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COOPERATIVE INTERACTIONS BETWEEN THE CONTRACTILE PROTEINS OF CARDIAC AND SKELETAL MUSCLE

GARY BAILIN, MU-JU SHEN and ARNOLD M. KATZ *

Department of Physiology and Biophysics and Department of Medicine, Mount Sinai School of Medicine of the City University of New York, Fifth Avenue and 100th Street, New York, N.Y. 10029 (U.S.A.)

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Summary

The calcium activation of the ATPase (ATP phosphohydrolase, EC 3.6.1.3) activity of cardiac actomyosin reconstituted from bovine cardiac myosin and a complex of actin-tropomyosin-troponin extracted from bovine cardiac muscle at 37°C was studied and compared with similar proteins from rabbit fast skeletal muscle. The proteins of the actin complex were identified by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Half-maximal activation of the cardiac actomyosin was seen at a calcium concentration of 1.2 ± 0.002 (S.E. of mean) μM. A hybridized reconstituted actomyosin made with cardiac myosin and the actin-tropomyosin-troponin complex extracted from rabbit skeletal muscle was also activated by calcium but the half-maximal value was shifted to 0.65 ± 0.02 (S.E. of mean) μM Ca^{2+}. Homologous rabbit skeletal actomyosin showed half-maximal activation at 0.90 ± 0.01 (S.E. of mean) μM Ca^{2+} and the value for a hybridized actomyosin made with rabbit skeletal myosin and the actin-complex from cardiac muscle was found at 1.4 ± 0.03 (S.E. of mean) μM Ca^{2+} concentration. Kinetic analysis of the Ca^{2+}-activated ATPase activity of reconstituted bovine cardiac actomyosin indicated some degree of cooperativity with respect to calcium. Double reciprocal plots of reconstituted actomyosins made with bovine cardiac actin complex were curvilinear and significantly different than those of reconstituted actomyosins made with the rabbit fast skeletal actin complex. The Ca^{2+}-dependent cooperativity was of a mixed type as determined from Hill plots for homologous reconstituted bovine cardiac and rabbit fast skeletal actomyosin. The results show that cooperative interactions in reconstituted actomyosins were greater when the actin-tropomyosin-troponin complex was derived from cardiac than skeletal muscle.

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* Philip J. and Harriet L. Goodhart Professor of Medicine (Cardiology)

The abbreviations used are: ATPase, ATP phosphohydrolase; EGTA, ethylene glycolbis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

Introduction

Contraction of cardiac and skeletal muscle is now believed to be effected by the interaction of six major proteins: myosin, actin, tropomyosin and three components of troponin [1—3]. The actin and myosin interaction is generally recognized to be directly responsible for the contractile process, whereas those interactions involving tropomyosin, the troponin components and actin in the thin filament [1—4] appear to be of primary importance in the mediation of excitation-contraction coupling, and possibly in controlling the intensity of the contractile process. Calcium functions as a trigger for the initiation of interactions between myosin and actin, a phenomenon which can be studied as the regulation of ATPase * activity in vitro [5—7]. One of the important interacting sites for calcium is within troponin, where calcium binds to a protein component of troponin termed Tn-C that has a molecular weight of 18 000 in both cardiac and skeletal muscle [1,3,8,9]. Four Ca^{2+} binding sites have been identified in Tn-C from rabbit skeletal muscle; these have been classified as two low and two high affinity sites [3,7,10,11]. There is some question as to whether all four sites are required for full activation of the ATPase activity of rabbit skeletal actomyosin [12] or whether the low affinity sites alone are involved in the activation process [11]. The binding of calcium to troponin probably initiates cooperative interactions between the proteins of the thin filament [3, 12—14] and negative cooperativity has been demonstrated for the two classes of Ca^{2+}-binding sites in rabbit skeletal troponin [3,7,10]. Studies with other muscle and non-muscle systems indicate that cooperativity also exists in cardiac muscle [15,16], insect flight muscle [17] and blood platelets [18,19].

The present study was undertaken to characterize and compare the cooperative interactions between the contractile proteins of cardiac and fast skeletal muscle.

