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Down-regulation of fast-twitch skeletal muscle fiber with cardiac troponin-C and recombinant mutants

Structure/function studies with site-directed mutagenesis

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Structure/function relationships in troponin C are studied with vertebrate fast-twitch fibers by exchanging the skeletal troponin C with two bacterially synthesized recombinant proteins designed by site-directed mutagenesis of cardiac troponin C. One mutant (CBM1) contained an additional active site, by deleting Val-28 and converting Leu-29, Gly-30, Ala-31 and Glu-32 to Asp, Ala, Asp and Gly, respectively, in the normally inactive trigger site 1 in the N-terminus. In another mutant (CBM2A), the normally active site 2 was inactivated by conversion of Asp-65 to Ala. The fibers were found to be down-regulated with recombinant cardiac troponin C (CTnC3), as with tissue-cardiac-troponin-C. With mutants, in one case (CBM1) the regulation was unmodified despite Ca^{2+} coordination by all sites. In contrast, regulation was found to be completely blocked with the mutant (CBM2A) where both trigger sites were inactive. The results provide the first indication that structural specification of the entire EF-hand motif of site 1, and not just Ca^{2+} coordination, is needed to operate fully the Ca^{2+} switch in fast-twitch fibers.

Ca^{2+}; Troponin C; Regulation; Muscle contraction; (Slow-twitch muscle, Heart)

1. INTRODUCTION

Cardiac and fast-twitch skeletal troponin Cs are nearly equal in size (159–161 amino acid residues, with about 70% sequence homology), and have the same overall supersecondary structures (4 EF-hand Ca^{2+}-binding loops, two each in the N- and C-termini) [1]. The two EF-hand motifs in the N-terminus are potential trigger sites. However, because of sequence modifications in the first motif (residues 16–46 in the N-terminus, containing site 1), site 1 in cardiac TnC is inactivated, leaving one trigger site active compared to two in skeletal TnC. Consistent with this, using protein-engineering techniques to modify specific residues for altering the Ca^{2+}-binding properties of bacterially synthesized cardiac TnC, Putkey et al. [2] found that the active site 2 is essential for triggering contraction in slow muscle. However, the functional implications of the extra active trigger site in fast-twitch skeletal troponin C are not well established.

Recently, differences in the switching mechanisms of skeletal and cardiac muscles have come to light from TnC-exchange studies on skinned myocytes [3]. Skeletal TnC inserted into cardiac muscle produced full regulation of force development and gave its native (skeletal-type) performance. However, in the converse study with fast-twitch fiber, cardiac TnC could regulate only partially, suggesting that the inactive site has significant functional consequences for grading the contraction in skeletal muscle. The down-regulation of generated tension is an important mechanism, since cardiac TnC is found in develop-

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ing chicken muscle, with skeletal TnC appearing later in the adult stages [4,5].

To gain new insights at the molecular level into the structure/function relationships of the extra active site for modulating tension development in skeletal TnC, the combined methodologies of TnC exchange in the myocyte and site-directed mutagenesis were utilized in the present study. The results with specific mutants of cardiac TnC, one with restored metal ion coordination in site 1 and the other with inactivated site 2, are described. They provide the first evidence that structural specification of the entire EF-hand motif, and not just Ca^{2+} coordination, is critical for the switching mechanism in fast-twitch muscle.

2. MATERIALS AND METHODS

2.1. *Animals and tissues*

Adult Syrian hamsters (6–12-month-old) were used. Skinned fast-twitch fibers were selected from psoas muscle on the basis of sarcomere homogeneity and tension response to activation by Sr^{2+} [6]. Skinned cardiac muscle preparations were of the right ventricular trabeculae. Typical experimental tissue preparations were 50–100 μm in width and 500–2000 μm in length.

