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FUNCTIONAL INTEGRITY OF THE SH_1 SITE IN MYOSIN FROM HYPERTROPHIED MYOCARDIUM

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Summary

The hypothesis that an alteration in the SH_1 site of hypertrophy myosin is responsible for the reduced Ca^{2+}-stimulated ATPase activity is examined. The functional integrity of the SH_1 site was evaluated by measurement of the (K^+)-EDTA-stimulated and Mg^{2+}-inhibited ATPase activities. Neither activity differed from control although the Ca^{2+}-stimulated ATPase of the same preparations was significantly reduced. The reduction in Ca^{2+}-activated ATPase was independent of ionic strength. Titration with *N*-ethylmaleimide elevated the Ca^{2+}-stimulated ATPase of hypertrophy myosin to the same peak activity as control. Actin-stimulated ATPase activity of hypertrophy myosin was also reduced. The results indicate that the SH_1 of hypertrophy myosin is functionally intact for (K^+)EDTA-stimulated ATPase and Mg^{2+} inhibition, but functionally deficient with regard to Ca^{2+}-stimulated and actin-activated ATPase activities. This implies a partition of the functional aspects of SH_1.

Introduction

Subjecting the heart to a chronic pressure overload causes the myocardium to hypertrophy. Myosin [1,2] and actomyosin [3,4] isolated from hypertrophied myocardium exhibit a decreased ATPase activity. A possible basis for the reduced activity was suggested by data reported by Shiverick et al. [2]. Although the Ca^{2+}-stimulated ATPase activity of hypertrophy myosin was initially half the activity of control myosin, the difference in activities was eliminated during titration with *p*-chloromercuribenzoate. Titration with the sulfhydril reagent produced an increase in the Ca^{2+}-stimulated ATPase activity of both control and hypertrophy myosin, but the increase was significantly greater for hypertrophy myosin. The Ca^{2+}-activated ATPase of hypertrophy myosin was elevated to the same peak activity as control.

The biphasic response of Ca^{2+}-stimulated ATPase to sulfhydryl titration is well characterized for skeletal myosin [5]. Reaction with one class of reactive sulfhydryl groups (SH_1) per subunit of myosin produces an initial increase in activity [6,7], while subsequent reaction with a second class of sulfhydryl groups (SH_2) results in loss of activity [8]. Coincident with the increase in Ca^{2+}-activated ATPase is loss of (K^+)EDTA-stimulated ATPase [6,7] and an increase in the Mg^{2+}-stimulated ATPase of myosin [6,9]. Similar responses are also observed for Ca^{2+}-stimulated and (K^+)EDTA-stimulated ATPase activities of rabbit cardiac myosin [2,10]. The increase in Ca^{2+}-stimulated ATPase activities of control and hypertrophy myosin during titration with *p*-chloromercuribenzoate [2] was therefore attributed to reaction of the sulfhydryl reagent with the SH_1. The observation that the Ca^{2+}-stimulated ATPase activities of control and hypertrophy myosin were elevated to the same peak activity indicated that the activities were identical following modification of the SH_1. This suggested that an alteration in the SH_1 site was responsible for the reduced Ca^{2+}-activated ATPase of hypertrophy myosin.

This hypothesis is examined in the present study utilizing the known requirements of Ca^{2+}-, (K^+)EDTA-, and Mg^{2+}-stimulated ATpase activities for the SH_1 as well as the more specific reaction of *N*-ethylmaleimide with the SH_1. In addition, the contribution of hypertrophy myosin to the decreased Mg^{2+}-activated ATPase of hypertrophy actomyosin is examined.

Methods

Animal model. Right ventricular hypertrophy was induced in male Canadian albino rabbits (1.8—2.0 kg) by pulmonic banding [2]. Hemodynamic evaluation of this model indicated that hypertrophy develops without evidence of failure [2]. Operated animals were killed 6 weeks postoperatively. The ratio of right ventricular to total heart weight as well as dry weight analyses for right ventricular, left ventricular and liver samples were routinely determined. Values were within the ranges previously reported [2].

