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Effects of Ca^{2+} and Mg^{2+} on the Actomyosin Adenosine-5′-triphosphatase of Stably Phosphorylated Gizzard Myosin†

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ABSTRACT: There are conflicting reports on the effect of Ca^{2+} on actin activation of myosin adenosinetriphosphatase (ATPase) once the light chain is fully phosphorylated by a calcium calmodulin dependent kinase. Using thiophosphorylated gizzard myosin, Sherry et al. [Sherry, J. M. F., Gorecka, A., Aksoy, M. O., Dabrowska, R., & Hartshorne, D. J. (1978) *Biochemistry 17*, 4417–4418] observed that the actin activation of ATPase was not inhibited by the removal of Ca^{2+}. Hence, it was suggested that the regulation of actomyosin ATPase activity of gizzard myosin by calcium occurs only via phosphorylation. In the present study, phosphorylated and thiophosphorylated myosins were prepared free of kinase and phosphatase activity; hence, the ATPase activity could be measured at various concentrations of Ca^{2+} and Mg^{2+} without affecting the level of phosphorylation. The ATPase activity of myosin was activated either by skeletal muscle or by gizzard actin at various concentrations of Mg^{2+} and either at pCa 5 or at pCa 8. The activation was sensitive to Ca^{2+} at low Mg^{2+} concentrations with both actins. Tropomyosin potentiated the actin-activated ATPase activity at all Mg^{2+} and Ca^{2+} concentrations. The calcium sensitivity of phosphorylated and thiophosphorylated myosin reconstituted with actin and tropomyosin was most pronounced at a free Mg^{2+} concentration of about 3 mM. The binding of ^{125}I-tropomyosin to actin showed that the calcium sensitivity of ATPase observed at low Mg^{2+} concentration is not due to a calcium-mediated binding of tropomyosin to F-actin. The actin activation of both myosins was insensitive to Ca^{2+} when the Mg^{2+} concentration was increased above 5 mM. The data indicate that, at low Mg^{2+} concentrations, Ca^{2+} regulates the actomyosin ATPase not only by its effect on the phosphorylation of light chain by calcium calmodulin dependent kinase but also by a direct effect on the actin-activated ATP hydrolysis.

Phosphorylation of the 20 000-dalton (Da)[1] light chain of smooth muscle myosin is associated with severalfold increase in its actin-activated ATPase activity (Gorecka et al., 1976; Sobieszek, 1977; Chacko et al., 1977; Ikebe et al., 1978; Rees & Frederiksen, 1981). Phosphorylation of the light chain is catalyzed by a calcium calmodulin dependent kinase called myosin light chain kinase (Frearson & Perry, 1975; Dabrowska et al., 1978; Adelstein et al., 1978). Hence, calcium activates the actomyosin ATPase activity via its effect on the phosphorylation of the myosin light chain. Using myosin isolated from vas deferens, Chacko et al. (1977) reported that calcium regulates the actomyosin ATPase not only through its effect on myosin light chain kinase but also through a direct effect on actin-activated ATP hydrolysis. This direct effect on the actomyosin ATPase activity by Ca^{2+} was shown only for phosphorylated myosin. Subsequent studies utilizing myosins isolated from aorta (Rees & Frederiksen, 1981), pulmonary artery (Chacko & Rosenfeld, 1982) and gizzard (Nag &

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid; ATP, adenosine 5′-triphosphate; ATPase, adenosine-5′-triphosphatase; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); P_i, inorganic phosphate; Da, dalton.

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Seidel, 1983) confirmed the direct effect of Ca^{2+} on actomyosin ATPase.

In order to study the direct effect of Ca^{2+} on the actomyosin ATPase activity of myosin, Sherry et al. (1978) made stably phosphorylated gizzard myosin using ATPγS. Since thiophosphorylated protein is not dephosphorylated by phosphatase (Gratecos & Fischer, 1974), they eliminated the possibility of dephosphorylation by contaminant phosphatase. Using the thiophosphorylated myosin, they found no evidence for a direct effect of Ca^{2+} and concluded that the gizzard myosin is regulated by Ca^{2+} only through myosin light chain phosphorylation. The biochemical observation by Sherry et al. (1978) was also supported by physiological experiments carried out using a skinned smooth muscle preparation in which ATPγS was used to phosphorylate the myosin (Hoar et al., 1979).

