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Role of Tropomyosin in Smooth Muscle Contraction: Effect of Tropomyosin Binding to Actin on Actin Activation of Myosin ATPase†

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ABSTRACT: The binding of gizzard tropomyosin to gizzard F-actin is highly dependent on free Mg^{2+} concentration. At 2 mM free Mg^{2+}, a concentration at which actin-activated ATPase activity was shown to be Ca^{2+} sensitive, a molar ratio of 1:3 (tropomyosin:actin monomer) is required to saturate the F-actin with tropomyosin to the stoichiometric ratio of 1 mol of tropomyosin to 7 mol of actin monomer. Increasing the Mg^{2+} could decrease the amount of tropomyosin required for saturating the F-actin filament to the stoichiometric level. Analysis of the binding of smooth muscle tropomyosin to smooth muscle actin by the use of Scatchard plots indicates that the binding exhibits strong positive cooperativity at all Mg^{2+} concentrations. Calcium has no effect on the binding of tropomyosin to actin, irrespective of the free Mg^{2+} concentration. However, maximal activation of the smooth muscle actomyosin ATPase in low free Mg^{2+} requires the presence of Ca^{2+} and stoichiometric binding of tropomyosin to actin. The lack of effect of Ca^{2+} on the binding of tropomyosin to actin shows that the activation of actomyosin ATPase by Ca^{2+} in the presence of tropomyosin is not due to a calcium-mediated binding of tropomyosin to actin.

Contraction in striated muscle is regulated by the interaction of Ca^{2+} and troponin–tropomyosin complex which lies along actin filaments (Ebashi & Endo, 1968). While calcium sensitivity of the actomyosin system in striated muscle resides in the complete complement of the troponin–tropomyosin complex, tropomyosin alone can either inhibit (Eaton et al., 1975) or potentiate (Bremel et al., 1972) the actin-activated adenosine-5′-triphosphatase (ATPase)[1] activity depending on the conditions of the assays.

† This work was supported by National Institutes of Health Grants HL 22264 and HL 28476 and NSF Grant PCM83-09139. H.M. is supported by a postdoctoral fellowship from the Muscular Dystrophy Association.

[1] Abbreviations: EGTA, ethylene glycol bis(aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; ATP, adenosine 5′-triphosphate; ATPase, adenosine-5′-triphosphatase; P_i, inorganic phosphate.

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Though troponin is not present in smooth muscle [for review, see Adelstein & Eisenberg (1980), Chacko et al. (1983), and Hartshorne & Mrwa (1982)], tropomyosin lies along the thin actin filaments at locations similar to its position on the thin filament of striated muscle (Parry & Squire, 1973; Ebashi et al., 1966). Tropomyosin has been shown to potentiate the actin-activated ATP hydrolysis of smooth muscle myosin under conditions at which it inhibits skeletal muscle actomyosin ATPase (Chacko et al., 1977; Sobieszek & Small, 1977; Chacko, 1981). This potentiation is observed only when the 20000-dalton light chain of myosin is phosphorylated with the calcium–calmodulin-dependent kinase. At low concentrations of free Mg^{2+}, the maximal activation of actomyosin ATPase requires not only the presence of tropomyosin but also Ca^{2+} even after the myosin is stably phosphorylated (Chacko et al., 1977; Chacko & Rosenfeld, 1982; Nag & Seidel, 1983). A recent study by Heaslip and Chacko (1985) shows that the requirement for tropomyosin and calcium for optimal activation of stably phosphorylated myosin is evident irrespective of the source of actin or tropomyosin (i.e., skeletal or smooth muscle).

In this report we show that the binding of smooth muscle tropomyosin to smooth muscle actin is dependent on free Mg^{2+} concentration. Furthermore, the binding exhibits strong positive cooperativity at all Mg^{2+} concentrations, and it is independent of free Ca^{2+} concentration. The actin-activated ATPase activity is maximal when a stoichiometric amount of tropomyosin is bound to actin. The Ca^{2+}-independent binding of smooth muscle tropomyosin to smooth muscle actin indicates that the modulation of the actin-activated ATPase activity of phosphorylated myosin by Ca^{2+} in the presence of tropomyosin is not due to a direct effect of Ca^{2+} on tropomyosin binding to F-actin.

