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Calmodulin is intrinsically LESS effective than troponin C in activating skeletal muscle contraction

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Abstract Calmodulin (CaM) and troponin C (TnC) are evolutionarily and structurally homologous, yet they are not functionally interchangeable. In particular, CaM cannot effectively substitute for TnC as an activator of skeletal muscle contraction. To determine if this is a consequence of CaM's weak association with troponin T and I or the result of a more fundamental mechanistic defect, we have used CaM and a CaM[TnC] chimera, CaM[3,4 TnC], that stably associates with the thin filament. Replacement of TnC with CaM or CaM[3,4 TnC] reveals that CaM-like molecules reduce the Ca^{2+}-sensitivity and cooperativity of activation, as well as the maximal Ca^{2+}-activated tension. These observations indicate that CaM-like molecules are unable to continuously maintain the activated state of the thin filament.

Key words: Calmodulin; Troponin C; Skeletal muscle; Muscle contraction

1. Introduction

Two members of the superfamily of calcium-binding proteins, calmodulin (CaM) and troponin C (TnC), are compared as activators of skeletal muscle. Despite their homology, these proteins play distinctly different roles in cells. CaM is a cytosolic protein that acts as a generalized second messenger for calcium, while TnC is an integral component of the thin filaments of striated muscle that functions as the calcium-binding activator of contraction. The binding of TnC to thin filaments is largely stabilized by divalent metal ions bound to the high affinity binding sites III and IV [1]. In CaM these are low affinity calcium-specific sites and are believed to be unoccupied at the low calcium concentrations characteristic of relaxed muscle.

Studies on TnC's ability to replace CaM show that TnC is relatively ineffective as an activator of many of the cytosolic enzymes regulated by CaM [2–4]. But studies on CaM's ability to substitute for TnC have been more equivocal. Diamond et al. [5] reported that CaM was unable to substitute for TnC. However, Babu et al. [6] suggested that this was due largely to the dissociation of CaM from the thin filament at the low calcium concentrations characteristic of relaxed muscle. They found that, despite its weak binding, CaM would support partial tension recovery at pCa 4, and they proposed that it would be a fully competent TnC replacement, if it were stably associated with the thin filament.

Because CaM and TnC are homologous, differences in their ability to activate contraction provide a way to investigate structure–function relationships in these proteins [2–4]. Here, we have determined the effect of saturating concentrations of CaM on the maximal Ca^{2+}-activated tension of fibers. In addition, we have used a molecular chimera of CaM and TnC, CaM[3,4 TnC], which contains the N-terminal helix, domains I and II, and the central helix of CaM, and the C-terminal domains III and VI of TnC [3], that stably associates with the thin filament. The properties of CaM[3,4 TnC] have enabled us to determine whether CaM's low affinity for TnC-extracted sites on the thin filament is its primary defect as an activator of contraction or whether it is deficient in other, more fundamental, aspects of thin filament activation.

2. Materials and methods

2.1. Solutions and physiological analysis

Solution and equipment for tension measurements on individual fibers have been described [7–9]. The pCa 8 'relaxing' solution contains (all are sodium salts in mM): 9.8 mM EGTA, 0.2 mM CaEGTA [ethylene glycol bis(β-aminoethylether) *N,N,N'N'*-tetraacetic acid], 5 mM MgATP, 5 mM ATP, 7.5 mM PO_4, 40.1 mM propionate, 17.2 mM sulfate, 10 mM MOPS (morpholinopropane sulfonic acid), pH 7.00. The 'activating' solutions for pCa/tension data collection contain: 10 mM total EGTA (CaEGTA : EGTA ratio according to the pCa), 5 mM MgATP, 5mM free ATP, 10 mM MOPS, 7.5 mM phosphate, propionate and sulfate to adjust the final solution to our standard ionic strength (0.200). Wash solution contains 195 mM NaPropionate and 10 mM MOPS adjusted to pH7.0. The rigor solution is an EDTA based pCa 8 solution without ATP or Mg^{2+}. The TnC extraction solution contains 10 mM EDTA and 5 mM MOPS at pH 7.2.

