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CALCIUM REGULATION OF CARDIAC MYOFIBRILLAR ACTIVATION: Effects of MgATP

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In 2 mM MgATP, 0.08 ionic strength and 1 mM free Mg^{++} cardiac myofibrils bound 3.5 nmoles Ca/mg protein at maximal ATPase activation. Significant amounts of Ca were also bound to cardiac myosin with these same conditions. By subtraction of this myosin-bound Ca we obtained an estimate of 4 moles Ca bound per mole of myofibrillar troponin at maximal ATPase. We found, however, that Ca activation of myofibrillar ATPase could be estimated assuming that only two of troponin's Ca-binding sites are engaged in regulation of crossbridge activity. Increases in MgATP from 0.3 to 5.0 mM raised the free Ca, giving half-maximal isometric tension or ATPase. Although part of this shift is most probably due to changes in the number of rigor (nucleotide-free) actin-myosin linkages, the rightward shift of the free Ca^{++}-activation relation with increase in MgATP from 2 to 5 mM appears to be due to effects of active (nucleotide-containing) actin-myosin linkages.

INTRODUCTION

Although there is ample evidence that calcium activates skeletal (1) and cardiac (2) myofibrillar ATPase and tension development, the stoichiometry and nature of calcium activation are not yet clear. The sequence of reactions leading to myofibrillar activation most probably begins with calcium binding to troponin and ends with the generation of a thin filament site to which thick filament crossbridges may attach. Troponin contains four binding sites for calcium (3, 4) but whether all or part of these sites are necessary for the transduction of the calcium binding signal to crossbridge connection is unknown. Moreover recent results of Bremel and Weber (3, 5) have raised the possibility that crossbridge connections themselves influence the nature and stoichiometry of calcium activation. The results of Bremel and Weber thus suggest that energy transduction at the crossbridge level may influence calcium activation by a sort of positive feedback mechanism.

In the experiments described here, we measured myofibrillar activation and calcium binding and examined the influence of MgATP, the substrate for energy transduction on the calcium-activation process. Results of these experiments show: (a) that increases in the concentration of MgATP raise the free Ca^{++} concentration giving half-maximal tension and ATPase; and (b) that the calcium-activation process most probably involves the binding of two calciums to troponin.

METHODS

Preparations

Canine cardiac myofibrils were prepared essentially as described by Solaro et al. (6) except that the purified myofibrils were washed with 10 pellet vol of 2 mM ethyleneglycol bis (β-aminoethylether) -N, N′ tetraacetic acid (EGTA), 2 mM $MgCl_2$, 60 mM KCl, and 30 mM imidazole at pH 7.0. The EGTA and $MgCl_2$ were removed by three washes in 10 pellet vol of 60 mM KCl, 30 mM imidazole, pH 7.0. The final pellet was suspended in this same solution to a protein concentration of 20–30 mg/ml. Myosin was prepared from dog hearts by slight modification of the procedure of Huszar and Elzinga (7). Chemically skinned right papillary muscles were prepared as previously described (2).

Assays

Contaminant calcium in buffer, salt, ATP, and creatine phosphate stock solutions was determined with an Instrumentation Laboratories (model 353) atomic absorption spectrophotometer fitted with a high solids burner. Unknown and standard calcium solutions contained 1% $LaCl_3$. Calcium in myofibrillar suspensions was solubilized with 2 mM $MgCl_2$, 2 mM EGTA, 60 mM KCl, 30 mM imidazole at pH 7.0. Under these conditions, skeletal myofibrils bind trivial amounts of calcium. Thus, the supernatant fraction obtained from 15 min centrifugation of this suspension at 17,000 × g contains rapidly exchangeable myofibrillar calcium. Calcium in this fraction was determined as described above. Calcium contamination of myosin preparations was determined from a perchloric acid digest of the protein. Standards and unknown calcium solutions were treated identically. Total calcium contamination in binding experiments was 2–5 μM.

Myofibrillar adenosinetriphosphatase (ATPase) was determined as previously described (2) by measuring inorganic phosphate liberation. The reaction was stopped at various times by adding samples of the incubation mixture to an equal volume of ice-cold 10% trichloroacetic acid. Inorganic phosphate was determined by the method of King (8). Experimental conditions are given in the figure legends.