Unoperated weight and sex-matched rabbits were used as controls. Two control rabbits were killed with each operated rabbit and the two control right ventricles pooled to provide comparable amounts of tissue.

Myosin preparation. Myosin was prepared according to the method of Shiverick et al. [11] and used immediately following preparation. The myosin was consistently free of protein contaminants on the basis of sodium dodecyl sulfate polyacrylamide gel electrophoresis and routinely had an A_{280nm}/A_{260nm} greater than 1.50. Prior to use, dithiothreitol was removed by dialysis at room temperature for 4 h under nitrogen against 0.5 M KCl, 0.05 M Tris · HCl (pH 7.6). For actin-activation studies, 1 mM imidazole (pH 7.0) was substituted for Tris · HCl.

Actin preparation. Actin was extracted by the method of Rees and Young [12] from an acetone powder of the back and leg muscles of rabbits [13]. A minor protein band at 35 000 daltons, thought to be tropomyosin, was sometimes evident in dodecyl sulfate-polyacrylamide gels of the actin preparations but had no apparent influence on the results. Actin was freshly prepared for each experiment.

Reaction of myosin with N-ethylmaleimide. Myosin, at a concentration of 3.0 mg/ml in 0.5 M KCl, 0.05 M Tris · HCl (pH 7.6), was allowed to react for 2 h at 0°C (10) with molar excesses of *N*-ethylmaleimide as indicated. The reaction was complete within 2 h for all concentrations of *N*-ethylmaleimide as judged by changes in Ca^{2+}-stimulated ATPase activity. The reaction was stopped by the addition of myosin to the ATPase assay mixture, which resulted in a 1 : 40 dilution of the *N*-ethylmaleimide.

Determination of ATPase activity. The Ca^{2+}-stimulated ATPase activity was assayed in 0.05 M KCl, 9 mM $CaCl_2$, 4 mM ATP, and 0.05 M Tris · HCl (pH 7.6). The KCl concentration was increased to 0.5 M [6] for assay following modification with *N*-ethylmaleimide and varied at other times as indicated in the text. The assay mixture for (K^+)EDTA-activated ATPase contained 0.67 M KCl, 1 mM EDTA, 5 mM ATP, and 0.05 M Tris · HCl (pH 7.6). Myosin concentration for both assays was 0.1 mg/ml. The Mg^{2+}-inhibited ATPase of myosin was determined in 0.1 M KCl, 2 mM ATP, 0.05 M Tris · HCl (pH 7.6) and $MgCl_2$ as indicated. Myosin concentration was 0.2 mg/ml. Final volume for each assay was 2.0 ml and reactions were initiated by addition of myosin to the assay mixtures.

Actin-activated ATPase was measured using skeletal F-actin. The F-actin, polymerized in 0.1 M KCl, 1 mM $MgCl_2$ for 2 h at room temperature and collected by centrifugation for 2 h at 105 000 × *g* [12], was suspended in 0.1 M KCl, 0.02 M imidazole (pH 7.0) at a concentration of 5 mg/ml. Myosin, 1 mg ml in 0.5 M KCl, 1 mM imidazole (pH 7.0), was mixed with amounts of F-actin as indicated and allowed to react for 20 min at room temperature [14]. The protein solution was then diluted slowly with 0.02 M imidazole (pH 7.0) to a KCl concentration of 0.07 M and aliquots transferred to incubation vessels. Following a 10 min preincubation, reactions were initiated by addition of substrate. Final assay conditions were 0.07 M KCl, 2.5 mM $MgCl_2$, 2.5 mM ATP, 0.02 M imidazole (pH 7.0), 0.1 mg/ml myosin, and actin as indicated in a final volume of 3.0 ml.

All reactions were carried out at 25°C and stopped by the addition of 1 ml of cold 20% $HClO_4$. Reactions were linear with time. Inorganic phosphate was determined by a modified Fiske-SubbaRow method [15] using a Technicon autoanalyzer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Electrophoresis was performed on 7.5% sodium dodecyl sulfate-polyacrylamide gels prepared according to the method of Weber and Osborn [16]. Protein solutions were prepared as previously described [11]. Gels were stained with Coomassie Brilliant Blue.

