^{2+}$ concentration reached 2 mM. Further increase in $Mg^{2+}$ concentration decreased the $Ca^{2+}$ sensitivity. The ATPase activity was not $Ca^{2+}$ sensitive at 6 mM $Mg^{2+}$.

fect of $Mg^{2+}$ on the $Ca^{2+}$ sensitivity of actin-activated ATPase activity of arterial myosin. $Ca^{2+}$ sensitivity was maximal between 2 and 3 mM $Mg^{2+}$ and it disappeared when the free $Mg^{2+}$ concentration was increased to 6 mM.

**Effect of $Mg^{2+}$ Concentration on $Ca^{2+}$ Binding.** To determine whether the $Ca^{2+}$ sensitivity observed was associated with $Ca^{2+}$ binding to myosin, the $Ca^{2+}$ bound by the column-purified, phosphorylated myosin was determined at various $Mg^{2+}$ concentrations. Fig. 6 shows the mol of $Ca^{2+}$ bound per mol of myosin at $Mg^{2+}$ concentrations used for actin-activation. At 0 $Mg^{2+}$ concentration, 1 mol of myosin bound about 4 mol of $Ca^{2+}$. The myosin bound 2 mol of $Ca^{2+}$ per mol at $Mg^{2+}$ concentrations between 2 and 3 mM. The $Ca^{2+}$ binding decreased as the $Mg^{2+}$ concentration was increased.

Actin and tropomyosin did not alter the $Ca^{2+}$-binding characteristics of myosin because the myosin that was reconstituted with actin and tropomyosin also bound 2 mol of $Ca^{2+}$ per mol at 2–3 mM $Mg^{2+}$. Varying the KCl concentration from 40 mM to 100 mM also had no effect on $Ca^{2+}$ binding.

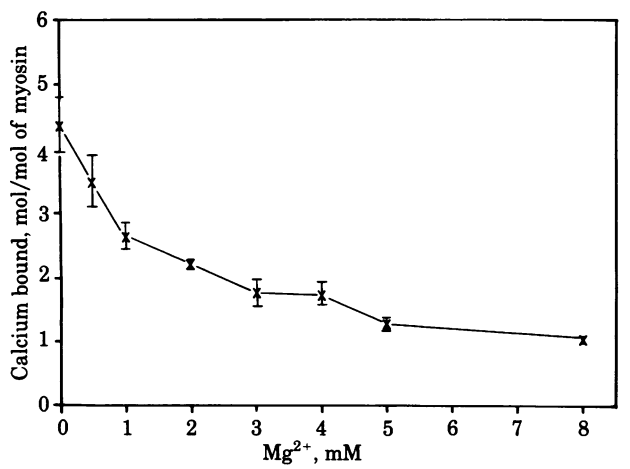


FIG. 6. Effect of $Mg^{2+}$ concentration on $Ca^{2+}$ binding: mol of $Ca^{2+}$ bound per mol of myosin at pCa 5 is shown at various $Mg^{2+}$ concentrations. At 0 $Mg^{2+}$ the myosin bound about 4 mol of $Ca^{2+}$. Myosin bound 2 mol of $Ca^{2+}$/mol of myosin at $Mg^{2+}$ concentrations between 2 and 3 mM. Notice that the $Ca^{2+}$ binding decreased as the $Mg^{2+}$ concentration was increased. Values are means ± SEM.

## DISCUSSION

The calcium dependence of myosin light chain kinase from arterial smooth muscle has been reported (19, 29–31). Data presented here extend previous observations (19, 31) that the phosphorylation of myosin light chain increases the actin-activated ATP hydrolysis by arterial myosin. Using myosin that was phosphorylated to various degrees, we showed that the actin-activated ATPase activity was linearly correlated with the phosphate, covalently bound to the myosin. Fully phosphorylated myosin (2 mol of phosphate per mol of myosin) that was reconstituted with rabbit skeletal muscle actin that contained tropomyosin had the highest ATPase activity. The tropomyosin potentiates the actin-activated ATPase activity (5, 6). The maximal activity was observed when the actin was complexed with tropomyosin at a molar ratio of 6:1 (actin/tropomyosin). Actin used for these experiments was complexed with turkey gizzard tropomyosin to obtain the maximal activity. In low salt (20 mM KCl), the fully phosphorylated arterial myosin that was reconstituted with actin (molar ratio of myosin/actin, 1:20) that contained tropomyosin had a lower activity (0.1 μmol of $P_i$/mg·min) than did myosins from mammalian visceral smooth muscles (0.25 μmol of $P_i$/mg·min) assayed under the same conditions (4–6). However, the $K_2EDTA$-stimulated, $Ca^{2+}$-activated, and $Mg^{2+}$-activated ATPase activities of arterial myosin assayed in 0.5 M KCl were the same as those of myosins isolated from other mammalian smooth muscles (3, 5).

The actin-activated ATPase activity of phosphorylated arterial myosin was dependent on $Mg^{2+}$ concentration. A similar effect on actin-activation of ATPase activity of *Acanthamoeba* myosin II caused by $Mg^{2+}$ has been reported (32). The $Mg^{2+}$ effect was observed over a range (13–80 mM) of KCl concentrations. When the KCl concentration was increased higher than 80 mM, the actin-activated ATP hydrolysis decreased, perhaps by dissociation of the actomyosin complex (33).