Experimental Procedure

Bovine cardiac and rabbit skeletal myosin were prepared by methods described previously (20,21]. A complex of actin, tropomyosin and troponin was isolated from acetone-dried cardiac muscle powder at 37° C by the method of Katz [22] with the following modification: the extracting solution contained 0.5 mM ATP, pH 7.0, and 1.0 mM dithiothreitol. For the isolation of the actin-tropomyosin-troponin complex from acetone-dried rabbit skeletal muscle powder, the extracting solution contained 0.1 mM ATP, pH 7.0, but no dithiothreitol. Reconstituted actomyosins were prepared by combining myosin and the actin-tropomyosin-troponin complex at a 3 : 1 weight ratio. Rabbit skeletal actin [23], tropomyosin and troponin [24] were isolated by established procedures.

The ATPase activity of the actomyosins was determined at 25° C in 0.08 M KCl, 5 mM $MgCl_2$, 25 mM histidine and 2 mM ATP at pH 6.8. Reaction media contained $1.5 \cdot 10^{-4}$ M $CaCl_2$ and various amounts of EGTA to attain specific ionized Ca^{2+} concentrations. A Ca-$EGTA^{4-}$ binding constant of $2.2 \cdot 10^6$ at pH

* See second footnote p. 469

6.8 [25] was used to calculate the ionized Ca^{2+} concentration of the Ca-EGTA buffers. The Ca^{2+} concentrations were varied between $2 \cdot 10^{-8}$ and 10^{-4} M; for Ca^{2+} concentrations above 10^{-5} M, a $CaCl_2$ solution was used instead of a Ca-EGTA buffer. Myosin concentration was 0.24 mg per ml, and that of the actin-tropomyosin-troponin complex was 0.08 mg per ml. Reactions were initiated by the addition of ATP and stopped by the addition of 1 ml of 10% trichloroacetic acid. Inorganic phosphate liberation was determined as described earlier [26]. In view of the non-linearity of ATP hydrolysis by actomyosin, conditions were chosen so that reaction velocities could be measured when less than 22% of the ATP was hydrolyzed.

Gel electrophoresis

Polyacrylamide gel electrophoresis was performed with 10% gels in 12.5-cm tubes at a current of 4 mA per tube for 18 h. A 0.1 M phosphate buffer (pH 7.1) containing 0.1% sodium dodecyl sulfate [27] was used according to procedures described recently [28]. Densitometer scanning was accomplished with an E-C Transmission Densitometer equipped with an integrator and a 550—570-nm filter for Coomassie Blue. Molecular weights were estimated by co-electrophoresis of a mixture of standard proteins: cytochrome *c* (12 400); myoglobin (17 800); chymotrypsinogen (25 000); ovalbumin (45 000); bovine serum albumin (67 000); and phosphorylase 'a' (90 000). The purified proteins were obtained from Schwarz-Mann with the exception of phosphorylase, which was obtained from Boehringer-Mannheim.

Protein concentration was determined by the biuret method; bovine serum albumin was used as a standard [29]. All chemicals were of reagent grade and were used without further purification.

Results

Comparison of actin complexes extracted from cardiac and skeletal muscles

The protein complex extracted from the acetone-dried powder of bovine cardiac or rabbit skeletal muscle at 37° C consisted of actin, tropomyosin and the troponin components as judged by gel electrophoresis in sodium dodecyl sulfate (Fig. 1). A 45 000-dalton actin component of the complex of actin-tropomyosin-troponin had the same mobility as that of the rabbit skeletal actin complex (compare gel scan and insert of Figs. 1A and B). The protein component of the cardiac actin complex with a molecular weight of 34 000 resembled tropomyosin and the same protein component was found in the rabbit skeletal actin complex (compare tropomyosin protein bands in gel scan and insert of Figs. 1A and B). At least three additional proteins were present in the complex of actin-tropomyosin-troponin. First, a protein of molecular weight 38 000 was found in the bovine cardiac actin complex that corresponded to the tropomyosin binding component of troponin Tn-T. It is not clear whether this protein was present in the rabbit skeletal actin complex (compare region containing Tn-T protein band in Fig. 1A with Fig. 1B). Secondly, a 28 000-dalton protein in the cardiac actin complex migrated more slowly than a protein that resembled the inhibitory component of troponin, Tn-I, of molecular weight 24 000 in the rabbit skeletal actin complex (compare Tn-I protein bands in gel scan