Materials and Methods

Fully phosphorylated myosin from chicken gizzard was prepared and purified by gel filtration on Sepharose 4B-CL agarose column as reported (Chacko, 1981; Chacko & Rosenfeld, 1982) with minor modifications. Protease inhibitors were added to the following buffers: wash and extraction buffer, 1 μM antipain, 1 mM EGTA, 50 μM 1-*trans*-epoxysuccinylleucylamido(4-guanidino)butane (E-64) (a gift from Dr. Y. Nonomura, University of Tokyo), 1 μM pepstatin A, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF); phosphorylation buffer, 2.0 μM antipain, 2.0 μM pepstatin A, and 0.2 mM PMSF; magnesium precipitation buffer, 0.5 μM antipain and 0.5 μM pepstatin A.

Smooth muscle actin was prepared following the method of Ebashi (1985). Trace amounts of tropomyosin present in the actin prepared by this method was removed according to Spudich and Watt (1971). The F-actin obtained by this method was highly viscous and free of tropomyosin.

Tropomyosin was prepared from the alcohol ether powder (Bailey, 1948) of the residue after myosin extraction. Tropomyosin was extracted and purified to homogeneity on a hydroxylapatite column according to the method of Eisenberg and Kielley (1974).

The protein concentration was determined according to Lowry et al. (1951). The level of phosphorylation of the 20000-dalton light chain of myosin was determined by urea gel electrophoresis (Perrie et al., 1973). Sodium dodecyl sulfate ($NaDodSO_4$) ($7^1/_2$%)/polyacrylamide gel electrophoresis (Fairbanks et al., 1971) was used to ascertain protein purity.

ATPase activity was assayed as previously described (Chacko et al., 1977). A Ca/EGTA buffer (Fabiato & Fabiato, 1979) was used for experiments involving varying free Ca^{2+} and Mg^{2+} concentrations. The following protein concentrations were used: myosin, 0.2 mg/mL; smooth muscle actin and smooth muscle tropomyosin to obtain the required molar ratios. The assay mixture was incubated at 25 °C; aliquots were removed at zero time and two additional intervals. The inorganic phosphate released was measured according to Martin and Doty (1949).

Tropomyosin binding to smooth muscle F-actin was carried out according to the procedure used by Yang et al. (1979) for determining the binding of tropomyosin to Acanthamoeba actin and Eaton et al. (1975) for studying the binding of skeletal muscle tropomyosin to skeletal muscle actin. In order to iodinate the tropomyosin, 4 mL of smooth muscle tropomyosin (20 mg/mL) in 0.37 M KCl and 0.063 M phosphate buffer (pH 6.9) was mixed with 0.1 mg/mL lactoperoxidase solution (1 mg/mL in water) and 0.35 μg of $Na^{125}I$ (ICN; specific activity 0.5 mCi, dissolved in 0.03 mL of water), and the reaction was initiated by adding 0.02 mL of 0.03% hydrogen peroxide. Five additional 0.02-mL aliquots of hydrogen peroxide was added at 10-min intervals. Following the fourth aliquot of hydrogen peroxide, 0.01 mL of 0.01 M KI was added as a carrier. The reaction was stopped by adding DTT to obtain a final concentration of 0.5 mM. The mixture was dialyzed against 0.3 M KCl, 20 mM imidazole hydrochloride (pH 7.0), and 0.5 mM DTT with several changes of dialysate until the dialysate became free of radioactivity. The binding of ^{125}I-tropomyosin to F-actin was carried out as described by Kominz and Maruyama (1967) but using an air-driven ultracentrifuge (airfuge, Beckman). The ionic condition was similar to that of the conditions used for the ATPase assay. Actin and tropomyosin was mixed at various molar ratios in Ca/EGTA buffer made according to Fabiato and Fabiato (1979). The buffer contained 15 mM imidazole hydrochloride, 2 mM ATP, 2.5 mM DTT, $MgCl_2$ as indicated, and free calcium concentration of either pCa 4 or pCa 8. The ionic strength was adjusted to 50 mM for all experiments by adding KCl unless stated otherwise. The protein solutions were mixed in centrifuge tubes and incubated at 25 °C for 15 min and centrifuged for 20 min at 25 °C by using an airfuge. Two 20-μL aliquots from each mixture was transferred into scintillation vials prior to centrifugation. After centrifugation, two 20-μL aliquots were removed from the supernatant of each tube. These samples were mixed in 10 mL of ACS (Amersham) and counted in a Beckman liquid scintillation counter. The difference in the counts between samples before and after centrifugation at a given condition represented the amount of tropomyosin bound.