The concentrations of multivalent ionic species are calculated after solving the multiple equilibria of two metals (Ca^{2+}, Mg^{2+}) and two ligands (EDTA or EGTA and ATP), by using the following apparent association constants (log values at pH 7.00): CaEGTA 6.3, MgEGTA 1.6, CaATP 3.7, MgATP 4.1. All experimental solutions are adjusted to pH 7.00 ± 0.01, and the temperature is maintained at 20.0 ± 0.1°C.

Single fibers were dissected from strips of psoas muscle 1–2 mm in diameter × 30 mm long that were chemically skinned as described [8,10]. To extract TnC, skinned fibers were washed twice with wash solution and then with rigor solution at room temperature and then incubated in extraction solution for 5–10 min at 30°C [8]. The pCa/tension data are collected on an apparatus which automates solution mixing and data recording, and the parameters of the pCa/tension relation fit by a least squares regression as described [7–9].

2.2. Protein purification

TnC was purified as described [8]. CaM and the CaM[3,4 TnC] chimera were expressed in bacteria and purified using Phenyl-Sepharose chromatography as described in George et al. [2,3]. Protein concentrations were determined by Bradford assays [2] and by Coomassie brilliant blue staining of purified CaM on SDS-polyacrylamide gels [11]. The protein standard was a 1 mg/ml solution of CaM whose concentration was determined by amino acid analysis.

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3. Results

3.1. Extraction of TnC and replacement by CaM

The maximal Ca^{2+}-activated tension of skinned rabbit psoas fibers is reduced in direct proportion to the TnC extracted from the thin filament, and this lost tension is fully restored by incubation of fibers in TnC solutions at pCa 8 [8,9,12]. In contrast, incubation of TnC-extracted fibers in CaM at pCa 8 fails to restore tension [5]. However, CaM will support partial tension recovery at pCa 4 [6]. Because it was inferred that CaM had failed to saturate the TnC-extracted sites in that study, we determined the CaM concentration needed to saturate the TnC-extracted sites. Following a 5 min extraction (which results in approximately a 70% reduction in maximal Ca^{2+}-activated tension), fibers were exposed at pCa 4 to a series of solutions containing increasing concentrations of CaM. As is evident in Fig. 1, CaM binds to TnC-extracted fibers and saturates those sites at concentrations between 3 and 5 μM (75 to 100 μg/ml). Increasing CaM in the external solution to concentrations as high as 500 μg/ml failed to increase tension above that recorded at 100 μg/ml. The exchange and saturation binding is also evident on SDS-polyacrylamide gels of control, extracted and CaM-substituted fibers (Fig. 1, insert).

To determine whether saturating concentrations of CaM effectively recovered the Ca^{2+}-activated tension lost by TnC extraction, fibers were extracted for 5 mins, incubated in pCa 4 solutions containing 200 to 400 μg/ml of CaM, and their maximal Ca^{2+}-activated tensions compared to controls (Fig. 2). The extraction reduced the maximal Ca^{2+}-activated tension by approximately 67%. Following incubation in CaM, only 40 ± 8% of the tension lost by extraction was recovered. In contrast, when the substituted CaM was removed by washing the fiber in a pCa 8 solution, and replaced by TnC following an incubation in 50 μg/ml TnC, full tension was recovered (Fig. 2).