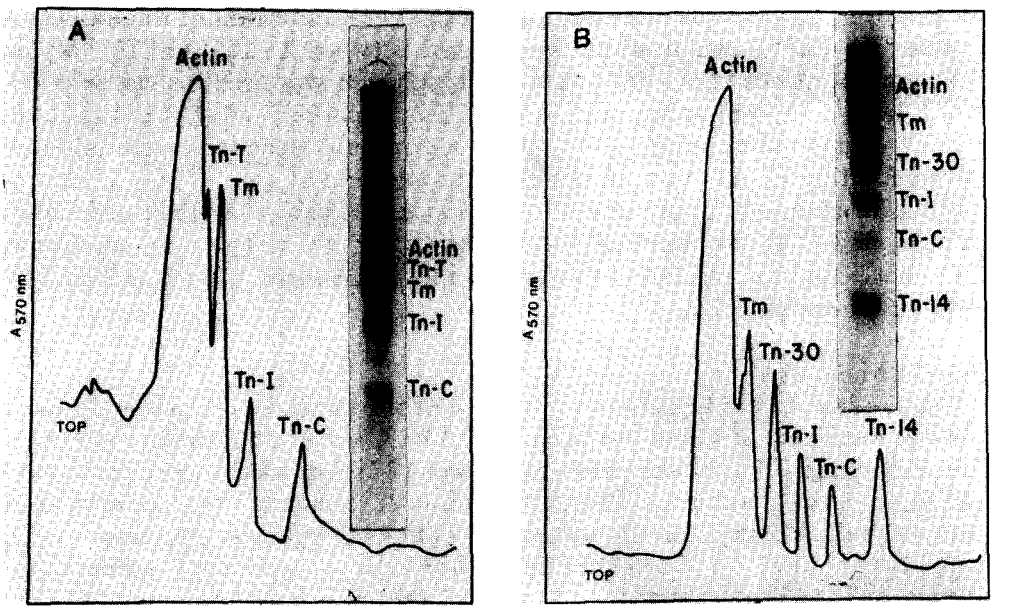

Fig. 1. Gel pattern and densitometric scan of a complex of actin-tropomyosin-troponin. The protein complex was obtained by extracting acetone-dried muscle powder at 37° C. Samples of the actin complex were dialyzed against the electrophoresis buffer solution and 150—200 μg were subjected to polyacrylamide gel electrophoresis under the same conditions. For further details, see Experimental Procedure. A, bovine cardiac actin complex, insert: gel pattern; B, rabbit skeletal actin complex, insert: gel pattern, Tm, tropomyosin; Tn-T, Tn-I and Tn-C, components of troponin; Tn-30 and Tn-14, proteolytic digestion products of Tn-T.

and insert of Figs. 1A and B). The third protein in the cardiac actin complex had a molecular weight of 18 000 and corresponded to the calcium-binding component of troponin Tn-C, that was found in the rabbit skeletal actin complex (compare Tn-C protein bands in gel scan and insert of Figs. 1A and B). The complex of rabbit skeletal actin-tropomyosin-troponin also contained proteins with molecular weights of 30 000 and 14 000 that were not found in the bovine cardiac actin-tropomyosin-troponin complex (Tn-30 and Tn-14 protein bands in Fig. 1B).

Adenosinetriphosphatase activity

Hydrolysis of ATP by homologous reconstituted bovine cardiac actomyosin, made from cardiac myosin and the cardiac actin-tropomyosin-troponin complex, was activated by free Ca^{2+} concentrations in the micromolar range when reactions were carried out as described in Experimental Procedure. The activation response followed a sigmoid pattern (Fig. 2A). When cardiac myosin alone was tested in the assay system, there was no activation of the ATPase activity in the free Ca^{2+} concentration range of $2 \cdot 10^{-8}$ to 10^{-4} M. The ATPase activity of homologous reconstituted cardiac actomyosin was activated by Ca^{2+} with half-maximal activation occurring at 1.2 μM Ca^{2+} (Fig. 2A). A hybridized reconstituted actomyosin made from bovine cardiac myosin and the rabbit skeletal actin-tropomyosin-troponin complex was also activated by Ca^{2+}, but the half-maximal value was reduced to 0.65 μM Ca^{2+}. This difference was significant ($P < 0.01$). However, the ATPase activities of homologous or hy-