In the present study, we determined the effects of Ca^{2+} and Mg^{2+} on actin-activated ATP hydrolysis by purified gizzard myosin which had been phosphorylated by ATPγS. Furthermore, the effects of divalent cations on the actin activation of thiophosphorylated myosin are compared with that of myosin phosphorylated stably with ATP by using both smooth and skeletal muscle actin. The actin-activated ATP hydrolysis of both myosins is found to be modulated by Ca^{2+} and Mg^{2+}.

Materials and Methods

Actomyosin containing the endogenous kinase was extracted from fresh chicken gizzard as previously described (Chacko, 1981). Myosin was phosphorylated with either 2.5 mM ATP or 0.5 mM ATPγS and purified by gel filtration on a Sepharose 4B-CL agarose column (Chacko et al., 1977). The myosin peak was located by ATPase assay of the column fractions, pooled, and dialyzed against 20 mM imidazole hydrochloride, pH 7.0, 1 mM DTT, and 10 mM $MgCl_2$ until the KCl concentration was lowered to 20 mM. The precipitated myosin was collected by centrifugation at 48000*g* for 15 min, dissolved in a buffer containing 0.4 M KCl, 20 mM imidazole hydrochloride, pH 7.0, 2 mM DTT, 2 mM EDTA, and 2 mM EGTA, and dialyzed for 10 h against the same buffer. The sample was again dialyzed for 6 h against the above buffer without EDTA and EGTA.

Rabbit skeletal muscle actin was prepared according to the method of Spudich & Watt (1971). Chicken gizzard actin was prepared from acetone-washed fibers of gizzard according to the method of Ebashi (1985). Tropomyosins from rabbit skeletal muscle and from turkey gizzard were prepared from alcohol–ether powder (Bailey, 1948) according to the procedure of Eisenberg & Kielley (1974). Each of the proteins was determined to be pure by electrophoresis on 1% SDS–7.5% polyacrylamide gel (Fairbanks et al., 1971).

Protein concentrations were determined according to Lowry et al. (1951) with bovine serum albumin as a standard.

The actin-activated ATPase assays were carried out in 15 mM imidazole hydrochloride, pH 7.0, 2 mM ATP, 2.5 mM DTT, and $MgCl_2$ as indicated and either at pCa^{2+} 4 or at pCa^{2+} 8 (Chaberek & Martell, 1959). The ionic strength of each buffer was maintained at 0.05 M with KCl. The protein concentrations used were the following: myosin, 0.134 mg/mL; actin, 0.244 mg/mL; tropomyosin, 0.073 mg/mL. Samples were incubated at 37 °C, and aliquots were removed at zero time and at two other times (usually 10 and 20 min) to ascertain linearity of phosphate release. P_i was measured according to Martin & Doty (1949).

Binding of tropomyosin to F-actin was conducted as described by Eaton et al. (1975). The iodination of tropomyosin with $Na^{125}I$ was catalyzed by lactoperoxidase (Marchalonis, 1969). The iodination reaction was carried out in 0.37 M KCl and 0.063 M phosphate buffer (pH 6.9). After iodination the protein was dialyzed against 0.3 M KCl, 0.063 M phosphate buffer, and 0.5 mM DTT with several changes of the buffer until the dialysate was free of radioactivity. The ^{125}I-tropomyosin was then dialyzed against 15 mM imidazole hydrochloride (pH 7.0) and 1 mM DTT to obtain a KCl concentration of 10 mM and to remove all the phosphate. The F-actin was mixed with ^{125}I-tropomyosin (molar ratio of Tm:A = 1:6) under the conditions of the ATPase assay (described above) in the presence of myosin. The Ca^{2+} concentration was kept constant either at pCa 4 or at pCa 8 and the free Mg^{2+} concentrations varied between 0.5 and 6 mM. After a 15-min incubation, 20-μL aliquots were removed for counting, and the tubes each containing 0.3 mL were centrifuged in an airfuge (Beckman) at 25 °C by using an A-95 rotor. After centrifugation 0.1-mL aliquots of the supernatants were removed and mixed with 10 mL of scintillation fluid (ACS, Amersham) and counted in a liquid scintillation counter (Beckman LS). The difference in counts before and after centrifugation represented the amount of tropomyosin bound.