Protein determination. Myosin and actin concentrations were calculated using molar extinction coefficients, $E^{280}_{1\%}$ of 5.60 and 10.9, respectively.

Materials. Dithiothreitol was a product of Calbiochem. *N*-Ethylmaleimide was purchased from Eastman Organics and ATP from Sigma Chemical Co. Electrophoresis grade reagents for polyacrylamide gels were obtained from Bio-Rad. All other reagents were analytical grade. Solutions were prepared in deionized distilled water.

Results

Ca^{2+}-stimulated and (K^+)EDTA-stimulated ATPase activities

The dependence of (K^+)EDTA-stimulated ATPase activity on the functional integrity of the SH_1 [6,7] suggested that an alteration in the SH_1 site of hypertrophy myosin would also influence this activity. Measurement of (K^+)-EDTA-activated ATPase for hypertrophy myosin indicated, however, that the activity was not different from control (Table I). The Ca^{2+}-activated ATPase determined simultaneously for the same hypertrophy myosin preparations was decreased by approx. 30% ($P < 0.01$).

It was recognized that the similar (K^+)EDTA-stimulated ATPase activities may have resulted from the high ionic strength at which the assay was carried out. The effect of increasing ionic strength on the Ca^{2+}-stimulated ATPase activity of control and hypertrophy myosin was therefore examined (Fig. 1). Each myosin showed the characteristic decrease in activity with increasing ionic strength [6] and the difference in activities for the two myosins remained constant at all ionic strengths. The difference in Ca^{2+}-activated ATPase of control and hypertrophy myosin persisted at high ionic strength.

Inhibition of myosin ATPase by Mg^{2+}

At low ionic strength and in the absence of Ca^{2+}, myosin exhibits a low level of ATPase activity, analogous to (K^+)EDTA-activated ATPase at low ionic strength. Millimolar concentrations of Mg^{2+} inhibits the activity [6] and this inhibition has been shown to require a functionally intact SH_1 [6,9]. Measurement of this activity for control and hypertrophy myosin over a range of 10^{-6}—10^{-3} M Mg^{2+} yielded superimposable ATPase activity profiles for the two myosins (Fig. 2). Both exhibited a low level of activity in the absence of Mg^{2+} and the activity of each became fully inhibited at 10^{-4} M Mg^{2+}. The Ca^{2+}-stimulated ATPase activities of the control and hypertrophy myosin preparations used in these determinations were similar to the activities indicated in Table I.

Titration with N-ethylmaleimide

N-Ethylmaleimide at low concentrations reacts only with the most reactive sulfhydryl groups of a protein and it was with this reagent that the concept of SH_1 and SH_2 groups in myosin was developed [6,8]. Titration of control and

TABLE I

Ca^{2+}-STIMULATED AND (K^+)EDTA-STIMULATED ATPase ACTIVITIES

The activities were determined simultaneously for the same myosin preparation according to conditions described in Methods. Values are mean ± S.E. for six experiments. The difference in Ca^{2+}-stimulated ATPase activities is statistically significant ($p < 0.01$) by the paired Student's *t*-test.

Myosin	ATPase activity (μmol $P_i \cdot mg^{-1} \cdot min^{-1}$)	
	Ca^{2+}-stimulated	(K^+)EDTA-stimulated
Control	0.286 ± 0.019	0.580 ± 0.015
Hypertrophy	0.197 ± 0.007	0.636 ± 0.025