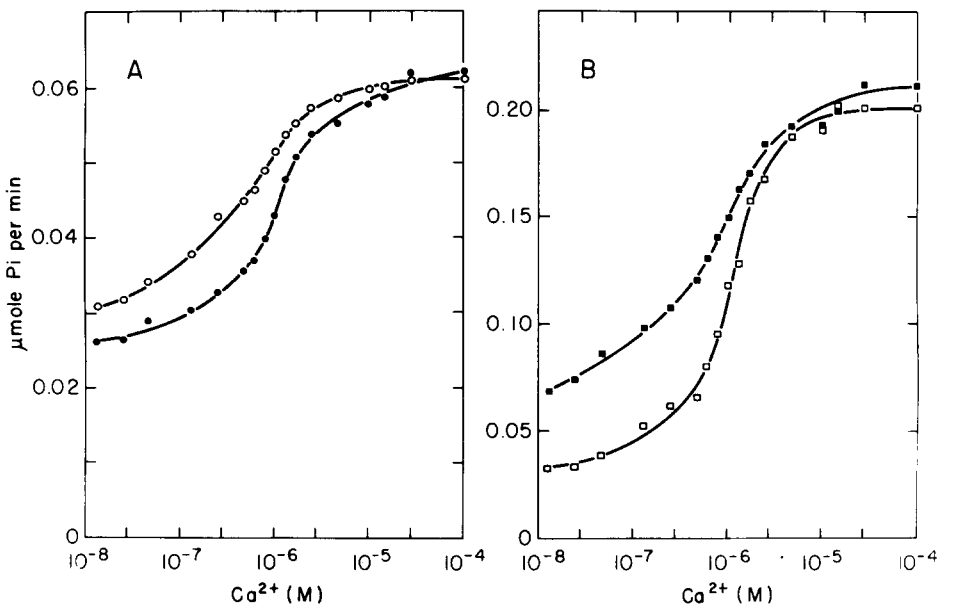

Fig. 2. Effect of Ca^{2+} on the hydrolysis of ATP by reconstituted actomyosins. The assay procedure and determination of ionized Ca^{2+} concentrations are described under Experimental Procedure. A, bovine cardiac myosin complexed with: ●, bovine cardiac actin-tropomyosin-troponin; ○, rabbit skeletal actin-tropomyosin-troponin. B, rabbit skeletal myosin complexes with: ■, rabbit skeletal actin-tropomyosin-troponin; □, bovine cardiac actin-tropomyosin-troponin.

bridized actomyosins at 10^{-4} M Ca^{2+} showed no significant differences (Table I).

Similar experiments performed with reconstituted actomyosins made from rabbit skeletal myosin and the rabbit skeletal actin-tropomyosin-troponin complex (homologous rabbit skeletal actomyosin) or the rabbit skeletal myosin and the complex of bovine cardiac actin-tropomyosin-troponin (hybridized actomyosin) also showed sigmoidal activation of ATPase activity by Ca^{2+} (Fig. 2B). For homologous reconstituted rabbit skeletal actomyosin, the half-maximal activation by Ca^{2+} occurred at 0.9 μM Ca^{2+}. There was a rightward shift to 1.4 μM Ca^{2+} for hybridized reconstituted actomyosin prepared with cardiac actin-tropomyosin-troponin, the difference being significant ($P < 0.01$). There was no significant difference ($P < 0.05$) between the ATPase values obtained

TABLE I

EFFECT OF Ca^{2+} ON THE ATPase ACTIVITY OF RECONSTITUTED ACTOMYOSINS

Reconstituted actomyosin made from		Actomyosin ATPase activity at 10^{-4} M Ca^{2+} (μmol P_i/min per mg)	Half-maximal Ca^{2+} activation (μM Ca^{2+})	Number of experiments
Myosin	Actin-tropomyosin-troponin complex			
Bovine cardiac	Bovine cardiac	0.062 ± 0.001 *	1.2 ± 0.002 *	4
Bovine cardiac	Rabbit skeletal	0.065 ± 0.002	0.65 ± 0.02 **	4
Rabbit skeletal	Rabbit skeletal	0.211 ± 0.027	0.9 ± 0.01	5
Rabbit skeletal	Bovine cardiac	0.20 ± 0.008	1.4 ± 0.03 **	5

* Standard error of the mean

** Students' t-test: $P < 0.01$.

for homologous or hybridized reconstituted rabbit skeletal actomyosins at 10^{-4} M Ca^{2+} (Table I, lower part). The ATPase activity of rabbit skeletal myosin alone was not activated in the range between $2 \cdot 10^{-8}$ and 10^{-4} M Ca^{2+} concentration.