Protein concentrations were determined by the method of Lowry et al. (9) with bovine serum albumin as a standard. Calcium binding by myofibrils was assayed by a centrifugation technique. Two ml of a batch containing myofibrillar protein, [^{3}H]-glucose $^{45}CaCl_2$, and imidazole were added to preweighed conical centrifuge tubes containing 3 ml of a solution containing desired concentrations of $MgCl_2$, creatine phosphate, creatine phosphokinase, KCl, NaCl, EGTA, and calcium. The suspension was mixed well and immediately centrifuged at 2,000 g_{max} for 20 min. Aliquots of the supernatant fraction were counted and the rest was discarded. The tubes were reweighed to determine the pellet volume. The pellet was then suspended in 50 mM $CaCl_2$. Samples of this suspension were added to 2 ml of 2% sodium dodecyl sulfate (SDS) and placed in a boiling water bath for 15–20 min. Pilot studies showed that dissolution of the pellet in SDS produced results identical with those obtained by extraction of the radioactivity in 1.4 M perchloric acid or in NaOH. SDS caused no quenching in the Beckman liquid scintillation system we used. Myofibrillar bound calcium was computed from the total added and contaminant-exchangeable calcium in each experiment and the fraction of myofibrillar-bound $^{45}CaCl_2$.

Corrections for supernatant counts in the pellet were made by using 5 mM [^{3}H]-glucose as a solvent space marker.

Myosin calcium binding was measured essentially as described above except that the mixtures were centrifuged at 100,000 g_{max} for 20 min, and the resultant pellets were dissolved in 0.6 M KCl, and 50 mM $CaCl_2$.

Calculation of Mg^{++}, Ca^{++}, and $MgATP^{2-}$

The system of simultaneous equations describing the multiple equilibria in various incubation solutions was solved either by digital computer, or with a Wang 700 A/B programmable calculator. The ionic strength, pH, temperature, Mg^{++}, Ca^{++}, and $MgATP^{2-}$ were specified for each solution and the computer program calculated total concentrations of $CaCl_2$, $MgCl_2$, Na_2ATP, and KCl to be added to solutions containing constant concentrations of EGTA, imidazole, creatine phosphate, and creatine phosphokinase. NaCl in each experiment was held constant and ionic strength was adjusted with KCl. All experiments contained 2 mM creatine phosphate and 0.1 mg/ml creatine phosphokinase. Free calcium in binding, ATPase, and tension experiments were adjusted by varying both EGTA (0–3 mM) and calcium. Details of the computation method and the constants employed will be published elsewhere.

Solutions and Reagents

All solutions were prepared with distilled deionized water. Stock $CaCl_2$ solutions were prepared from $CaCO_3$ and HCl and neutralized with KOH. Na_2ATP (low in calcium), creatine phosphate, creatine phosphokinase, and imidazole were obtained from Sigma Chemical Co. EGTA was obtained from Eastman Organic Chemicals, Div., Eastman Kodak Co. $^{45}CaCl_2$ and [^{3}H]-glucose were obtained from New England Nuclear. All other chemicals were analytical grade reagents.

RESULTS

A problem in determining the stoichiometry of calcium activation of myofibrils is that calcium binding is most accurately measured with isolated myofibrils which split ATP but do not generate force. We therefore first wished to examine whether calcium activation of cardiac myofibrillar ATPase was closely related to calcium activation of force development. We measured ATPase and isometric tension development with two different experimental conditions: 5 mM MgATP and 0.14 M ionic strength and 2.0 mM MgATP and 0.08 M ionic strength. Free Mg^{++}, temperature, and pH were the same. The results of these experiments, which are shown in Fig. 1A and B, show that within experimental scatter, tension and ATPase are closely related functions of free Ca^{++}. Data in Fig. 1 also show that increasing MgATP not only causes a rightward shift of the free Ca^{++}-activation relation but also an increased steepness. Changes in ionic strength from 0.10 to 0.14 M cause no change in the free Ca^{++} activation relation (10, 11), so that the rightward shift noted is most likely to be due to the different MgATP concentration. To test this more directly we measured the activation of myofibrillar ATPase by free Ca^{++} with 0.3, 2.0, and 5.0 mM MgATP and a constant ionic strength. Fig. 2 shows results of this series of experiments. Again there was a rightward shift of the free Ca^{++} relation.