Myosin 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 (Perrie et al., 1973) in order to determine the extent of myosin phosphorylation. The gels were stained with Coomassie brilliant blue R and scanned at 584 nm with a gel scanner/integrator (Transidyne, Ann Arbor, MI), and the percent of areas under the fast migrating (phosphorylated) and slow migrating (unphosphorylated) bands of 20000-Da light chain was determined.

Results

In this study we achieved stable phosphorylation by phosphorylating the myosin with either ATP or ATPγS and then separating it from the kinase and phosphatase by gel filtration. Under the conditions used in this investigation, gel filtration on Sepharose 4B-CL yields myosin free of contaminating kinase and phosphatase. The absence of kinase is demonstrated in Figure 1A. Unphosphorylated myosin was not phosphorylated when incubated with either thiophosphorylated or phosphorylated myosin in the presence of ATP, Ca^{2+}, and Mg^{2+} (compare well 5 with 4 and well 9 with 8). The absence of phosphatase is demonstrated in Figure 1B, where it can be seen that the level of phosphorylation is stable when thiophosphorylated or phosphorylated myosin, or a mixture of the two, is incubated in the absence of ATP (compare well 3 with 2, well 6 with 5, and well 9 with 8).

Gizzard actin and tropomyosin were also incubated under the conditions of the ATPase assay with unphosphorylated and phosphorylated myosin to determine if these proteins were also free of kinase and phosphatase activities. Maintenance of the phosphorylated state of the 20000-Da light chain of phosphorylated myosin (Figure 1B, wells 4, 7, and 10) and lack of phosphorylation of the unphosphorylated myosin in the presence of actin and tropomyosin (Figure 1A, wells 2, 6, and 10) confirmed that these proteins were free of kinase and phosphatase activities.

Since the myosins were obtained free of kinase and phosphatase, it was possible to vary the Ca^{2+} and Mg^{2+} concentration in the ATPase assay without an effect on the phosphorylated state of the myosin. Panels A and B of Figure 2 depict the effects of free Mg^{2+} and Ca^{2+} on the ATPase activity of thiophosphorylated myosin and myosin reconstituted with either actin or actin and tropomyosin. Actin and tropomyosin isolated from gizzard (Figure 2A) or skeletal muscle (Figure 2B) were used for activation. On increasing the free Mg^{2+} concentration from 0.44 to 8 mM at pCa^{2+} 8, the specific