Evidence that the interaction of Ca^{2+} with bovine cardiac or rabbit skeletal reconstituted actomyosins are of a cooperative nature is shown in Fig. 3. Double reciprocal plots of initial velocity and Ca^{2+} concentration (corrected for basal myosin ATPase activity) gave curvilinear patterns for the reconstituted bovine cardiac actomyosins (Fig. 3A). Curvilinearity was more pronounced in the case of homologous reconstituted cardiac actomyosin than for the hybridized reconstituted cardiac actomyosin. Similarly, curvilinear patterns were obtained for the reconstituted rabbit skeletal actomyosins (Fig. 3B). These phenomena are criteria of cooperative interactions [30]; in this case, between Ca^{2+} and the respective actomyosins. It is noteworthy that the patterns of the reconstituted actomyosins made with the cardiac actin complex were more cuvilinear than those made with the rabbit skeletal actin complex (compare Figs. 3A and B).

Cooperativity in the homologous actomyosin was also observed when a graphical solution to the Hill equation was used [30]. The Hill plot of the data in Fig. 2 was curvilinear for the homologous actomyosins (Fig. 4). This pattern was also found for the hybridized actomyosins (not shown).

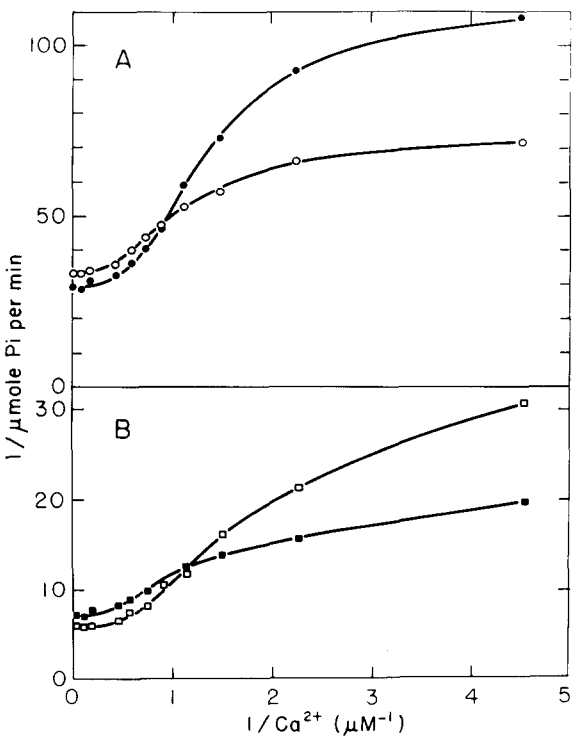

Fig. 3. Double reciprocal plot of the effect of Ca^{2+} on the hydrolysis of ATP by reconstituted actomyosins. A, bovine cardiac myosin complexed with: ●, bovine cardiac actin-tropomyosin-troponin; ○, rabbit skeletal actin-tropomyosin-troponin. B, rabbit skeletal myosin complexed with: □, rabbit skeletal actin-tropomyosin-troponin; ▫, bovine cardiac actin-tropomyosin-troponin.

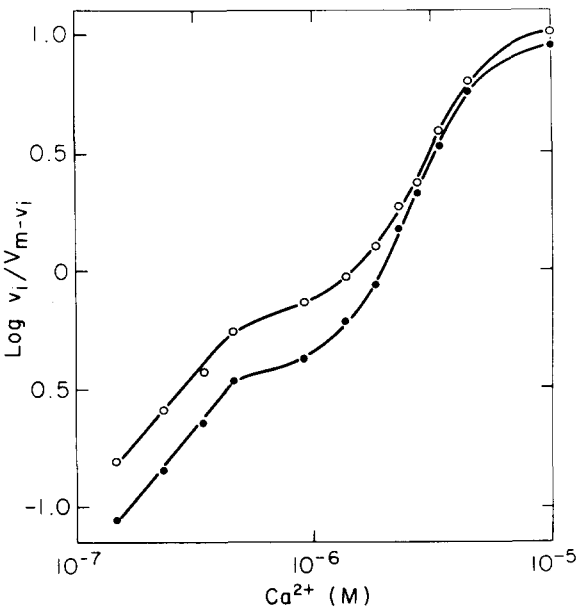

Fig. 4. Hill plot of data from Fig. 2 for homologous reconstituted actomyosins. ●———●, bovine cardiac actomyosin; ○———○, rabbit skeletal actomyosin.