2.2. *TnC extraction*

70–80% of the native TnC was extracted by treating the fiber with an extremely low salt-EDTA solution (5 mM EDTA and 10 mM imidazole, pH 7.2), as described by Babu et al. [3,6]. The treatment yielding 100% deletion from the fiber was not used in order to avoid the extraction of another putative cofactor essential for activation [7]. TnC or the mutated bacterial proteins could be inserted into the extracted fiber by 30 min incubation with 0.2–2 mg/ml protein in the relaxing solution. Following this incubation, fibers were thoroughly rinsed in protein-free relaxing solution to remove free, unanchored, protein from the interstitial spaces. The composition of the relaxing solution was: 100 mM K propionate, 20 mM imidazole, 6.06 mM $MgCl_2$, 6.5 mM ATP, 5 mM EGTA, 20 mM phosphocreatine and 250 U/ml creatine phosphokinase. Activating solution contained CaEGTA in place of EGTA and had free [Ca^{2+}] equal to 10^{-4} M (referred to as pCa_4). Ionic strength of the activating and relaxing solutions was close to 190 mM.

2.3. *Bacterial proteins and site-directed mutagenesis*

A complete description of the chicken cDNA for CTnC, construction of expression plasmids, mutagenesis, protein isolation, and the technique used to determine the Ca^{2+}-binding properties of mutants is given elsewhere by Putkey et al. [2,8]. Functional characteristics of the mutated proteins were initially tested in slow muscle fibers, which have TnC analogous to cardiac TnC [9]. The critical tests are performed here on fast-twitch fibers, because one of the mutants was made specifically to achieve Ca^{2+} binding equal to that of fast muscle TnC. Bacterially synthesized CTnC is termed CTnC3 to distinguish it from the CTnC derived from cardiac tissue. The two mutated TnCs used here are designated CBM1 and CBM2A. In CBM1, Ca^{2+} coordination in site 1 was restored by deletion of Val-28 and conversion of Leu-29, Gly-30, Ala-31 and Glu-32 to Asp, Ala, Asp and Gly, respectively. In CBM2A, Ca^{2+} coordination in site 2 was inactivated by conversion of Asp-65 to Ala.

2.4. *SDS-PAGE*

After the tension measurements had been completed, the tissues were carefully detached from the transducers and stored at −70°C for analysis by gel electrophoresis. SDS-PAGE runs were made by adding 1 mM EGTA in SDS sample buffer [10]. In quantitative estimations for uptake of various TnCs into the fiber, silver-stained gels were scanned with an LKB laser densitometer. On analysis of the results obtained with purified tissue and recombinant proteins, we found that CTnC, CTnC3, and CBM2A stained 1.4-times more intensely than STnC and CBM1 for the same amount of protein. This was used as a correction factor for normalizing the data. All protein concentrations in solution were determined by BioRad protein assay.

3. RESULTS AND DISCUSSION

The experimental protocol is shown in the top set of force traces (labelled a) in fig.1A. In each case the fiber was activated with a pCa_4 solution, and the first trace represents the force response of the fiber in its native state. Force was diminished after TnC extraction, and the fiber was fully resensitized with skeletal TnC. We have previously shown that a fast-twitch fiber loaded with bovine or rabbit cardiac troponin C gives a submaximal force response under maximal activation [3]. In the present article, we show that the fiber is similarly down-regulated on loading with bacterially synthesized cardiac TnC (CTnC3). The physiological force response with recombinant CTnC3 is shown in the last trace of set b in fig.1A. Traces in set e show that the bacterial CTnC3 was fully effective in cardiac muscle, as was the case previously [3] for bovine and rabbit proteins. These data on CTnC3 in cardiac muscle correlate well with its ability to regulate force generation in slow fibers [2]. Similarly, the gel runs indicate that CTnC3 was firmly anchored to the TnC-denuded sites in the fiber (fig.1B, lane 2), and that the uptake of CTnC3 corresponded to the amount deleted of skeletal TnC in fast twitch fibers (fig.2, cf. 2nd and 3rd bars, lower panel).

