Published in final edited form as:

Biochem Biophys Res Commun. 2011 May 20; 408(4): 697–700. doi:10.1016/j.bbrc.2011.04.092.

The $\text{Ca}^{2+}/\text{Mg}^{2+}$ Sites of Troponin C Modulate Crossbridge-Mediated Thin Filament Activation in Cardiac Myofibrils[†]

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Abstract

The $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites (III and IV) located in the C-terminal domain of cardiac troponin C (cTnC) have been generally considered to play a purely structural role in keeping the cTnC bound to the thin filament. However, several lines of evidence, including the discovery of cardiomyopathy-associated mutations in the C-domain, have raised the possibility that these sites may have a more complex role in contractile regulation. To explore this possibility, the ATPase activity of rat cardiac myofibrils was assayed under conditions in which no Ca^{2+} was bound to the N-terminal regulatory Ca^{2+} -binding site (site II). Myosin-S1 was treated with N-ethylmaleimide to create strong-binding myosin heads (NEM-S1), which could activate the cardiac thin filament in the absence of Ca^{2+} . NEM-S1 activation was assayed at pCa 8.0–6.5 and in the presence of either 1mM or 30 μM free Mg^{2+} . ATPase activity was maximal when sites III and IV were occupied by Mg^{2+} and it steadily declined as Ca^{2+} displaced Mg^{2+} . The data suggest that in the absence of Ca^{2+} at site II strong-binding myosin crossbridges cause the opening of more active sites on the thin filament if the C-domain is occupied by Mg^{2+} rather than Ca^{2+} . This finding could be relevant to the contraction-relaxation kinetics of cardiac muscle. As Ca^{2+} dissociates from site II of cTnC during the early relaxing phase of the cardiac cycle, residual Ca^{2+} bound at sites III and IV might facilitate the switching off of the thin filament and the detachment of crossbridges from actin.

Keywords

troponin; thin filament; myosin crossbridges; magnesium; NEM-S1

Introduction

Cardiac troponin C (cTnC) has three functional Ca^{2+} -binding sites, namely, site II in the N-terminal domain and sites III and IV in the C-terminal domain. Site II is specific for Ca^{2+} , but with a lower Ca^{2+} affinity ($K \sim 10^5 \text{ M}^{-1}$) than sites III and IV, and binding to this site activates crossbridge cycling and force development. Sites III and IV can bind either Ca^{2+} or Mg^{2+} and have been generally considered to play a purely structural role in keeping cTnC bound to the thin filament [1]. However, suggestions have been made that sites III and IV might have a more complex role in contractile regulation [2; 3]. This idea is reinforced by the recent discovery of cardiomyopathy-associated mutations [4; 5] in the C-domain of

[†]This work was supported by the National Institutes of Health (grant HL-91162)

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cardiac troponin C. Evaluation of the possible regulatory role of the C-domain of cTnC must take into account the more complex cation-protein interactions at this site, as compared to the N-terminal domain. .

The free intracellular $[Mg^{2+}]$ in intact cardiac myocytes has been found to be in the range of 0.5-0.7 mM [6]. The Mg^{2+} binding constant to sites III and IV of cTnC is $\sim 5 \times 10^3 M^{-1}$ [7] and the binding constant for Ca^{2+} at these sites is $\sim 3 \times 10^8 M^{-1}$. Thus in the relaxed state, with $pCa > 7.0$, sites III and IV would be primarily occupied by Mg^{2+} (cTnC·2 Mg^{2+}). During activation the stronger binding Ca^{2+} ions would displace Mg^{2+} as well as bind to vacant site II (cTnC·3 Ca^{2+}). A regulatory role for the C-domain of cTnC has generally been discounted on the grounds that, in contrast to the rapid Ca^{2+} binding to site II, the Ca^{2+} - Mg^{2+} exchange at sites III and IV is very slow relative to the kinetics of contraction and relaxation [7]. Thus in a single twitch very little Ca^{2+} would bind to the C-domain, even as site II is occupied. However with the repetitive activity characteristic of cardiac muscle the Ca^{2+}/Mg^{2+} binding ratio should change as a function of heart rate. It has been suggested that the C-domain of cTnC may have a role in modulating contractile activity in accordance with activity pattern [2]. To explore this possibility we have assayed thin filament activation of rat cardiac myofibrils under conditions in which no Ca^{2+} was bound to the N-terminal regulatory site (site II). Myofibrils were reacted with myosin S1 treated with N-ethylmaleimide (NEM-S1) to create strong binding myosin heads that can activate the thin filament in the absence of Ca^{2+} . We have found that NEM-S1 induced significantly higher activation of myofibrillar ATPase when sites III and IV of cTnC were occupied by Mg^{2+} rather than Ca^{2+} , suggesting a role for the C-domain of cTnC in modulation of crossbridge dependent thin filament activation.