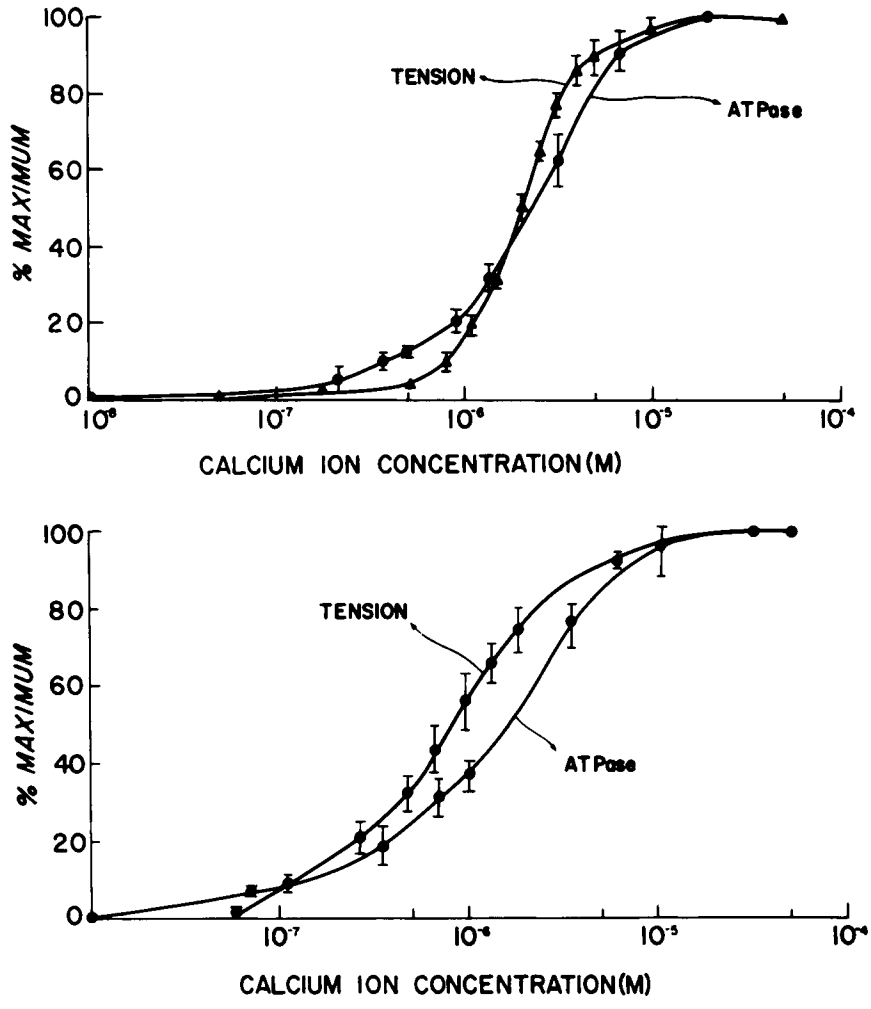

Fig. 1. Effect of calcium ion concentration on myofibrillar ATPase and isometric tension of "chemically skinned" papillary muscle. ATPase and tension at 10^{-8} M Ca^{++} was subtracted from total ATPase and tension before normalizing the data. Bars indicate SEM of three experiments. A; 5 mM MgATP, 1 mM free Mg^{++}, 20 mM imidazole pH 7.0, 25°C, ionic strength 0.14 M. B; 2 mM MgATP, 1 mM free Mg^{++}, 20 mM imidazole pH 7.0, temperature 25°C ionic strength 0.08 M. See Methods for details.

Fig. 3 shows the relation between myofibrillar-bound calcium, myosin-bound calcium, and myofibrillar ATPase. Myofibrillar ATPase and calcium binding were measured with 2 mM MgATP. Myosin calcium binding studies were made in the absence of MgATP, since nucleotide does not change calcium binding (5, 10). The dotted line in Fig. 3 is the difference between myofibrillar bound calcium and myosin-bound calcium, assuming that 50% of the myofibrillar protein is myosin (11). We feel it is not unreasonable to conclude