3.1. *Effect of mutants made with site-directed mutagenesis*

Results concerning two mutants are described.

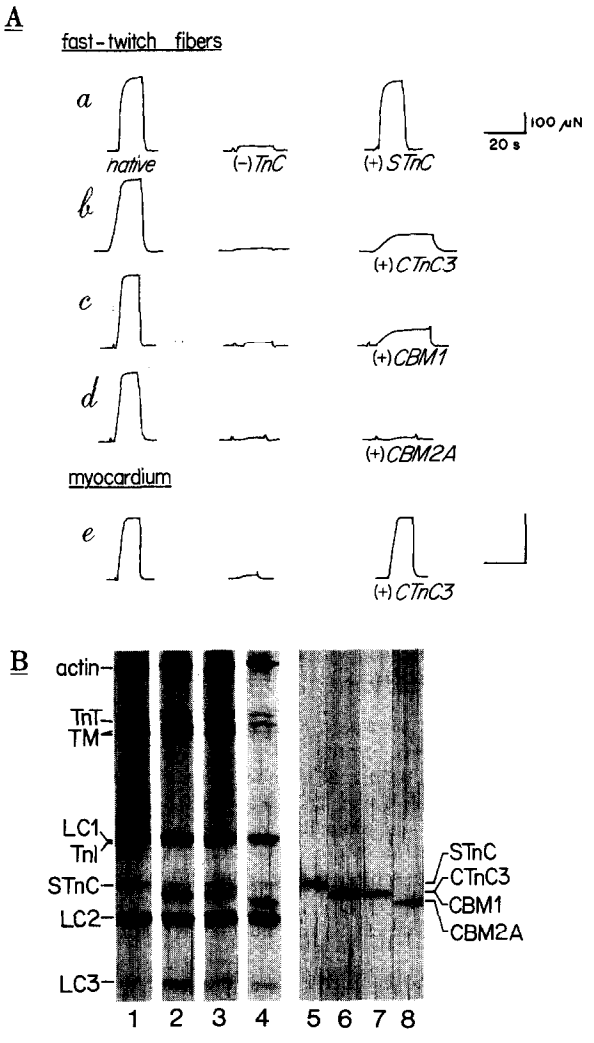

Fig.1. (A) Force response of isolated skinned single fibers to activation with pCa_4 after loading with skeletal troponin C (a) and various analogs of cardiac troponin C (b–d). Trace e shows the response of cardiac muscle with the bacterial cardiac troponin C (CTnC3). (B) Silver-stained gel lanes of the above fibers. Lanes: 1, non-extracted native fiber; 2–4, extracted fibers loaded with CTnC3, CBM1, and CBM2A, respectively; 5–8, purified proteins as indicated.

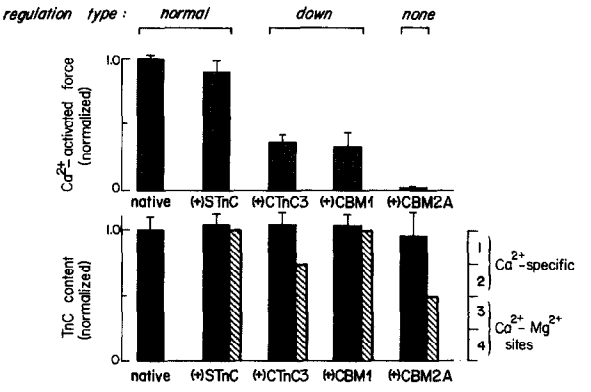

Fig.2. (Top) Normalized force response of fibers to maximal activation. The tension level of the fiber loaded with a specified protein was normalized to the tension prior to extraction. Data are shown on 4 fibers, ± SE. (Bottom) Shaded boxes, relative intensities of the various proteins normalized to the LC1 + TnI bands in the particular lane; filled boxes, residual skeletal TnC in the extracted fiber; lined boxes, estimated amount of Ca^{2+} bound (mol/mol) to the various classes of binding sites in the proteins loaded into the fiber.