Materials and Methods

Myofibrils were prepared from rat ventricle essentially as described by Solaro, et al [8], with minor modifications. They were stored at $-20^{\circ}C$ in a solution of 50% glycerol, 40mM KCl, 10 mM MOPS, pH 7.0, 0.5 mM $MgCl_2$, and 0.25mM dithiothreitol. Rabbit skeletal myosin S-1 was prepared according to Weeds and Taylor [9]. The reaction of S1 with N-ethylmaleimide and the separation of the purified NEM-S1 was carried out according to Swartz and Moss [10]. However, instead of dialysis, repeated cycles of dilution and ultrafiltration with Amicon Ultra 10 kDa filters were used for purification and concentration of the final product. Myofibril protein concentration was determined with BCA reagent (Pierce).

For assay of myofibrillar ATPase activity, aliquots (50 μ l) containing 0.2 mg/ml myofibril protein, $\pm 1 \mu$ M NEM-S1, 100 mM KCl, 20 mM MOPS (pH 7.0), 1 mM EGTA, 1 mM NTA, and required concentrations of $MgCl_2$ and $CaCl_2$ were incubated at $30^{\circ}C$ in Eppendorf centrifuge tubes. ATPase activity was assayed with free Mg^{2+} set at 1 mM or 30 μ M. The program MAXCHELATOR [11] was used to calculate the concentrations of free Mg^{2+} and Ca^{2+} . The reaction was initiated with the addition of 1 mM ATP and it was terminated after 10 min by the addition of 50 μ l of 10% trichloroacetic acid. Following centrifugation at 14000 RPM for 5 min in an Eppendorf microcentrifuge, 50 μ l of supernatant was transferred to a glass tube, and inorganic phosphate was determined with malachite green reagent as described by Lanzetta, et al [12], with minor modifications. All measurements were carried out in a pCa range in which myofibrillar ATPase activity is at the lowest. Since the NEM-S1 has some residual ATPase activity, each assay involved a measurement of NEM-S1 alone, myofibrils alone, and myofibrils + NEM-S1, so that each measurement could be corrected for the contribution of the NEM-S1.

Results

Thin filament activation was brought about by addition of strong-binding NEM-S1 and ATPase activity was assayed with the free Mg^{2+} set at either 1 mM or 30 μM . Based on binding and kinetic studies [7] it is assumed that in the isolated myofibril exposed to 1 mM free Mg^{2+} sites III and IV will be occupied exclusively by Mg^{2+} at very low Ca^{2+} levels (pCa 8.0). As the Ca^{2+} concentration is elevated in the intermediate range (pCa 7.5-6.5) progressively more Mg^{2+} will be displaced by Ca^{2+} . On the other hand, if the free Mg^{2+} is 30 μM these sites should be largely empty at pCa 8.0 and occupied solely by Ca^{2+} as the Ca^{2+} concentration is elevated. Variation in free $[\text{Ca}^{2+}]$ was restricted to a range in which little or no Ca^{2+} would be bound at the regulatory site II.

As shown in Fig 1, the addition of 1 μM NEM-S1 to a myofibril suspension in the presence of 1mM free Mg^{2+} at pCa 8.0 produced an ~3.5-fold increase in myofibrillar ATPase activity. Further additions of Ca^{2+} up to pCa 6.5 produced no substantial change in baseline ATPase activity but the effect of NEM-S1 was attenuated with the increase in $[\text{Ca}^{2+}]$, until at pCa 6.5 the degree of activation was ~2-fold. The stability of the baseline ATPase activity, obtained in the absence of NEM-S1, indicates that no Ca^{2+} binding to the regulatory site II in the N-domain of cTnC occurs in the pCa range studied and provides strong support for the assumption that Ca^{2+} binding at the C-domain is responsible for the observed results. With 30 μM free Mg^{2+} there was only a slight increase in baseline ATPase activity over the pCa range 8.0-6.5 (Fig. 2). Addition of NEM-S1 caused an ~2-fold increase in ATPase activity at all pCa values. In Fig. 3 the percent NEM-S1 activation is plotted as a function of pCa at 1 mM Mg^{2+} and 30 μM Mg^{2+} . The plots clearly differ at pCa 8.0 but converge at pCa 6.5.