Results

Binding of Gizzard Tropomyosin to Gizzard Actin. The binding curves of tropomyosin to F-actin at varying concentrations of free Mg^{2+} and either in the presence (pCa 4) or in absence of Ca^{2+} (pCa 8) are shown in Figure 1A, plotted as the ratio of tropomyosin bound to actin monomer vs. free tropomyosin concentration. The actin concentration for this experiment was kept at 20 μM, and the tropomyosin concentration was varied from 0.2 to 10 μM. The tropomyosin binding to actin at 0.5 mM free Mg^{2+} is negligible even at very high ratios of tropomyosin:actin. The binding curve at 8 mM Mg^{2+} plateaus around a value which corresponds to 1 mol of tropomyosin to 7 mol of actin monomer when the tropomyosin to actin ratio reached 1:6. However, at 2 and 4 mM, full saturation (i.e., 1 mol of tropomyosin to 7 mol of actin monomer) is reached only at higher tropomyosin concentration. The bindings are similar in the presence and absence of Ca^{2+}

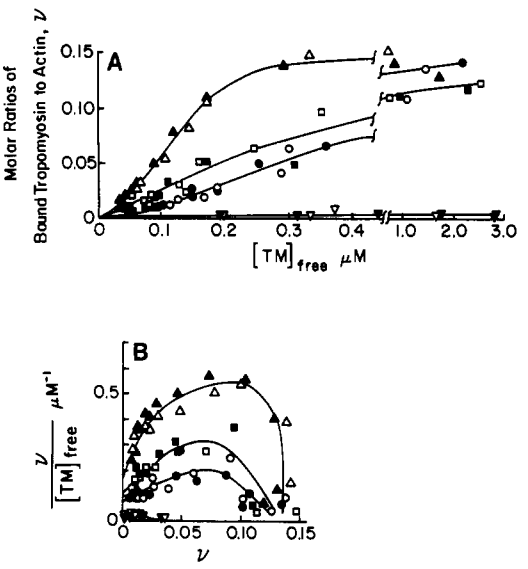

FIGURE 1: Binding of gizzard tropomyosin to gizzard actin at various concentrations of free Mg^{2+} ions in the presence and absence of Ca^{2+}. Upper figure (A) shows the binding as a function of free tropomyosin concentration. Actin concentration was 20 μM. Free Mg^{2+} concentrations were 0.5 (▽, ▼), 2 (○, ●), 4 (□, ■), and 8 mM (△, ▲). Open symbols show the result at pCa $^{2+}$ 8, and closed symbols show the result at pCa 4. Buffer conditions were the following: ionic strength 0.05 M; imidazole hydrochloride 15 mM (pH 7.0); ATP 2 mM; DTT 2.5 mM. Lower figure (B) shows the Scatchard plot. Meaning of the symbols is the same.

Table I: Effect of Mg^{2+} on the Binding of Gizzard and Rabbit Skeletal Muscle Tropomyosins to Gizzard F-Actin[a]

	1:3 tropomyosin:actin	
free Mg^{2+} (mM)	gizzard tropomyosin	rabbit skeletal tropomyosin
0.5	0.015	0.041
1.0	0.014	0.105
2.0	0.123	0.130
3.0	0.130	0.136
6.0	0.140	0.148

[a] Buffer conditions were the same as described in the legend to Figure 1. Data are represented with the molar ratio of bound tropomyosin to one actin monomer.

at all Mg^{2+} (apparent binding contant $K_{app} = 2.5 \times 10^6$ M^{-1} in the presence of 2 mM free Mg^{2+}, 3.0×10^6 M^{-1} at 4 mM free Mg^{2+}, and 8.0×10^6 M^{-1} at 8 mM free Mg^{2+}). The Scatchard plot (Figure 1B) of the binding is very convexed at all Mg^{2+} concentrations, confirming that the binding of tropomyosin to F-actin is highly cooperative. The Ca^{2+} has no effect on the binding characteristics; when the binding begins to plateau, 1 mol of tropomyosin is bound to 7 mol of actin monomer both in pCa 4 and in pCa 8. In order to determine if the Mg^{2+} dependence for the binding of gizzard tropomyosin to gizzard actin is typical only for smooth muscle tropomyosin, binding of skeletal muscle tropomyosin to gizzard actin is carried out at varying free Mg^{2+} concentrations. The result of these experiments are shown in Table I. Both skeletal muscle and gizzard tropomyosin bind to gizzard actin at the same level at free Mg^{2+} concentration above 2 mM. However, at free Mg^{2+} concentration below 2 mM, the binding of skeletal tropomyosin to gizzard actin is 3–8-fold higher than the binding of gizzard tropomyosin to gizzard actin.