In the first, CBM1 (see section 2), site 1 had been modified with a limited number of amino acid replacements to bind Ca^{2+} like skeletal TnC (fig.2; see [2]). When CBM1 was loaded into fast-twitch fiber in exchange for skeletal TnC (fig.1B, lane 3), the force response was found to be submaximal as with CTnC3 (fig.1A, set c). This indicates that the triggering mechanism with CBM1 was also only partially effective. The force of cardiac muscle was full with CBM1, as with CTnC3 (not shown); also, CBM1 is fully effective in slow muscle fibers [2]. The second mutant, CBM2A, in which the normally active loop 2 was modified to eliminate Ca^{2+} binding (see fig.2), was tested here. This mutant was found to occupy (by gels) the denuded sites in fast-twitch fiber to exactly the same extent as other analogs (fig.1B, lane 4). However, tension development by the CBM2A-loaded fiber in the activating solution was completely blocked (fig.1A, set d), showing that the triggering mechanism was now totally ineffective.

The present results of tension development and protein uptake are summarized in fig.2 for a number of fibers. In addition, the data on Ca^{2+} binding by tissue skeletal TnC [11] and the recombinants [2] are also included for comparison. These binding data indicate that CBM1 is similar to skeletal TnC regarding Ca^{2+} coordination, and that only the putative Ca^{2+}-Mg^{2+} sites are active in CBM2.

3.2. *Structure/function implications*

The recently demonstrated ability to make TnC exchange in cardiac muscle and skeletal fibers has turned out to be a useful approach for distinguishing between the physiological functions

of cardiac and skeletal TnCs. For instance, we have previously shown that higher sensitivities for activation with Ca^{2+} and with Sr^{2+} in cardiac muscle over fast-twitch fiber can be fully explained by the properties of TnC moieties [3,12]. Further, it was found that skeletal TnC is critical in setting higher cooperativity in muscle, as judged by the slope of p*Ca*-force curves in cardiac muscle with TnC exchange [12]. As mentioned above (section 1) we have also shown that cardiac TnC is unable to restore full regulation in fast-twitch fiber, and this result is confirmed in this paper with recombinant CTnC3. The technical advancements in protein-engineering are now making possible systematic investigations of the structure/function relations of TnC in the fiber, as described here. Such studies should eventually provide a detailed understanding of the Ca^{2+}-switching mechanisms in muscle contraction as well as in other cellular processes.

Two major conclusions are derived from this study. Between cardiac and skeletal TnCs, 48 amino acids are different and nearly half are confined to the first EF-hand motif [1]. Although these differences are accompanied by loss of Ca^{2+} coordination in the first site in the N-terminus of cardiac TnC, it is unknown whether the residues responsible for Ca^{2+} coordination per se could account for the physiological activities of the two types of TnCs or if other sequence changes were also important for some of the functions. The present study of down-regulation of fast-twitch fiber by CTnC and by the mutant CBM1 provides the most direct evidence that Ca^{2+} coordination in site 1 is not sufficient to restore fully the trigger activity of cardiac TnC. This is then the first indication that structural specification of the entire EF-hand motif of site 1 is essential for the switching mechanism of contraction by TnC molecules.

Another major conclusion of the fiber studies is drawn from the results with the CBM2A mutant in which both trigger sites were inactivated. The findings that CBM2A retains the ability to anchor in fast-twitch fiber, as in slow muscle [2], and that despite this it is totally ineffective in function, provide additional support for the paradigm that Ca^{2+}-binding sites in the N-terminus of TnC are the trigger sites. The combined results on slow and fast fibers also provide the best indication that the presence of two active, Ca^{2+}-Mg^{2+} type, sites in the C-terminus half are sufficient to anchor the regulatory protein in striated muscles.

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