Discussion

Although suggestions have been made that the C-domain of cTnC might have a modulatory role in contractile regulation [2; 3] experimental data in support of this assertion are sparse. Such a role is suggested by studies showing that the structure of the C-domain and the strength of the interaction with cardiac troponin I (cTnI) depends on whether sites III and IV are occupied by Ca^{2+} or Mg^{2+} [2; 3]. The discovery of mutations in the C-domain which can cause either hypertrophic cardiomyopathy (D145E) or dilated cardiomyopathy (G159D) also suggest a more complex function for this region [4]. Another recent development relates to the effects of modifiers of Ca^{2+} sensitivity which act through the C-domain. Thus EMD 57033 sensitizes skinned cardiac fibers to Ca^{2+} [13], whereas the green tea polyphenol epigallocatechin 3-gallate reduces Ca^{2+} sensitivity [14]. Both bind to the C-domain [15; 16] but how they produce opposite effects remains to be determined.

By using NEM-S1 to activate the thin filament we could directly compare apo-cTnC, cTnC·2 Mg^{2+} , and cTnC·2 Ca^{2+} with respect to any modulating effect on crossbridge-mediated thin filament activation. As Ca^{2+} is added in the range below that needed to activate ATPase activity (pCa 8.0 – 6.5) it is likely that there will be a variable population of mixed species (cTnC· Mg^{2+} · Ca^{2+}). However, based on known binding constants [7], it seems reasonable to assume that at pCa 8.0 and 1 mM Mg^{2+} the dominant species will be cTnC·2 Mg^{2+} and at pCa 6.5, with either 1 mM or 30 μM Mg^{2+} , the dominant species will be cTnC·2 Ca^{2+} . At 30 μM Mg^{2+} and pCa 8.0, the C-domain sites should be largely empty. Maximum ATPase activation by NEM-S1 occurred when Mg^{2+} was the only cation bound at sites III and IV. The level of activation declined as Mg^{2+} was displaced by Ca^{2+} .

Our result can be explained in terms of the 3-state model of thin filament activation in which strong binding crossbridges can displace tropomyosin from its blocking position on F-actin

[17; 18]. By employing NEM-S1 the Ca^{2+} -dependent transition from the blocked state to the closed state is bypassed. It is less clear why the presence of Mg^{2+} at the C-domain of TnC would promote this effect. The high-resolution structure of the core domain of troponin suggests that the effect might involve the inhibitory segment of TnI. This segment binds to F-actin in the blocked state and to the N-terminal α -helix of site III of TnC in the activated state of the filament [19]. Since NEM-S1 induced thin filament activation requires the inhibitory segment of TnI to dissociate from its binding site on actin, the implication is that the interaction of that segment with TnC is stronger when sites III and IV are occupied by Mg^{2+} rather than Ca^{2+} . Evidence for the $\text{Ca}^{2+}/\text{Mg}^{2+}$ dependent differences in the interaction between the C-domain of TnC and TnI have been presented based on NMR [3] and microcalorimetric studies [2]. Stereospecific metal-ligand interaction requirements and structural constraints may provide the basis for the difference in the Mg^{2+} vs. Ca^{2+} bound structure of TnC [20].

It is of interest to compare these results with data obtained in skinned skeletal and cardiac muscle fibers where NEM-S1 was added in the pCa range in which Ca^{2+} alone produced either no activation or just slight activation of force [21]. In these studies steady state force and the rate constant for force redevelopment following quick release (K_{tr}) were measured as a function of pCa. There is ample evidence that K_{tr} reflects the level of thin filament activation [22; 23]. NEM-S1 increased the steady-state force over the entire low Ca^{2+} range. It also increased K_{tr} but, unlike steady state force, this effect was diminished as $[\text{Ca}^{2+}]$ was increased. That is, K_{tr} was maximal when only Mg^{2+} was bound to TnC and apparently decreased as Ca^{2+} displaced Mg^{2+} from the C-domain. In fact, with only the cTnC· Mg^{2+} complex present (1 mM Mg^{2+} , pCa 9.0) the K_{tr} value for skinned cardiac muscle was the same as that recorded at full Ca^{2+} saturation (pCa 4.5) when only the cTnC·3 Ca^{2+} was present.