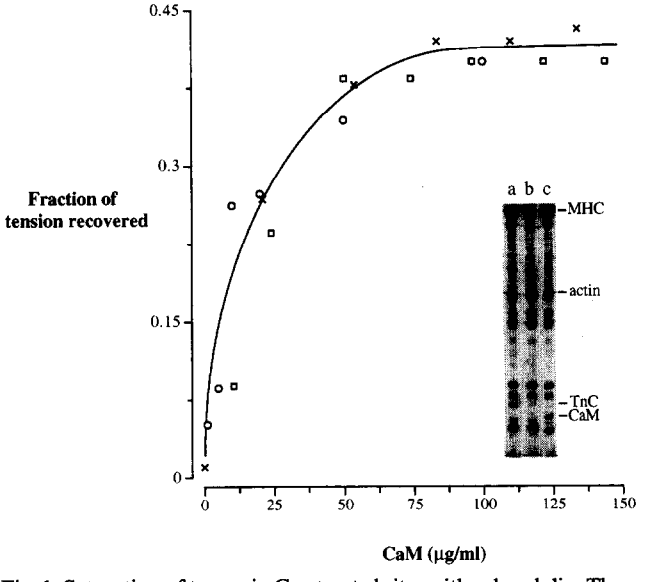

Fig. 1. Saturation of troponin C-extracted sites with calmodulin. Three fibers (○, ×, □) were extracted, incubated at pCa 4 in solutions with increasing CaM concentrations, and the maximal Ca^{2+}-activated tension determined. To calculate the fraction of tension recovered, the residual tension following TnC extraction was subtracted from the Ca^{2+}-activated tension measured at each CaM concentration, and that number was divided by the tension lost in TnC extraction. The insert shows a 10.5% SDS-polyacrylamide gel electrophoretogram of a silver stained control (a), a TnC extracted (b) and a CaM substituted (c) fiber.

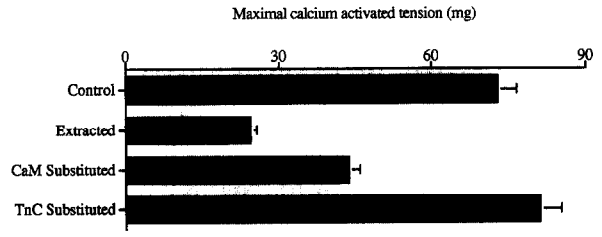

Fig. 2. Tension recovery following incubation of troponin C-extracted fibers in calmodulin. The maximal calcium tension of 9 control fibers is compared to that of 10 fibers extracted for 5 min, and 10 fibers incubated in saturating concentrations of CaM. Also shown is the tension recovered when 5 of the fibers incubated in CaM were washed in the pCa 8 relaxing solution and then incubated with TnC. Error bars represent the S.E.M.

3.2. Reconstitution with a CaM-TnC chimera that stably associates with the thin filament

The ability to restore maximal Ca^{2+}-activated tension measures only one aspect of skeletal muscle activation. Parameters that give more insight into the mechanism of activation are the Ca^{2+}-sensitivity and cooperativity of tension generation. Rabbit psoas fibers are maximally activated at pCa 5.5 and exhibit high cooperativity, with Hill coefficients (n_Hs)between 5 and 7 [7,8].

Because CaM binding and contractile activation take place over the same calcium concentration range, the Ca^{2+}-dependence of tension generation cannot be straightforwardly separated from the Ca^{2+}-dependence of CaM association with the thin filament.

To better assess the adequacy of CaM as a TnC replacement, we employed a chimera of CaM and TnC, CaM[3,4 TnC] [2], that would be expected to stably associate with the thin filament at pCa 8. In CaM[3,4 TnC] the low affinity calcium-specific sites III and IV of CaM are replaced with the high affinity divalent metal ion binding sites III and IV of TnC. This results in a molecule that exhibits properties of both its precursors. Like TnC, CaM[3,4 TnC] binds to TnC-extracted sites at pCa 8 and remains associated at pCa 8, based on the maximal Ca^{2+}-activated tension following a wash in the pCa 8 relaxing solution (Fig. 3). However, like CaM, it only partially restores maximal calcium activated tension (Fig. 3). To assure that the changes in the chimera had no effects other than stablizing the association with the thin filament, serial replacement studies with CaM and CaM[3,4 TnC] were conducted. As shown in Fig. 4, they reveal no significant difference between CaM or CaM[3,4 TnC] in activating thin filaments at saturating calcium concentrations.