Effect of Tropomyosin Binding to Actin on the Actin Activation. The actin-activated ATPase activity of phosphorylated myosin as a function of increasing tropomyosin at 10 μM actin (molar ratio of myosin:actin = 1:20) is shown in Figure

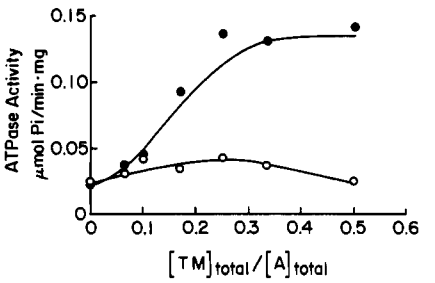

FIGURE 2: Effect of binding of gizzard tropomyosin to gizzard actin on actin-activated ATP hydrolysis by phosphorylated gizzard myosin. Actin concentration was 10 μM, and myosin concentration was 0.5 μM (molar ratio, M:A 1:20). Assay conditions were the same as described in Figure 1. Free Mg^{2+} concentration was 2 mM. Open circles, pCa 8; closed circles, pCa 4.

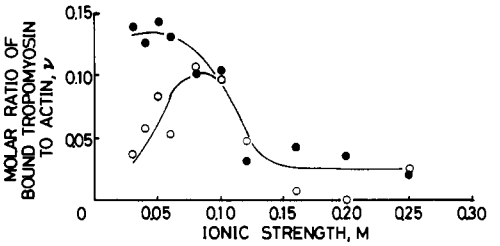

FIGURE 3: Binding of gizzard tropomyosin to gizzard F-actin as a function of ionic strength at two different free Mg^{2+} concentrations and in the presence of Ca^{2+} (pCa 4). Actin concentration was 10 μM. Tropomyosin concentration was 3.3 μM. Molar ratio of tropomyosin:actin = 1:3. Free Mg^{2+} concentrations were 0.5 (○) or 2 mM (●). Buffer conditions were the same as described in the legend to Figure 1. Ionic strength was adjusted by adding appropriate amounts of KCl according to the calculation based on the procedure described by Fabiato and Fabiato (1979).

2. In the presence of Ca^{2+}, the actin-activated ATPase activty is maximal when 1 mol of tropomyosin is bound to 7 mol of actin monomer. Only a slight increase in the ATPase activity is observed on increasing the tropomyosin in the absence of Ca^{2+}. The ATPase activity is 5-fold higher in the presence of Ca^{2+} when the tropomyosin to actin ratio reaches 1:3, a ratio at which F-actin becomes saturated to the stoichiometric ratio (Figure 1A). Similar results are obtained when the actin concentration is raised to 20 μM; the ATPase activity at 20 μM actin is slightly higher than that at 10 μM at all tropomyosin concentrations.

Effect of Ionic Strength on Tropomyosin Binding. Figure 3 depicts the binding of tropomyosin to F-actin as a function of ionic strength. At 0.5 mM free Mg^{2+}, the binding is very low at an ionic strength lower than 0.04 M. On increasing the ionic strength (I), the binding increases steeply to 70% at 0.1 M I; further increase in ionic strength is associated with a decrease in binding. The binding levels off to 30% binding at 0.25 M I. The binding at 2 mM free Mg^{2+} is close to saturation at 0.04 M I and remains at that level until the ionic strength reaches 0.06 M. Similar to the binding at 0.5 mM Mg^{2+}, further increasing in the ionic strength from 0.06 M is associated with a decrease in the binding. The binding at 0.5 and 2 mM Mg^{2+} is similar between 0.1 and 0.12 M I.

The effect of ionic strength on the tropomyosin potentiated actomyosin ATPase activity at 0.5 and 2 mM free Mg^{2+} is shown in Figure 4. The ATPase activity at free Mg^{2+} concentration of 2 mM is severalfold higher than that at 0.5 mM between ionic strengths 0.03 and 0.1 M. At both 0.5 and 2 mM free Mg^{2+}, the ATPase increases rapidly on raising the ionic strength from 0.05 M. Raising the ionic strength from 0.08 M caused the ATPase activity to fall. The activity at 0.5 mM Mg^{2+} also shows similar dependence for ionic strength, although the activity is low at all ionic strengths compared