Discussion

Composition of actin complexes extracted from cardiac and skeletal muscles

The gel electrophoresis pattern of the protein complex isolated after extraction of acetone powders of bovine cardiac or rabbit skeletal muscle indicated the presence of actin, tropomyosin, and the subunit components of troponin (Fig. 1A and B). The migration of an 18 000-dalton component in the bovine cardiac and rabbit skeletal actin complex is similar to the migration of the calcium binding component of troponin, Tn-C, in bovine cardiac or rabbit skeletal muscle [9]. This 18 000-dalton component is probably not a contaminating light chain removed from cardiac [31] or rabbit skeletal myosin [31,32] because the actin complex was extracted from the acetone-dried powders under mild conditions, whereas the 18 000 dalton light chain of bovine cardiac myosin is not released even when the myosin is modified extensively with reagents such as 5,5.-dithiobis-(2-nitrobenzoic acid) [31,34] or 1-fluoro 2,4-dinitrobenzene [35]. Moreover, less than 50% of this light chain of rabbit skeletal myosin is removed when the myosin is modified by sulfhydryl reagents [34,36,37].

A 28 000- and a 24 000-dalton protein component was found in the bovine cardiac and rabbit skeletal actin complexes, respectively (compare Tn-I bands in Figs. 1A and B). This is in good agreement with other reports on the molecular weight of the inhibitory component of troponin, Tn-I, in cardiac [9, 38—40] and skeletal muscle [9]. The proteins cannot be contaminating light chains of either bovine cardiac [31] or rabbit skeletal [32,33] myosin, which have a molecular weight in the range of 25 000 daltons. The removal of the 25 000-dalton light chain subunit of rabbit skeletal myosin, for instance, usually requires alkali at high pH or denaturation reagents (ref. 32 and referen-

ces therein). The inhibitory and calcium binding components of troponin did not stain intensely with Coomassie Blue when compared to actin or tropomyosin. Nevertheless, a mass ratio of 1 : 1 was estimated by densitometry for the Tn-I and Tn-C components of the bovine cardiac actin complex. The same stoichiometry has been found in rabbit skeletal troponin [41].

The rabbit skeletal actin complex also contained protein components with molecular weights of 30 000 and 14 000 (Tn-30 and Tn-14 protein bands in gel scan and insert of Fig. 1B). These proteins may be proteolytic digestion products of the tropomyosin binding component of troponin, Tn-T, of molecular weight 37 000 [9,42] or of tropomyosin. These putative proteolytic digestion products, however, were not found in the cardiac actin complex (compare Figs. 1A and B), suggesting that under nearly similar conditions, bovine cardiac Tn-T in the cardiac actin complex is more resistant to proteolytic digestion than is rabbit skeletal Tn-T in its actin complex. These findings are reminiscent of earlier studies regarding the relatively greater resistance of cardiac myosin to trypsin digestion (see for example refs. 20 and 43—45) which reflects structural differences between bovine cardiac and rabbit skeletal myosin [20].

Effects of Ca^{2+} on actomyosin ATPase activity

The ATPase activity of homologous reconstituted bovine cardiac actomyosin made from cardiac myosin and the complex of cardiac actin-tropomyosin-troponin was activated by free Ca^{2+} concentration in the micromolar range (Fig. 2A). The activation by Ca^{2+} followed the sigmoidal pattern which is associated with cooperative behavior in proteins and enzymes [30]. The ATPase activities (0.062 μmol of P_i per min per mg protein) measured at saturating Ca^{2+} concentrations and at a half-maximal activation value of 1.2 μM Ca^{2+} are similar to those obtained previously for cardiac natural actomyosin [46—48], myofibrils [15,49], and reconstituted actomyosin [47,50]. The activation pattern of hybridized reconstituted actomyosin, made from cardiac myosin and the complex of rabbit skeletal actin-tropomyosin-troponin, was sigmoidal, but the half-maximal value was shifted to 0.65 μM Ca^{2+} concentration (Fig. 2A).