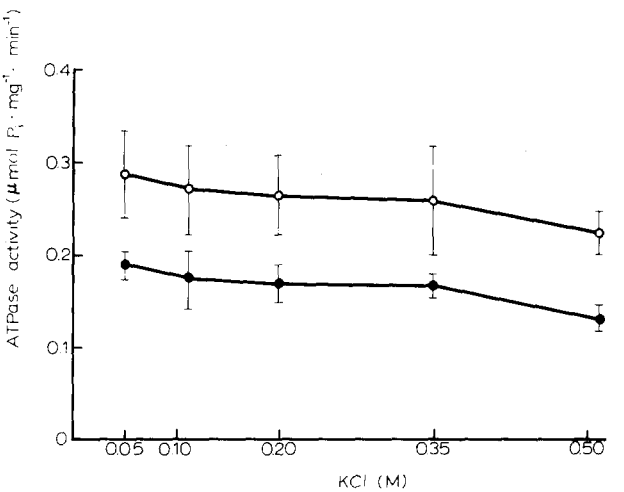

Fig. 1. Effect of increasing KCl concentration on Ca^{2+}-stimulated ATPase activity. The concentration of KCl in the assay mixture was increased as indicated. Each point represents the mean ± S.D. for 2—5 experiments, with a equal number of determinations for control (○) and hypertrophy (●) myosin at a given concentration.

hypertrophy myosin with *N*-ethylmaleimide confirmed the results obtained previously with *p*-chloromercuribenzoate [2] (Fig. 3). Although the Ca^{2+}-stimulated ATPase of hypertrophy myosin was significantly reduced prior to reaction with *N*-ethylmaleimide ($P < 0.01$) and following reaction with a 2-fold molar excess ($P < 0.05$), the difference was abolished following reaction with increased molar ratios of the sulfhydryl reagent. Maximal Ca^{2+}-stimulated ATPase activity occurred at an 8-fold molar excess for both myosins, coincident with near complete loss of (K^+)EDTA-activated ATPase. This response is similar to that previously reported for skeletal [5,6] and cardiac [10] myosin. The similar loss of (K^+)EDTA-activated ATPase by the control and hypertrophy

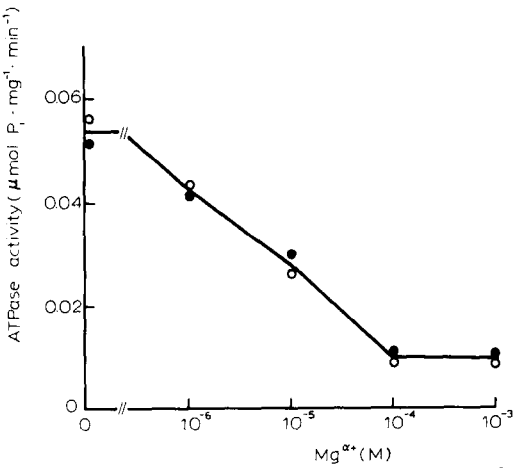

Fig. 2. Inhibition of myosin ATPase activity by Mg^{2+}. Activity was measured as a function of Mg^{2+} concentration by adjusting the concentration of $MgCl_2$ in the assay mixture. The ATP concentration was held constant at 2 mM. The results of one experiment are shown. Two additional experiments gave the same profile. Symbols are the same as in Fig. 1.