These data call attention to the possible differences between cTnC·3 Ca^{2+} , cTnC·2 Ca^{2+} , and cTnC·2 Mg^{2+} with respect to the influence of each on myosin-mediated thin filament activation. In the latter two complexes the N-terminal regulatory site is unoccupied and, based on the data shown above, it would appear that the cTnC·2 Mg^{2+} complex is more effective than cTnC·2 Ca^{2+} at promoting myosin-mediated activation of the thin filament. With respect to physiological relevance, it is important to know which are the predominant cTnC complexes formed under a given set of conditions. Unfortunately, direct measurement in the intact muscle fiber is not feasible at the present time. It is agreed that very little Ca^{2+} will exchange with Mg^{2+} at the C-terminus during the time course of a single twitch [7]. However, in a beating heart the interval between contractions is relatively short and it becomes shorter still as the heart rate increases. Along with the shorter interval between beats and the increased action potential frequency there is an increased Ca^{2+} influx into the myocyte [24]. Thus as the heart rate increases the $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio at the C-terminus should increase. An important property of cardiac muscle is that as the heart rate increases the rate of relaxation increases as well [24]. This rate-dependent acceleration of relaxation plays an important role in optimizing ventricular filling in the presence of a reduced diastolic interval.

During activation at higher heart rates the formation of the cTnC·3 Ca^{2+} complex will play the dominant role, leading to a rapid thin filament activation and force development. Relaxation begins with the dissociation of Ca^{2+} from site II, thus increasing the population of cTnC·2 Ca^{2+} . However, there is now agreement that the rate of relaxation is determined by processes intrinsic to the cardiac sarcomere rather than by the rate of Ca^{2+} removal from site II [24; 25; 26]. The mechanisms responsible for the fast relaxation kinetics are still being investigated and probably involve several different steps in the crossbridge cycle. Among the factors implicated in rapid relaxation are lengthening-induced strain on cross-bridges,

leading to their more rapid detachment from actin, and phosphate-induced reversal of the crossbridge power stroke [26]. Also, evidence has been presented indicating that increase in heart rate is associated with augmented levels of myofilament protein phosphorylation and reduced myofilament Ca^{2+} sensitivity [27]. We suggest that the C-domain of cTnC may influence relaxation rate as a consequence of the formation of cTnC- 2Ca^{2+} early in the relaxation phase. By reducing thin filament activation, the Ca^{2+} bound to sites III and IV, acting in conjunction with factors listed above, might promote a faster rate of relaxation. As suggested previously [2], variation in the $\text{Ca}^{2+}/\text{Mg}^{2+}$ binding ratio at sites III and IV has the potential to provide an on-going measure of the frequency and intensity of Ca^{2+} activation in cardiac muscle. To determine whether the $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites do indeed serve such a function will require a more extensive investigation of the kinetic and structural correlates of C-terminal cation binding.

Acknowledgments

We would like to thank Dr. Sam Lehrer for critical reading of the manuscript.

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Figure 1.

The effect of NEM-S1 on myofibrillar ATPase activity at varying free Ca²⁺ in the presence of 1 mM free Mg²⁺. Assay mixtures contained: 0.2 mg/ml protein, $\pm$ 1 μ M NEM-S1, 100 mM KCl, 20 mM MOPS (pH 7.0), 1 mM EGTA, 1 mM NTA, 1mM ATP, and added MgCl₂ and CaCl₂ to set the required free metal ion concentrations. Temperature, 30°C; incubation time 10 min. Each point is mean $\pm$ SEM of 3-12 measurements. The maximum Ca²⁺-activated ATPase activity was 159 $\pm$ 15 nmol Pi/mg/min

Figure 2.

The effect of NEM-S1 on myofibrillar ATPase activity in the presence of 30 μ M free Mg^{2+} . Conditions were as in Figure 1, except for differences in added total Mg^{2+} and Ca^{2+} . Each point is mean $\pm$ SEM of 4-14 measurements.

Figure 3.

Effect of magnesium binding to site III and IV of cTnC on the relative activation of myofibrillar ATPase by NEM-S1. The % activation was calculated from data in figures 1 and 2 using the equation:

$$\% \text{Activation} = (A_{\text{NEM-S1}}/A - 1) \times 100,$$

where A and $A_{\text{NEM-S1}}$ are the ATPase activities in the absence and presence of NEM-S1, respectively at each free [Ca²⁺].