Reconstituted actomyosins made from rabbit skeletal myosin and the actin-tropomyosin-troponin complex from rabbit skeletal or bovine cardiac muscle also showed a difference in the Ca^{2+}-activated hydrolysis of ATP (Fig. 2B). The half-maximal value of 0.9 μM Ca^{2+} for homologous reconstituted rabbit skeletal actomyosin is consistent with reports in the literature [6,13,50—53]. The rightward shift of the activation curve for homologous reconstituted rabbit skeletal actomyosin to a half-maximal value of 1.4 μM Ca^{2+} for hybridized reconstituted rabbit skeletal actomyosin (Fig. 2B) appears to be due to effects of the actin complex as suggested by the finding that the half-maximal values for hybridized and homologous actomyosins made with the bovine cardiac actin complex are nearly the same (Table I). This is also the case for hybridized and homologous actomyosins made with the rabbit skeletal actin complex (Table I).

A cooperative role for Ca^{2+} is indicated by double reciprocal plots of the Ca^{2+}-dependence of initial velocity (Fig. 3). Curvilinear patterns, indicative of a cooperative interaction [30], were more pronounced in the case of reconstituted actomyosins made with the cardiac actin complex than those made with

the rabbit skeletal actin complex (compare Figs. 3A and B). This would support the idea that the cooperativity is governed by the complex of actin-tropomyosin-troponin. Differences in the degree of cooperativity were also shown in a Hill plot for homologous reconstituted bovine cardiac and rabbit skeletal actomyosin (Fig. 4). The non-linear patterns are characteristic of mixed types of cooperative interactions, both positive and negative [54,55].

The cooperativity seen in the rabbit skeletal system may be underestimated as proteolytic digestion products of troponin were found in the rabbit skeletal actin complex (Fig. 1B). However, the bovine cardiac actin complex (free of troponin digestion products) when combined with rabbit skeletal myosin showed less cooperativity than the bovine cardiac actin complex combined with bovine cardiac myosin (homologous cardiac actomyosin) (Compare Figs. 3A and B). This also indicates that myosin may play a role in the cooperative interactions.

The differences in cooperativity found in the present study are consistent with the findings of Ebashi et al. who have shown that cardiac and rabbit skeletal troponin differ with respect to their affinities for Sr^{2+} or Ca^{2+} [56] and in the formation of Ca^{2+}-sensitive homologous or hybridized complexes made by essentially reconstituting a troponin from components of cardiac and skeletal sources [9,56]. Binding of Ca^{2+} to troponin and especially its subsequent interaction with the other regulatory proteins, have also been bound to exhibit cooperativity [4,12,13]. Negative cooperativity has been demonstrated for two classes of Ca^{2+} binding sites (high and low affinity) in purified troponin [4,8, 10,16]. The Ca^{2+} binding constant of the low affinity sites is approximately $5 \cdot 10^5$ M^{-1} ($K_{Ca} = 2 \cdot 10^{-6}$ M) as compared to values of $5—25 \cdot 10^6$ M^{-1} ($K_{Ca} = 4 \cdot 10^{-8}$ to $2 \cdot 10^{-7}$ M) for the high affinity sites [4,10] of rabbit skeletal troponin. The cooperativity found in the present study of homologous reconstituted bovine cardiac and to a lesser extent of rabbit skeletal actomyosins is probably due to interactions at the low affinity sites because the major effects are seen in the range of Ca^{2+} concentrations between 10^{-7} and 10^{-6} M. The present data are, therefore, in accord with recent evidence that the low affinity sites may be the physiologically important Ca^{2+} binding regions which govern muscle contraction [11,12].

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Recently, positive cooperativity has been indicated from Ca^{2+} binding studies of glycerinated muscle fibers (Fuchs, F. and Bayuk, M. (1976) Biochim. Biophys. Acta 440, 448—455) and from Ca^{2+} binding data for a reconstituted actomyosin-tropomyosin-troponin system (Murray, J.M., Weber, A. and Bremel, R.D. (1975) in Calcium Transport in Contraction and Secretion (Carafoli, E., Clementi, E., Drabikowski, W. and Margreth, A., eds.), pp. 489—496, North Holland Publ. Co., Amsterdam).

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