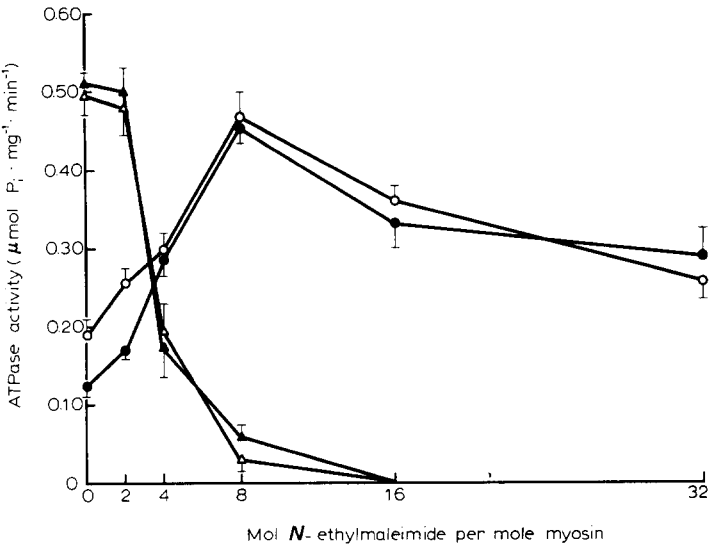

Fig. 3. Effect of titration with *N*-ethylmaleimide on Ca^{2+}-stimulated and (K^+)EDTA-stimulated ATPase activities. Myosin was allowed to react with the indicated molar excesses of *N*-ethylmaleimide for 2 h at 0°C [10]. The Ca^{2+}-stimulated and (K^+)EDTA-stimulated ATPase activities were determined simultaneously for the same myosin preparation: Ca^{2+}-stimulated ATPase of control (○) and hypertrophy (●); (K^+)-EDTA-stimulated ATPase of control (△) and hypertrophy (▲). The Ca^{2+}-stimulated ATPase was determined at 0.5 M KCl. Each point is the mean ± S.E. for five experiments. The Ca^{2+}-stimulated ATPase activities are significantly different prior to reaction with *N*-ethylmaleimide ($P < 0.01$) and following reaction with a 2-fold molar excess of *N*-ethylmaleimide ($P < 0.05$).

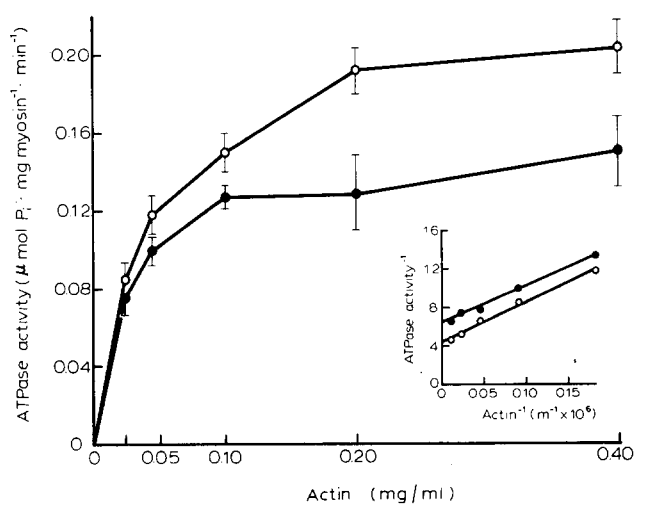

Fig. 4. Actin-stimulated ATPase activity as a function of actin concentration. The myosin was allowed to react for 20 min at room temperature [14] with skeletal F-actin and then diluted to 0.07 M KCl to give a myosin concentration of 0.1 mg/ml and actin concentration as indicated. Each point represents the mean ± S.E. for 3—7 experiments, with an equal number of determinations for control (○) and hypertrophy (●) myosin at a given actin concentration. The difference in activities is statistically significant ($P < 0.05$) at actin concentrations of 0.05 mg/ml and greater. The inset shows a Lineweaver-Burk plot of the mean activites following conversion of the actin concentrations to molar units.

myosin indicated that *N*-ethylmaleimide was equally reactive with both myosins and that the enhanced Ca^{2+}-activated ATPase was due to reaction of *N*-ethylmaleimide with the SH_1. Thus, the difference in Ca^{2+}-stimulated ATPase activities of control and hypertrophy myosin was absent following reaction of *N*-ethylmaleimide with the SH_1 site.

Actin-stimulated ATPase activity

Actin-stimulated ATPase activity of control and hypertrophy myosin was examined as a function of actin concentration. Hypertrophy myosin was activated significantly less than control ($P < 0.05$) at actin concentrations of 0.05 mg/ml and greater (Fig. 4). Actin concentrations were converted to molar units, assuming a molecular weight of 41 700 [17], and kinetic constants were obtained from a Lineweaver-Burk plot of the mean activities. The plot yielded values for V of 0.222 and 0.156 $\mu mol \cdot mg^{-1} \cdot myosin^{-1} \cdot min^{-1}$ and apparent K_m values for actin of $8.7 \cdot 10^{-7}$ and $5.9 \cdot 10^{-7}$ M for control and hypertrophy myosin, respectively. This experiment indicates that the alteration in hypertrophy myosin may be responsible for the reduced Mg^{2+}-activated ATPase of hypertrophy actomyosin [3,4].