The $Ca^{2+}$ sensitivity and the $Ca^{2+}$-binding property of myosin were affected by $Mg^{2+}$ concentration. The maximal $Ca^{2+}$ sensitivity (65%) was observed at 2–3 mM $Mg^{2+}$, a concentration at which myosin bound 2 mol of $Ca^{2+}$ per mol. Subsequent increase in $Mg^{2+}$ caused a decrease in $Ca^{2+}$ binding and $Ca^{2+}$ sensitivity. Turbidity measurements of the myosin at pCa 5 and pCa 8 that were carried out at various $Mg^{2+}$ concentrations (1–6 mM) gave similar values (data not shown), indicating that the difference in ATPase activity was not associated with a difference in aggregation properties. The effect of $Mg^{2+}$ on the $Ca^{2+}$ sensitivity of arterial myosin is similar to that observed for *Acanthamoeba* myosin II (32). The $Ca^{2+}$-binding characteristics of *Acanthamoeba* myosin are not known.

Rees and Frederiksen (19) reported that the actin-activated ATPase activity of phosphorylated myosin is 40% higher in the presence of 0.1 mM $CaCl_2$ than when the $Ca^{2+}$ is replaced by 2 mM EGTA, confirming our previous report of calcium's effect on phosphorylated myosin isolated from vas deferens (6). The data presented in this paper indicate that a direct correlation between $Ca^{2+}$ binding and $Ca^{2+}$ sensitivity of actomyosin ATPase exists for smooth muscle myosin. The observation that the $Ca^{2+}$ sensitivity of actin-activated ATPase activity of arterial myosin was present even after the myosin was phosphorylated indicates that $Ca^{2+}$ plays a dual role in the regulation of actomyosin ATPase in some mammalian smooth muscles. Myosins isolated from avian gizzard (34), porcine stomach (4), and bovine stomach (5) do not require $Ca^{2+}$ for actin activation once the myosin is phosphorylated.

The $Ca^{2+}$ sensitivity expressed by arterial myosin is similar to that of the scallop myosin because the latter also binds 2 mol of $Ca^{2+}$ when it expresses $Ca^{2+}$ sensitivity (18, 28). Although

$Ca^{2+}$ exerts its effect on scallop actomyosin ATPase by directly binding to myosin, it has a dual effect on the actin-activated ATP hydrolysis of arterial myosin. For arterial myosin, $Ca^{2+}$ was required for the activation of ATPase activity because of its effect on the phosphorylation system. $Ca^{2+}$ was also required for expression of the maximal ATPase activity of phosphorylated myosin, presumably when the $Ca^{2+}$-binding sites were not occupied by $Mg^{2+}$. Although binding of $Ca^{2+}$ is essential for actomyosin ATPase in molluscan myosin, $Mg^{2+}$ at higher concentration (presumably $Mg^{2+}$ binding to myosin) can replace the effect of $Ca^{2+}$ in the artery. The contractile system in arterial smooth muscle behaves similarly to that of vas deferens muscle (6). It is different from gizzard (1, 2), porcine stomach (4), and bovine stomach (5), though in all smooth muscles, phosphorylation is a prerequisite for actin-activation (however, see refs. 12 and 13).

Our findings show that the actin-activated ATPase activity is linearly correlated with the degree of phosphorylation; however, this does not rule out the possibility that additional factors (35) or leiotonins (12) may serve as alternative or complementary mechanisms for the regulation of actin-activated ATP hydrolysis. Addition of such factors or proteins to the phosphorylated myosin without altering the level of phosphorylation will enable us to understand how the various regulatory mechanisms interact in the regulation of actin–myosin interaction and ATP hydrolysis.

This report shows that the actin-activated ATP hydrolysis, $Ca^{2+}$ binding, and $Ca^{2+}$ sensitivity of smooth muscle myosin are affected by $Mg^{2+}$ concentration. The mechanisms by which $Mg^{2+}$ modulates the ATPase activity and $Ca^{2+}$ sensitivity are not clear at present. However, the $Mg^{2+}$ effects were seen at a $Mg^{2+}$ concentration reported as a range (2–5 mM) for free $Mg^{2+}$ in muscle (36, 37). No data are available for the free $Mg^{2+}$ concentrations of smooth muscle cells in the pulmonary artery. Using chemically skinned smooth muscle fibers, Saida and Nonomura (38) showed that >1 mM $Mg^{2+}$ was required for $Ca^{2+}$-induced contraction in skinned fibers as well as for the activation of ATPase and superprecipitation in smooth muscle actomyosin; $Mg^{2+}$ >2 mM caused a slow tension development by itself in the absence of $Ca^{2+}$. On the basis of these results they suggested that the mode of actin–myosin interaction in smooth muscle is substantially different from that in skeletal muscle.

The biochemical demonstration of a direct relationship between $Ca^{2+}$ binding and $Ca^{2+}$ sensitivity at the physiological range of $Ca^{2+}$ and $Mg^{2+}$ concentrations raises the possibility for the existence of a direct, myosin-mediated calcium regulation of contraction in some smooth muscles. Although the basic mechanism of the contractile process appears to be similar for various vertebrate smooth muscles (39), the regulation of contraction may be different for different smooth muscles.

We thank George Thomas for excellent technical assistance, Janet Flinn for typing the manuscript, and Peter D. Villari, Inc., for donating the pulmonary artery. This study was supported by Grants HL-23779 and HL-22446 from the National Institutes of Health.

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