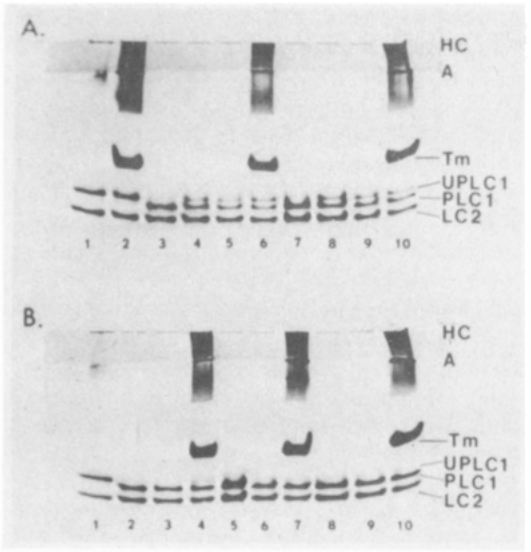

FIGURE 1: Urea gel electrophoresis of myosin. Myosin was assayed for contamination of light chain kinase (A) or phosphatase (B). After incubation under appropriate assay conditions samples were precipitated with 10% Cl_3COOH and dissolved in 8 M urea, 5% β-mercaptoethanol, and 0.005% Bromphenol Blue. Tris base (1 M) was added to raise the pH of the sample to 8.4. The samples (15–25 μg) were loaded to the gel and electrophoresed (Perrie et al., 1973). The unphosphorylated (UPLC1) and phosphorylated (PLC1) 20000-dalton light chain and the 15000-dalton light chain (LC2) of myosin enter the gel, as does the tropomyosin (Tm) and, to some degree, the actin (A). The myosin heavy chain (HC) remained at the top of the stacking gel. (A) Proteins were incubated under assay conditions (see Materials and Methods) with 2.5 mM free Mg^{2+} and pCa^{2+} 4. (1) Unphosphorylated myosin; 0-min incubation. (2) Unphosphorylated myosin plus gizzard actin and tropomyosin; 10-min incubation. (3 and 4) Thiophosphorylated myosin alone and with unphosphorylated myosin, respectively; 0-min incubation. (5 and 6) Thiophosphorylated plus unphosphorylated myosins without (5) and with (6) gizzard actin and tropomyosin; 10-min incubation. (7 and 8) Phosphorylated myosin alone and with unphosphorylated myosin, respectively; 0-min incubation. (9 and 10) Phosphorylated plus unphosphorylated myosin without (9) and with (10) gizzard actin and tropomyosin; 10-min incubation. (B) Proteins were incubated under assay conditions with 2.5 mM free Mg^{2+} and pCa^{2+} 8, except that ATP was omitted. (1) Unphosphorylated myosin. (2 and 3) Thiophosphorylated myosin before and after 15-min incubation, respectively. (4) Thiophosphorylated myosin plus gizzard actin and tropomyosin; 15-min incubation. (5 and 6) Thiophosphorylated plus phosphorylated myosin before and after 15-min incubation, respectively. (7) Thiophosphorylated plus phosphorylated myosin with gizzard actin and tropomyosin; 15-min incubation. (8 and 9) Phosphorylated myosin before and after 15-min incubation, respectively. (10) Phosphorylated myosin with gizzard actin and tropomyosin after 15-min incubation. Identical results are obtained when the assays of (B) are performed at pCa^{2+} 4 (not shown).

activity of myosin alone increased from 0.003 to 0.021 μmol of P_i mg^{-1} min^{-1} (Figure 2A). The presence of Ca^{2+} (pCa^{2+} 4) made only a slight difference in the ATPase activity. In the presence of pure gizzard or skeletal muscle actin (panels A and B of Figure 2, respectively), the myosin ATPase activity was increased 2–4-fold over the activity in the absence of actin. Raising the free Mg^{2+} concentration from 0.44 to 5 mM (pCa^{2+} 8) increased the actomyosin ATPase activity from approximately 0.01 to 0.04 μmol of P_i mg^{-1} min^{-1} for both types of actin. At low concentrations of free Mg^{2+}, Ca^{2+} produced a further stimulation of the ATPase activity.

The presence of tropomyosin further stimulated the ATPase activity of the actomyosin at all Mg^{2+} concentrations, irrespective of the source of actin and tropomyosin (Figure 2). This effect was most pronounced at free Mg^{2+} concentrations of 3–6 mM, where activity was increased 3–6.5-fold over that of myosin with actin alone. At pCa^{2+} 8 increasing free Mg^{2+}

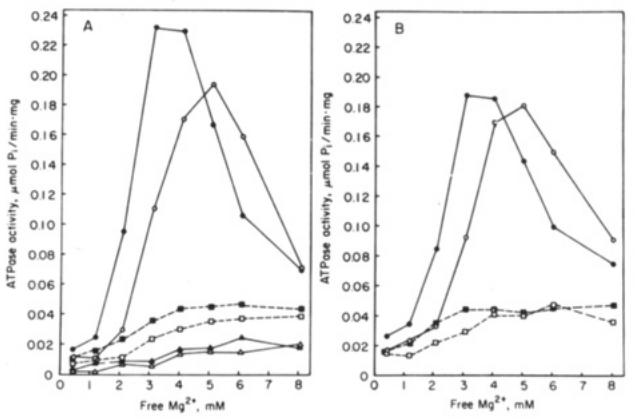

FIGURE 2: Calcium and magnesium dependence of the ATPase activity of thiophosphorylated gizzard myosin. Myosin was reconstituted with either gizzard (A) or skeletal muscle (B) actin or actin and tropomyosin. Symbols represent ATPase activity of thiophosphorylated gizzard myosin alone (△, ▲), myosin plus actin (□, ■), or myosin plus actin and tropomyosin (○, ●), at varying free Mg^{2+} concentrations and at either pCa^{2+} 4 (●, ▲, ■) or pCa^{2+} 8 (○, △, □). Myosin was determined to be 88% phosphorylated by urea gel electrophoresis. Assay conditions were as described under Materials and Methods. Free Mg^{2+} was determined according to Fabiato & Fabiato (1979), and pCa^{2+} was determined by using a calcium-selective electrode (Affolter & Sigel, 1979).

from 0.44 to 5 mM resulted in an increase in activity of the myosin reconstituted with actin and tropomyosin from 0.012–0.017 μmol of P_i mg^{-1} min^{-1} to a peak of 0.18–0.19 μmol of P_i mg^{-1} min^{-1} (Figure 2).