Discussion

The reduced Ca^{2+}-stimulated ATPase activity of myosin from hypertrophied hearts was attributed to an alteration of the SH_1 site because titration with *p*-chloromercuribenzoate elevated the activity of the hypertrophy myosin to the same peak activity as the control [2]. Although the dependence of (K^+)-EDTA-activated ATPase [6,7] and inhibition by Mg^{2+} [6,9] on an intact SH_1 suggested that these activities would also be altered with hypertrophy myosin, neither activity differed from control. This indicated that the SH_1 site of hypertrophy myosin is functionally intact with regard to these activities. The findings also indicated that the reduced Ca^{2+}-activated ATPase of hypertrophy myosin did not result from inactive myosin in the preparation. The results obtained for titration of control and hypertrophy myosin with *N*-ethylmaleimide, however, supported the hypothesis that the difference in Ca^{2+}-stimulated ATPase activities is absent following modification of the SH_1. The results obtained for actin-activation are also consistent with the hypothesis of an alteration associated with the SH_1 site of hypertrophy myosin. Studies using spin-labeled myosin have indicated the involvement of the SH_1 site in binding of actin to myosin [18]. It has also been shown that myosin is not activated by actin following modification of the SH_1 with *N*-ethylmaleimide [19,20].

Indirect evidence presently suggests the existence of isozymes of cardiac myosin. The Ca^{2+}-stimulated ATPase activities of rat and rabbit cardiac myosin differ intrinsically and in response to titration with *N*-ethylmaleimide [10]. These differences, however, are influenced by the thyroid state of the animal [21—23]. Cross-innervation studies have already revealed the isozymic nature of skeletal myosin [24,25]. Recent reports of reciprocal changes in cysteine-containing peptides [26] and *N*-methylhistidine content [27] of heavy chains following cross-innervation indicate that the structural alterations may not be limited to light chains. Reported increases in myocardial protein synthesis fol-

lowing onset of a pressure overload [28] and in rates of myosin synthesis and degradation with pressure-induced hypertrophy [29] are consistent with the appearance of an isozyme of cardiac myosin following pulmonic banding.

Although the results presented here are consistent with earlier reports of reduced Ca^{2+}-stimulated ATPase [1,2], there is not general agreement that the ATPase activity of myosin from hypertrophied myocardium is reduced. Wikman-Coffelt et al. [30,31] reported elevated Ca^{2+}-stimulated and (K^+)EDTA-stimulated ATPase activities 3 weeks following pulmonic banding and then an 11% decrease in the same activities 16 weeks after banding. This pattern is the reverse of that reported by Oganessyan et al. [4] for actomyosin ATPase activity following aortic banding. Although changes in light chain content have been reported for hypertrophy myosin with increased activity [30,32], no changes have been observed with a reduced activity [1,2]. Some of the variability may be due to differences in the severity and duration of the imposed stress. It should also be noted that, unlike the results presented here, others [1,30] have reported parallel changes in both the Ca^{2+}-stimulated and (K^+)EDTA-stimulated ATPase activities of hypertrophy myosin. The reason for this discrepancy is unknown.

The results imply a partition of the functional aspects of the SH_1 class of sulfhydryls in hypertrophy myosin. Although the SH_1 remains functionally intact for (K^+)EDTA-activated ATPase and Mg^{2+} inhibition, the SH_1 is functionally deficient with regard to Ca^{2+}-stimulated and actin-activated ATPase activities. It is believed that hypertrophy myosin may represent an isozyme of cardiac myosin, with possible amino acid substitutions and resultant conformational changes near the SH_1 site. Such changes could result in a slower rate of product release and reduced activation by actin. In addition, the reduced actin-activated ATPase of hypertrophy myosin suggests that the alteration may contribute to the depressed contractility observed in pressure-induced myocardial hypertrophy [33,34]. Because alkylation of myosin with *N*-ethylmaleimide also involves other non-essential sulfhydryl groups [35,36] the nature of the possible changes require identification of the reactive sulfhydryls comprising the SH_1 group in hypertrophy myosin. This is currently under investigation in our laboratory.

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