Because CaM[3,4 TnC] binds stably, the Ca^{2+}-sensitivity and cooperativity of fibers in which it has been substituted for TnC can be determined (Fig. 5). CaM[3,4 TnC] substituted fibers have a mean pK of 5.29 ± 0.05 and an n_H of 3.2 ± 0.3. This compares with pKs of 5.84 ± 0.09 and n_Hs of 5.4 ± 0.8 for control or TnC substituted fibers. Thus, as is true for the recovery of maximal calcium activated tension, the substitution of CaM-like molecules for TnC reduces the Ca^{2+}-sensitivity and cooperativity of striated muscle thin filaments. These observations clearly show that the inability of CaM to substitute for TnC is not related to its weak association with the thin filament,

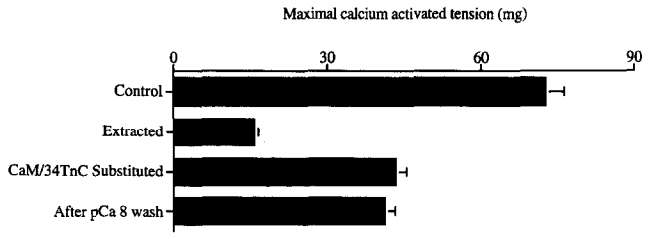

Fig. 3. Stability of CaM[3,4 TnC] association with the thin filament. The maximal calcium tension of 9 control fibers is compared to that of 28 fibers extracted for 5 min, and then incubated in saturating concentrations of CaM[3,4 TnC]. The stability of CaM[3,4 TnC]'s association with the thin filament at low [Ca^{2+}] is evident when the initial tension following replacement is compared with the tension recovered when 12 of the CaM[3,4 TnC] substituted fibers were washed for 2 min in pCa 8 relaxing solution and then activated at pCa 4 (labeled 'After pC 8 wash' on the graph). Error bars represent the S.E.M.

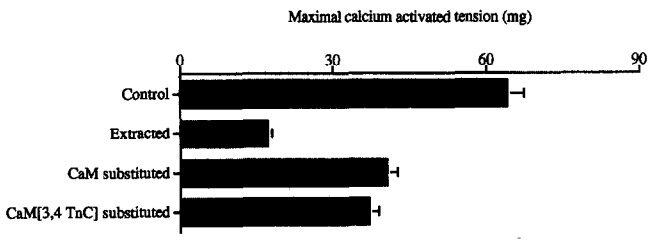

Fig. 4. Tension recovery following serial substitution of troponin C-extracted fibers with CaM and CaM[3,4 TnC]. The maximal calcium tension of 5 control fibers is compared to that of 5 fibers extracted for 5 min, and then incubated in saturating concentrations of CaM. Also shown is the tension recovered after washing the CaM substituted fibers at pCa 8 (which removes CaM) and then incubating them with CaM [3,4 TnC] in the pCa 4 activating solution. Error bars represent the S.E.M.

but is a consequence of a failure to effectively carry out other, more fundamental steps in thin filament activation.

4. Discussion

CaM exhibits many properties of TnC, including the ability to bind troponin I and T in a Ca^{2+}-dependent manner [13], and to support tension generation at pCa 4 [6]. These observations led to the attractive suggestion that CaM, the more generalized calcium second messenger could fully substitute for the more specialized TnC, if its low affinity for the thin filament could be overcome.

The weak association of CaM with TnC-extracted regulatory units on the thin filament certainly limits its usefulness as a TnC replacement. But, the two studies presented here show that CaM's deficiency as an activator of skeletal muscle contraction is not primarily a consequence of its weak association with the thin filament. In the first, TnC-extracted fibers incubated in saturating concentrations of CaM were found to recover less half of the tension lost by extraction. In the second, CaM[3,4 TnC], which stably associates with thin filaments, was found to be no more effective than CaM in tension recovery.

To more precisely determine the nature of CaM's deficiency as an activator of skeletal muscle contraction, the pCa/tension relation of CaM[3,4 TnC] substituted fibers was analyzed. Control and TnC reconstituted fibers exhibit highly cooperative pCa/tension relations with Hill coefficients (n_Hs) between 5 and 6. This cooperativity is due to two factors, interactions between Ca^{2+}-binding sites I and II, and extended interactions between troponin–tropomyosin (Tn–Tm) regulatory units that enable the thin filament to activate as a unit [8,9,14] Because pCa/tension curves can be generated with CaM[3,4 TnC], it was possible to consider which of these two components of cooperative activation was affected in CaM[3,4 TnC] substituted fibers.