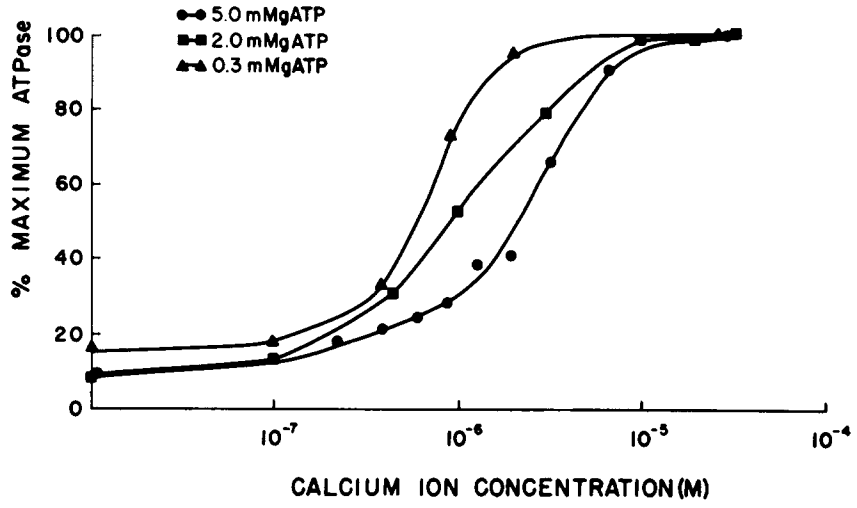

Fig. 2. Effect of MgATP on Ca^{++} activation of myofibrillar ATPase. MgATP as indicated, 1 mM free Mg^{++}, 20 mM imidazole pH 7.0, 25°C, ionic strength 0.14 M. See Methods for details.

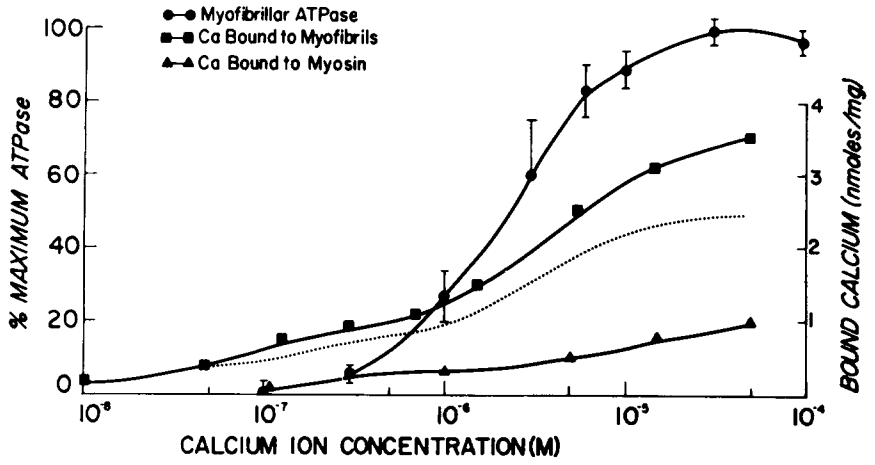

Fig. 3. Effect of calcium ion concentration on myofibrillar ATPase, myofibrillar calcium binding, and myosin calcium binding. Conditions for myofibrillar calcium binding and ATPase as in Fig. 1B. Bars indicate SEM of three experiments. Myosin calcium binding was done in the absence of ATP but all other conditions remained the same. See text for details.

that the dashed line in Fig. 3 thus represents calcium binding to troponin. We have previously (2) measured calcium binding to myofibrils with conditions similar to experiments reported in Fig. 1B. There are clearly different calcium requirements for myofibrillar activation in 2 mM vs. 5 mM MgATP. For example, at 100% activation there were nearly

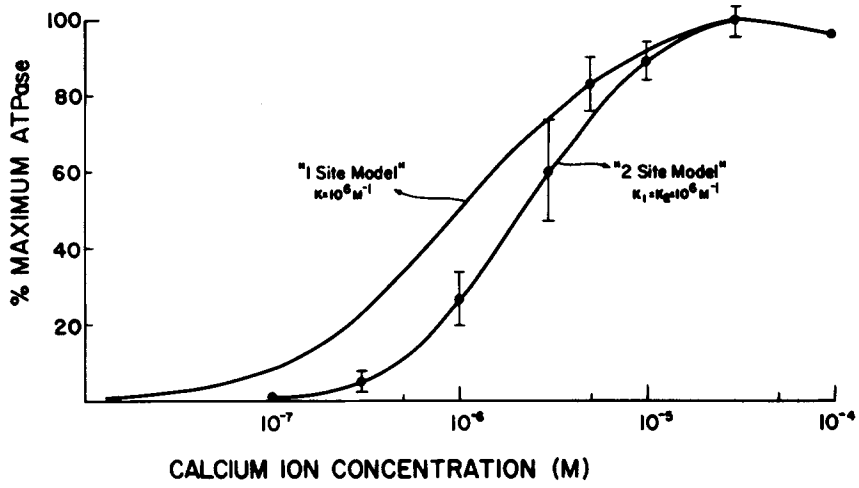

Fig. 4. Experimental and computed relation between calcium ion concentration and myofibrillar ATPase. See text for details.