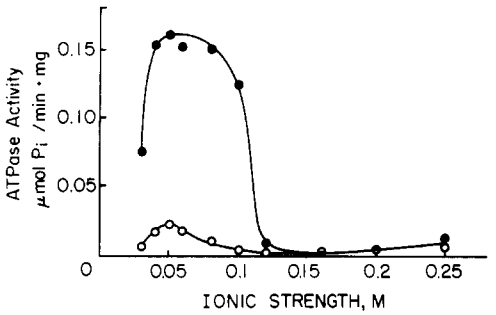

FIGURE 4: Effect of ionic strength on the actin-activated ATPase activity of myosin under conditions which are the same as in Figure 3. Appropriate amounts of KCl were added to adjust the ionic strength. Solid circles and open circles indicate 2 and 0.5 mM free Mg^{2+}, respectively. Myosin:actin molar ratio is 1:20. Actin-activated ATPase was determined as in Figure 2.

to the activity at 2 mM Mg^{2+}. At 0.1 M ionic strength the tropomyosin binding is the same both at 0.5 and 2 mM Mg^{2+} (Figure 3); however, the ATPase activity at 2 mM Mg^{2+} is severalfold higher than that at 0.5 mM (Figure 4).

DISCUSSION

Previous reports (Chacko et al., 1977; Sobieszek & Small, 1977; Chacko, 1981; Yamaguchi et al., 1984) have shown that the addition of smooth or skeletal muscle tropomyosin to actomyosin reconstituted with skeletal muscle actin and phosphorylated myosin is associated with a 2–3-fold potentiation of the actin-activated ATPase activity. The actin-activated ATPase activity of phosphorylated smooth muscle myosin is highly dependent on Mg^{2+} concentration and at low Mg^{2+} concentration, the maximal activation of ATPase activity by actin requires the presence of Ca^{2+} and tropomyosin (Chacko & Rosenfeld, 1982; Nag & Seidel, 1983; Heaslip & Chacko, 1985). It has not been reported if the Ca^{2+} and Mg^{2+} dependence for the actin activation of myosin in the presence of tropomyosin is due to differences in the physical binding of smooth muscle tropomyosin to smooth muscle actin. This study was carried out to correlate the physical binding of tropomyosin to F-actin and actin-activated ATP hydrolysis by phosphorylated smooth muscle myosin at various ionic conditions.

The binding of tropomyosin to F-actin is highly dependent on free Mg^{2+} concentration. A molar ratio of 1:3 (tropomyosin:actin monomer) is required to obtain F-actin filament saturated with tropomyosin to the stoichiometric ratio of 1 mol of tropomyosin to 7 mol of actin monomer at 2 mM free Mg^{2+}. The actin-activated ATPase activity was shown to be Ca^{2+} sensitive at this Mg^{2+} concentration (Chacko & Rosenfeld, 1982; Heaslip & Chacko, 1985). The requirement for high concentrations of tropomyosin to fully saturate the actin has also been observed by Sanders and Smillie (1984), who used skeletal muscle actin for the binding of gizzard tropomyosin. Our results indicate that increasing the Mg^{2+} concentration could decrease the amount of tropomyosin required for saturating the smooth muscle F-actin.

The binding of skeletal muscle tropomyosin to nonmuscle actin (Yang et al., 1979b) and the binding of smooth muscle tropomyosin to skeletal muscle actin (Sanders & Smillie, 1984) have been shown to be highly cooperative. In the present study the binding of smooth muscle tropomyosin to smooth muscle actin is found to be highly cooperative in all Mg^{2+} concentrations used as indicated by the convex nature of the Scatchard plots of the binding data.

The strongly positive cooperativity observed for the binding of smooth muscle tropomyosin to smooth muscle F-actin may be related to the end-to-end association of tropomyosin molecules on the F-actin (Wegner, 1979). Further studies using smooth muscle tropomyosin which does not show end-to-end association (Johnson & Smillie, 1977) may clarify if the cooperativity of tropomyosin binding is different from the cooperative effects on the ATPase activity once tropomyosin is bound to actin. However, experiments utilizing tropomyosin in which the end-to-end overlap has been removed (Johnson & Smillie, 1977; Walsh et al., 1984) showed that the presence of end-to-end interactions between tropomyosin molecules are not necessary for the cooperativity of ATPase in the skeletal muscle actomyosin system. Comparison of the effects of smooth and skeletal muscle tropomyosin on skeletal actomyosin subfragment 1 ATPase showed a greater cooperativity for the gizzard system, presumably a consequence of the greater rigidity of gizzard tropomyosin as indicated from conformational studies (Lehrer & Morris, 1984).