If individual regulatory units were unable to participate in extended cooperative interactions along the thin filament, the maximum n_H of the pCa/tension relation would be ≤ 2 (reflecting only the cooperative interactions between Ca^{2+}-binding sites I and II in TnC or CaM-like molecules [3,15] in individual regulatory complexes). This is observed experimentally when the cooperative interaction among regulatory units is eliminated by extensive TnC extraction [8]. Because the n_H of CaM[3,4 TnC] substituted fibers is significantly greater than 2, CaM[3,4 TnC] containing regulatory units must participate in the extended cooperative interactions that unify the regulatory strand. Yet, both the cooperativity and maximal tension of CaM[3,4 TnC] substituted fibers are half that of control or TnC extracted and replaced fibers.

The effect of CaM[3,4 TnC] on cooperativity and tension is similar to that observed when TnC is partially extracted. Extraction of as little as 5 to 10% of the TnC from a fiber results in n_Hs decidedly less than 4, as well as reduced Ca^{2+}-sensitivity and tension. In the case of TnC extraction, the decrease in Ca^{2+}-cooperativity, sensitivity and tension results from the inactivation of extracted regulatory units and their consequent inability to participate in extended cooperativity along the thin filament [8,9]. Because all the TnC-extracted sites are saturated in CaM[3,4 TnC] substituted fibers, we assume that all regulatory units can be activated, so CaM-like molecules are defective as TnCs because they either incompletely activate the regulatory complexes with which they are associated or they are unable to maintain the troponin–tropomyosin regulatory complex in the 'on-state' at saturating calcium concentrations. Because of the similarity of the effects of CaM[3,4 TnC] substitu-

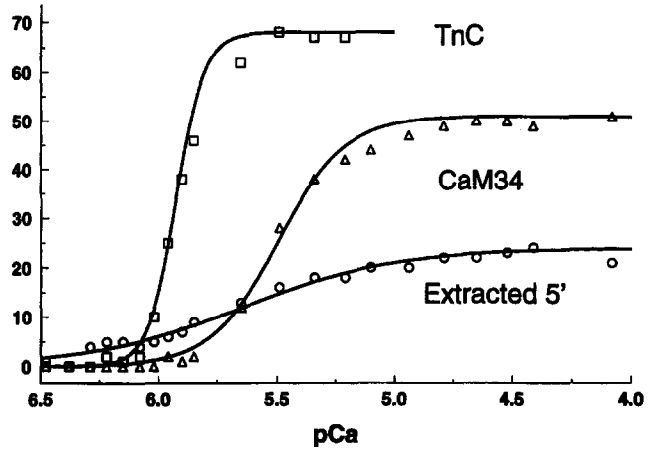

Fig. 5. pCa/tension relations of control, extracted, and CaM[3,4 TnC] substituted fibers. The pCa/tension relation of a TnC extracted and substituted fiber is compared with that of an extracted fiber and a CaM[3,4 TnC] substituted fiber. The pK and n_H of the TnC extracted and substituted fiber are 5.92 and 7.21; of the extracted fiber are 5.65 and 1.32; and of the CaM[3,4 TnC] substituted fiber are 5.49 and 3.03.

tion and partial TnC extraction on the pCa/tension relation, we suggest that the latter is the case and that the Ca^{2+}-dependent interactions between CaM-like molecules and TnI critical for activation [16, 17] are less effectively stabilized in the 'on-position' even at saturating calcium concentrations. This would result in a reduction in both the maximal Ca^{2+}-activated tension and the extended cooperativity along the regulatory strand. Thus it appears that the substitution of CaM-like molecules for TnC results in a thin filament regulatory complex that is unable to continuously maintain the activated state.

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