twice as many calciums bound in 2 mM MgATP than in 5 mM MgATP. Assuming that the actin (mol wt = 45,000) content of heart myofibrils is 20% and utilizing a molar ratio of actin to troponin of 7.0 (12), we calculated that at maximal tension with 2 mM MgATP, about 4.0 moles of calcium are bound per mole troponin and with 5 mM MgATP about 2.0 moles of calcium are bound per mole of troponin.

DISCUSSION

In agreement with tension (13) and ATPase (5) measurements made with skeletal myofibrils, we found that decreasing the concentrations of MgATP from 2.0 to 0.3 mM caused a leftward shift of the relation between free calcium concentration and myofibrillar activation (Fig 2). This shift is most easily explained by an increased number of rigor linkages at the lower MgATP concentrations. Bremel and Weber (3) have shown that the calcium affinity of thin filaments increases when occupied by a critical number of rigor linkages. Increasing the MgATP concentration from 2.0 to 5.0 mM caused a rightward shift of the free calcium dependence of isometric tension and ATPase but these data are not easily explained by a change in occupation of the thin filament with rigor linkages. It therefore seems conceivable that changes in the thick filaments or in thick filament-thin filament interactions induced by changes in MgATP in the millimolar range may alter the calcium activation in manner related more to active (nucleotide containing) than rigor links.

Weber and Murray (14) recently reviewed other evidence which suggests that co-operative interactions between myofibrillar proteins are functions of active as well as rigor linkages. Two such pieces of evidence are: (a) observations that actomyosin ATPase is higher in the presence than in the absence of regulatory proteins; and (b) observations

that myofibrillar ATPase increases hyperbolically with increases in MgATP concentration. These observations taken with Bremel and Weber's data (3) and our data reported here suggest that the steady state concentration of active complexes may alter activity and calcium regulation of myofibrils.

We found that more calcium was bound at maximal activation in 2 mM MgATP, 0.08 M ionic strength, vs. 5 mM MgATP, 0.14 M ionic strength. After correcting for calcium bound to myosin under our experimental conditions, we found that troponin in the myofibril bound about 4 moles Ca/mole troponin with 2 mM MgATP. These data suggest that only part of troponin's calcium binding sites are involved in activation at least under some experimental conditions.

Since we and others (5) have found that activation of the contractile apparatus could occur with the binding of two calciums to troponin under some conditions, we wished to determine whether the binding of two calciums to troponin could account for the activation data in Fig. 3. To do this requires estimates of the number and apparent affinity of binding sites regulating force or ATPase. We also assume that calcium binding to troponin and not myosin regulates generation of thin filament sites for crossbridge attachment. This seems a reasonable assumption in view of evidence that calcium between 10^{-7} and 10^{-5} M has no effect on ATPase of pure actin and myosin (5) in mM Mg^{++}.

A reasonable estimate of calcium saturation of troponin in a number of experiments could be obtained assuming four equivalent noninteracting sites with an apparent affinity of $\sim 10^6\ M^{-1}$. This means that calcium bound at each site at any free Ca^{++} could be calculated from equation (1),

$$CaTn = \frac{n_i\ K\ [Ca^{++}]}{1 + K\ [Ca^{++}]}, \quad (1)$$

where CaTn is the troponin-bound calcium, n is the number of binding sites, and K is the apparent affinity constant. If ATPase or tension were a simple function of calcium binding to a single site, one would obtain the curve-labeled "1-site model" in Fig. 4 for the calcium dependence of myofibrillar ATPase. If, however, myofibrillar ATPase required simultaneous occupation of two sites with calcium; then ATPase would be proportional to $[CaTn]^2$ (15). Figure 4 shows the relation between free Ca^{++} and ATPase one can compute for this "2-site model" provides an excellent fit of the experimental points. This result agrees well with the model of Asley and Moisesco (15) obtained from nonsteady state studies of calcium transients. Although the data presented here do not exclude a model requiring occupation of all four troponin sites by calcium in situ, we feel they provide evidence in favor of the view that only two of troponin's calcium binding sites function in regulation of crossbridge activity.

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