At high free Mg^{2+} concentration there is no difference between skeletal muscle and smooth muscle tropomyosin in their ability to bind to smooth muscle F-actin as shown in Table I. However, at low Mg^{2+} the skeletal muscle tropomyosin binds better than the smooth muscle tropomyosin. This is in agreement with the finding by Williams et al. (1984) that a higher free Mg^{2+} concentration is required for the maximum effect of smooth muscle tropomyosin on the skeletal muscle actomyosin subfragment 1 ATPase activity than in the case for skeletal muscle tropomyosin.

While free Mg^{2+} concentration has remarkable effect on the binding of tropomyosin to actin, Ca^{2+} has no effect on the binding since the bindings at pCa 4 and 8 are similar. At full saturation of the actin with tropomyosin, the actin activation is maximal in the presence of Ca^{2+}, and the inhibition of ATPase on removal of Ca^{2+} is slightly more pronounced. However, the finding that the actomyosin ATPase at varying levels of saturation of tropomyosin to F-actin is calcium sensitive implies that the role of tropomyosin is not to make the actomyosin system calcium sensitive but rather to potentiate the actin activation. Factors that affect the binding of tropomyosin to F-actin may affect the potentiation of actin-activated ATPase by tropomyosin. The effect of these factors on actomyosin ATPase would be expected to be supplementary to the regulation via phosphorylation–dephosphorylation since myosin phosphorylation is required for actin activation of the smooth muscle myosin (Chacko et al., 1977; Sobieszek & Small, 1977; Chacko, 1981; Merkel et al., 1984). Tropomyosin binding to actin may also have an important role in the regulation of actomyosin ATPase in nonmuscle cells in which phosphorylation–dephosphorylation have been shown to be important for actin activation (Adelstein & Conti, 1975; Scordilis & Adelstein, 1977; Scholey et al., 1980).

The ionic strength dependence of the tropomyosin is shifted to the left by increasing the free Mg^{2+} concentration from 0.5 to 2 mM. In spite of the 70% saturation of actin with tropomyosin at the ionic strength of 0.1 M in both 0.5 and 2 mM free Mg^{2+}, the actin-activated myosin ATPase at 0.5 mM free Mg^{2+} is lower than that at 2 mM free Mg^{2+}. This observation indicates that the free Mg^{2+} concentration has a direct effect on the actin-activated Mg^{2+} ATPase independent of its effect on tropomyosin binding to F-actin (Chacko & Rosenfeld, 1982; Nag & Seidel, 1983; Kaminski & Chacko, 1984; Heaslip & Chacko, 1985). The finding that there is less than the stoichiometric ratio (1 mol of tropomyosin to 7 mol of actin monomer) of tropomyosin binding to F-actin at ionic strength close to physiological is unexpected since Sanders and Smillie (1984) found that the gizzard tropomyosin remained bound

to skeletal muscle actin at ionic strength as high as 0.25 M. Raising the ionic strength up to 0.6 M is required to completely remove the skeletal muscle tropomyosin from skeletal muscle actin (Spudich & Watt, 1971). Though the actin used in the present study is from smooth muscle, it should not make any difference since it sediments well at ionic strength as high as 0.25 M (data not shown). It will be of interest to know if actin binding proteins (Lash et al., 1985; Hinssen et al., 1984) might play a role in the binding of smooth muscle tropomyosin to smooth muscle actin at physiological ionic strength. Several actin binding proteins have been reported to exist in smooth and nonmuscle cells (Lash et al., 1985; Hinssen et al., 1984; Sobue et al., 1981). It is not clear if their role is only in the modulation of microfilaments which are important for cytoskeletal structure or if they are also involved in the modulation of actin–myosin interaction in cell motility. The actin-binding protein caldesmon, first reported by Kakiuchi and his colleagues (Sobue et al., 1981), has recently been shown to have an effect on the actin-activated ATPase activity of smooth muscle actomyosin ATPase (Ngai & Walsh, 1984). Further studies to determine the effects of actin binding proteins on the interaction between actin and tropomyosin will enable us to understand if these proteins modulate the potentiation of actomyosin ATPase by tropomyosin.

ACKNOWLEDGMENTS

We thank Julia Heaslip for technical assistance, Cindy Cohen for secretarial assistance, and Dr. William S. Fillers for valuable discussions. The gizzards used for this study were donated by Weaver Co., New Holland, PA.

Registry No. ATPase, 9000-83-3; Mg, 7439-95-4; Ca, 7440-70-2.

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