The activity of myosin reconstituted with either gizzard actin and tropomyosin or skeletal muscle actin and tropomyosin was markedly stimulated by calcium at free Mg^{2+} concentrations of 1.2–4 mM, with a maximal effect (over 2-fold stimulation) occurring at about 3 mM free Mg^{2+}. This stimulation of ATPase activity by calcium was abolished by raising the free Mg^{2+} to 5 mM or higher, regardless of the source of actin and tropomyosin.

In order to compare the enzymatic characteristics of thiophosphorylated myosin with that of myosin phosphorylated with ATP, actin-activated ATPase activity was determined for both myosins, and its dependence on Ca^{2+} and Mg^{2+} was assessed (Figure 3). Data were expressed as a percentage of maximum activity since myosin preparations phosphorylated to different degrees possess different maximum ATPase activities. The ATPase of myosin phosphorylated with ATP (Figure 3B) was found to be dependent upon Ca^{2+} and Mg^{2+} concentrations in a manner similar to thiophosphorylated myosin (Figure 3A). At pCa^{2+} 8 the activity of each actomyosin increased to a maximum as free Mg^{2+} concentrations were raised from 0.44 to 5 mM. Both actomyosins were activated by calcium at free Mg^{2+} concentrations of less than 4 mM, but not at higher concentrations of Mg^{2+}. It might be noted, however, that thiophosphorylated myosin appeared to be slightly more sensitive to the effects of Mg^{2+} than phosphorylated myosin; the activity curves for Mg^{2+}-dependent stimulation of thiophosphorylated myosin (Figure 3A) are shifted leftward relative to those of phosphorylated myosin (Figure 3B) by 0.35–0.4 mM.

The effect of Ca^{2+} on the binding of gizzard tropomyosin to gizzard F-actin at varying Mg^{2+} concentrations was determined to see if the effect of Ca^{2+} on actin-activated ATPase activity at low Mg^{2+} concentrations was due to a Ca^{2+}-mediated binding of tropomyosin to F-actin. The binding of ^{125}I-tropomyosin to F-actin was carried out under the conditions of the ATPase assays. At the molar concentration of these proteins used for actin activation (Tm:A molar ratio =

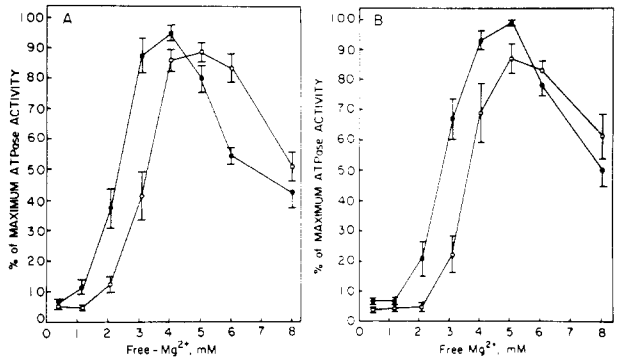

FIGURE 3: Comparison of the calcium and magnesium dependence of the ATPase activity of thiophosphorylated (A) and phosphorylated (B) gizzard myosins. Both myosins were reconstituted with gizzard actin and tropomyosin. Actomyosin ATPase activity for each magnesium concentration was determined at pCa^{2+} 4 (●) and at pCa^{2+} 8 (○) for preparations of myosin which had been 85–100% thiophosphorylated ($n = 7$) or phosphorylated ($n = 5$). For each preparation of myosin, activity at each Mg^{2+} and Ca^{2+} concentration was assayed and expressed as a percentage of the maximum activity observed for that myosin during the assay. Each point represents the mean ± SEM of the percentage of maximum activity obtained under the given assay conditions. Other assay conditions were as described under Materials and Methods. Free Mg^{2+} was determined according to Fabiato & Fabiato (1979), and pCa^{2+} was determined by using a calcium-selective electrode (Affolter & Sigel, 1979).

Table I: Effect of Mg^{2+} and Ca^{2+} on Gizzard Tropomyosin Binding to Gizzard F-Actin[a]

free Mg^{2+} (mM)	tropomyosin bound (%)	
	pCa 4	pCa 8
0.5	0	11.0
1.0	27.0	31.8
2.0	23.2	39.4
3.0	34.5	32.5
6.0	44.8	51.2

[a] Conditions: 2 mM ATP, 50 mM KCl, 20 mM imidazole hydrochloride (pH 7.0), 1 mM DTT, and Ca^{2+} concentration as shown. The amounts and ratios of myosin F-actin and tropomyosin as in Figures 2 and 3. Tropomyosin was iodinated with ^{125}I.

1:6), the amount of tropomyosin bound to F-actin is dependent on the Mg^{2+} concentration as shown in Table I. The binding of tropomyosin to F-actin is dependent on Mg^{2+} both in the presence and in the absence of Ca^{2+}. At 0.5 mM Mg^{2+} tropomyosin was not bound to F-actin at pCa 4, but 11% of the tropomyosin was bound at pCa 8. Upon increasing the Mg^{2+} to 6 mM the binding was raised to about 45% at pCa 4 and to 51% at pCa 8. At a free Mg^{2+} concentration of 3 mM, a concentration around which the maximum Ca^{2+} sensitivity of actin activation was observed, tropomyosin was bound only 2% more at pCa 4 than at pCa 8. At all other Mg^{2+} concentrations, the tropomyosin bound to F-actin is slightly higher in the absence of Ca^{2+}. The maximum binding obtained at the conditions used for the actin activation was around 50%.

Discussion

There is considerable agreement that phosphorylation of smooth muscle myosin is associated with increased actin-activated ATP hydrolysis (Gorecka et al., 1976; Sobieszek, 1977; Chacko et al., 1977; Ikebe et al., 1978; Rees & Frederiksen, 1981). On the other hand, there is some disagreement over the effect of Ca^{2+} on actin activation once the myosin is fully phosphorylated (Chacko et al., 1977; Rees & Frederiksen, 1981; Chacko & Rosenfeld, 1982; Nag & Seidel, 1983). Chacko et al. (1977) reported that the ATPase activity of phosphorylated vas deferens myosin was not fully activated by actin or actin containing tropomyosin unless Ca^{2+} was present. Using thiophosphorylated gizzard myosin reconstituted with rabbit skeletal muscle actin, Sherry et al. (1978) found that activation of the ATPase activity was not dependent on Ca^{2+}. Sobieszek and Small also reported that the phosphorylated gizzard myosin is not dependent on Ca^{2+} once the myosin was phosphorylated (Sobieszek & Small, 1977). Hence, it was suggested that the regulation of actomyosin ATPase of gizzard myosin by calcium depends only on the phosphorylation of the myosin light chain by the Ca^{2+}-dependent kinase (Sherry et al., 1978; Sobieszek & Small, 1977). On the basis of this report it may be argued that the additional calcium dependence observed for vas deferens myosin was either due to a difference in the type of smooth muscle or due to contamination of the actomyosin with kinase and phosphatase (Sherry et al., 1978; Hartshorne & Mrwa, 1982). Subsequent studies on myosin isolated from arterial muscle showed that the actin-activated ATPase activity of this myosin is also calcium dependent; however, the calcium dependence of phosphorylated myosin is shown only at low Mg^{2+} concentrations which favor the binding of Ca^{2+} by myosin (Chacko & Rosenfeld, 1982).

It has been previously reported that even after column purification the gizzard myosin is often contaminated with kinase and phosphatase; hence, measurement of actin-activated ATPase activity in the absence of Ca^{2+} is associated with dephosphorylation of myosin and decrease in the ATPase activity (Sherry et al., 1978). Figure 1 shows that it is possible to obtain myosin free of kinase and phosphatase and that phosphorylation of myosin prior to column purification yields myosin in a stably phosphorylated form. If column purified phosphorylated myosin was used, the level of phosphorylation remained the same throughout the ATPase assay. Addition of [γ-^{32}P]ATP in the ATPase assay mixture did not show the incorporation of ^{32}P into the myosin, indicating that dephosphorylation and rephosphorylation did not take place during the ATPase assay (data not shown).

The finding that actin alone produces a 2–4-fold increase in the ATPase activity of myosin is consistent with our previous observation on arterial myosin (Chacko & Rosenfeld, 1982). This activation of the myosin ATPase by actin was observed irrespective of the source of actin; the level of activation and the sensitivity to Ca^{2+} at low Mg^{2+} concentrations were not remarkably different.

Addition of tropomyosin to actin potentiated the actin-activated ATPase at both 25 and 37 °C at all Mg^{2+} concentrations used. The level of potentiation observed in this study (up to 6-fold) is higher than that has been reported previously (Chacko et al., 1977; Chacko & Rosenfeld, 1982; Nag & Seidel, 1983). This increased level of potentiation observed in this study may be attributed to the sources of actin and tropomyosin used. A recent study by Yamaguchi et al. (1984) using tropomyosins isolated from different sources showed differences in their abilities to potentiate the ATPase activity of gizzard myosin reconstituted with skeletal actin. The calcium stimulation that was most pronounced at about 3 mM free Mg^{2+} was observed irrespective of the source of actin and tropomyosin; however, gizzard actin and tropomyosin appeared to be a better activator of gizzard myosin. Unlike previous reports (Nag & Seidel, 1983; Nag et al., 1983), we found that the Mg^{2+} concentration required for maximal activation by skeletal muscle actin was not different from that required by gizzard actin, provided the gizzard actin was well polymerized at the time it was reconstituted with myosin.

The data presented in Figure 3 indicate that the actin-activated ATPase activity and its modulation by Ca^{2+} over a wide

range of Mg^{2+} concentrations were not remarkably different for phosphorylated and thiophosphorylated myosin. Both myosins gave high activities in the absence of Ca^{2+} when Mg^{2+} concentration was increased higher than 5 mM.

The actin-activated ATPase activity is greatly affected by assay conditions. Sherry et al. (1978) conducted actin activation of thiophosphorylated gizzard myosin at 10 mM (total) $MgCl_2$ in the presence of 2.5 mM ATP and 50 mM KCl and at pH 7.5. The free Mg^{2+} concentration under this condition, as calculated by the program of Fabiato & Fabiato (1979), is higher than 5 mM, a concentration at which Ca^{2+} does not increase the actin-activated ATPase activity as seen in the present study. Since Sherry et al. (1978) conducted the actin activation of thiophosphorylated gizzard myosin at free Mg^{2+} concentrations higher than 5 mM, it is not surprising that they did not observe a decrease in ATPase activity when Ca^{2+} was removed.

The Ca^{2+} dependence of actin-activated ATPase activity reported here resembles those reported by Ebashi and his colleagues (1979) in its requirement for tropomyosin. However, the thin filament mediated regulation proposed by these investigators requires leiotonins in addition to tropomyosin and Ca^{2+} for maximal activation, and it is independent of phosphorylation (Mikawa et al., 1978). Furthermore, the calcium effect they observed was obtained only in the presence of smooth muscle actin and at a Mg^{2+} concentration higher than 5 mM (Ebashi et al., 1979).

The present results indicate that Ca^{2+} regulates the actomyosin ATPase not only by its effect on the phosphorylation of myosin light chain by calmodulin-dependent light chain kinase but also by a direct effect on actin-activated ATP hydrolysis. Further kinetic analysis of this effect is needed to determine whether the Ca^{2+} acts directly on the active site of the enzyme or through the binding of the myosin to the actin.

The binding of ^{125}I-tropomyosin to F-actin under the conditions of the ATPase assay utilized in this study shows a dependence on Mg^{2+} concentration. Similar Mg^{2+} dependence for binding of skeletal muscle tropomyosin to skeletal muscle F-actin (Eaton et al., 1975) and *Acanthamoeba* F-actin (Yang et al., 1979) has been previously reported. A recent study (Sanders & Smillie, 1984) showed that the binding of gizzard tropomyosin to skeletal muscle F-actin is also dependent on Mg^{2+} and a 1:3 molar ratio of tropomyosin to F-actin is required for a 100% binding of tropomyosin at 25 °C and in 10 mM KCl. The difference in actin-activated ATPase activities between pCa 4 and pCa 8 (up to 65%) observed in this study cannot be attributed to a Ca^{2+}-mediated binding of tropomyosin to actin which then potentiates the actomyosin ATPase since the binding of tropomyosin to F-actin is in general higher at pCa 8. The 2% lower binding at 3 mM Mg^{2+} in pCa 8 does not appear to be significant.

The level of tropomyosin binding obtained in this study is similar to that observed by Sanders & Smillie (1984) at a 1:6 molar concentration of gizzard tropomyosin to skeletal muscle actin. Higher levels of tropomyosin binding and actin-activated ATPase activity are observed with increasing the ratio of gizzard tropomyosin to gizzard actin to 1:3; however, the calcium sensitivity of actomyosin ATPase is unaffected by the increased binding of tropomyosin to actin (H. Miyata and S. Chacko, unpublished observation). The apparent lack of relationship between tropomyosin binding and calcium sensitivity of actin activation is not surprising since previous studies have shown that the calcium sensitivity of actin-activated ATPase activity of phosphorylated myosin is observed in the absence of tropomyosin (Chacko et al., 1977; Reese & Frederiksen, 1981; Chacko & Rosenfeld, 1982; Nag & Seidel, 1983). Further studies on the binding of smooth muscle tropomyosin to smooth muscle F-actin under a variety of conditions are needed to fully understand the binding of tropomyosin to actin. In the presence of heavy meromyosin the binding of skeletal muscle tropomyosin (Eaton, 1976) and gizzard tropomyosin (Williams et al., 1984; Lehrer & Morris, 1984) to actin has been reported to be cooperative.

The effect of Ca^{2+} on smooth muscle actomyosin is clearly different from that of the myosin-mediated calcium regulation in the scallop since the latter does not require phosphorylation; activation of actomyosin is accomplished by cooperative binding of Ca^{2+} by the regulatory light chain (Kendrick-Jones et al., 1970). Further studies on scallop myosin by Chantler et al. (1981) showed that Ca^{2+} binds randomly to the two heads of myosin; however, both heads needed to have bound Ca^{2+} before activation of either head. In the smooth muscle system the phosphorylation of the 20 000 Da light chain appears to be cooperative (Persechini & Hartshorne, 1981; Ikebe et al., 1982; Sellers et al., 1983). However, the direct effect of Ca^{2+} on the actomyosin ATPase reported here is a modulation of the actomyosin system which is already "turned on" by phosphorylation.

The finding that the modulation of actin-activated ATPase activity by Ca^{2+} is observed at free Mg^{2+} concentrations thought to be present in smooth muscle cells (Somlyo et al., 1982) strongly suggests that the data obtained from isolated proteins are physiologically relevant. The results obtained from physiological studies carried out with intact smooth muscle strips also suggest an additional need for Ca^{2+} for the maintenance of tension (Gerthoffer & Murphy, 1983).

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

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Chemical Synthesis and Immunological Activities of Glycolipids Structurally Related to Lipid A†

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ABSTRACT: Complete chemical syntheses of a number of monosaccharides derived from 2-deoxy-2-[(3*R*)-3-hydroxytetradecanamido]-D-glucopyranose and structurally related to the hydrophobic moiety (lipid A) of several bacterial endotoxins are described. Selected humoral (complement activation) and cellular (mitogenicity and induction of interleukin 1 production) in vitro activities of a lipid A preparation obtained from the *Bordetella pertussis* endotoxin were compared with those of ten of these monosaccharides and with those of previously synthesized, analogous disaccharides. Results show that each of these in vitro activities of the lipid A preparation can be efficiently induced by at least one of the monosaccharide derivatives.

The isolated lipidic moiety (lipid A) of endotoxins obtained from Gram-negative bacteria expresses a great number of the in vivo and in vitro biological activities of the intact molecule (Galanos et al., 1977). Because of significant species variations, intrinsic microheterogeneity, occlusion of other membrane components, and unavailability of suitable techniques of purification, the exact structure of the natural lipid region is unknown. However, chemical analyses (Rosner et al., 1979; Wollenweber et al., 1982) revealed that this material contains a β-1,6-linked D-glucosamine disaccharide substituted by phosphate groups and by ester- and amide-bound fatty acids. In some lipid A preparations ethanolamine (Mühlradt et al.,

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