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Characterization of the Ca^{2+}-switch in skeletal and cardiac muscles

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To determine the significance of the global structure of the regulatory proteins in the mechanism of the Ca^{2+}-switch in cardiac and skeletal muscle contractions, the properties of a family of Ca^{2+}-binding proteins with 4 or 3 EF-hand motifs have been studied with desensitized skinned fiber preparations. Proteins with 4 EF hands (such as troponins C – TnCs) are dumb-bell shaped, those with 3 EF hands (parvalbumin) being ellipsoidal. The number of active sites varied between four and two. We find that the ability to anchor in the fiber is limited to proteins with 4 EF hands and, at least, two active Ca^{2+}-binding sites, one each in the N- and C-termini. The results suggest that the dumb-bell shaped global structure is critical for the switching action in muscular contraction, and a trigger site in the N-terminus and a structural site in the C-terminus need to be active in order to regulate contractility.

Troponin C; Calmodulin; Parvalbumin; Oncomodulin

1. INTRODUCTION

A multitude of processes are triggered by Ca^{2+} and a variety of specialized Ca^{2+}-binding proteins exist to carry out diversified functions in the cell [1]. The overall structure of these switching proteins varies, and the proteins differ in the amounts and affinities of Ca^{2+} binding. Thus, for instance, on the basis of comparison of amino acid sequences [2], skeletal and cardiac troponins C are both found to have the same number (four) of EF-hand motifs for Ca^{2+} binding (2 in the N-terminus half; 2 in the C-terminus half), but site 1 (N-terminus, residues 16–46) is able to coordinate Ca^{2+} only in the skeletal isoform [3]. By investigation of the high-resolution X-ray crystalline structure, regulators with 4 EF hands have a dumb-bell shaped structure [4–6]; others with 3 EF-hand domains assume an ellipsoidal form [7–9]. Functionally, also, cardiac troponin C (TnC) in the myocardium manifests higher apparent Ca^{2+} and Sr^{2+} affinities than skeletal TnC [10,11]. Using recombinant mutants with skinned fibers, it is now becoming possible to perform critical studies of the structure/function relations controlled by the sequence differences in TnC isoforms [3,12,13]. However, whether the dumb-bell shaped global structure, itself, presumably common to TnC isoforms, has a functional significance in the switching mechanism of muscular contraction has so far not been systematically attempted.

Here, this point is addressed by utilizing a variety of Ca^{2+}-binding modulators with either 4 or 3 EF-hand motifs available from different tissue sources. The results indicate that the dumb-bell structure may be essential for switching the contraction in muscle fiber. Additional insights into the mechanism of the C-terminus sites are also described.

2. MATERIALS AND METHODS

2.1. *Tissue and fiber preparation*

Small bundles (1–2 × 4–10 mm) of psoas muscle from adult rabbit and Syrian hamster were tied to sticks and stored overnight at −20°C for skinning in solution containing 150 mM

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potassium propionate, 5 mM Mg acetate, 5 mM EGTA, 5 mM ATP, 1 mM dithiothreitol and 50% (v/v) glycerol, at pH 7.00 [10]. The selection of single fibers for experiments was on the basis of sarcomere uniformity and p*Sr* activation [14]. For cardiac muscle, trabeculae from the right ventricle of hamster heart were used. Fiber was attached to the force transducer, and transferred to relaxing solution (typically 100 mM potassium propionate, 20 mM imidazole, 6.06 mM $MgCl_2$, 5 mM ATP, 5 mM EGTA, 20 mM phosphocreatine and 250 U/ml of creatine phosphokinase, pH 7.00; ionic strength, 190–200 mM; 1 mM free Mg^{2+}). The potassium salt was modified to alter the ionic strength, as needed. For the 40 mM salt solution used with *Limulus* TnC, the composition was modified to 10 mM imidazole, 5.41 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, 6 mM phosphocreatine and the same amount of creatine phosphokinase as above. Free Mg^{2+} was close to 4 mM to suppress the Ca^{2+}-free tension in low salt [15]. In each case, the fiber was briefly treated with 0.5% Lubrol wx (or Triton-X) detergent (2–5 min treatment at 10°C). Activating solutions were made by appropriately varying the Ca^{2+}- or Sr^{2+}-EGTA to EGTA ratio. Experiments were carried out at a sarcomere length of 2.5 μm (cardiac muscle: 2.2 μm) as monitored by laser diffraction. Hamster muscles were activated at 20°C, and at 5°C in the case of rabbit.

2.2. *TnC extraction from the fiber and reconstitution*

To extract TnC, the attached fiber was equilibrated in a Ca^{2+}-free rigor solution (165 mM potassium propionate, 20 mM imidazole, 2.5 mM EGTA; pH 7.0) at 4°C, and transferred to the extracting solution (5 mM EDTA, 10 mM imidazole; pH 7.2) at 30°C for 5–30 min duration [10]. Force with maximal activation (p*Ca*4) was checked periodically, and extraction was ended when the force had dropped to 10–20% P. SDS gel runs on such fibers indicated 70–75% TnC extraction. Extraction was performed for 60 min in experiments with *Limulus* TnC, which deleted nearly 100% of the fiber TnC [16].

To recombine TnC or the analogs with denuded sites in the fiber, 10–30 min incubation at 5°C was employed (0.2–5.0 mg/ml protein in the relaxing solution). Afterwards the free protein was washed out with several rinses, unless otherwise indicated. Calmodulin and *Limulus* TnC, when tested, were also included in the activating solutions. Rinsing was necessary to run the gels, and was also carried out in the activating solution [17]. All experimental fibers were stored at −70°C for quantitative analysis by gel electrophoresis. In the present experiments, skeletal fast muscle TnC was from rabbit, cardiac TnC from bovine, and CBM1 and CBM2A were bacterially synthesized mutants of chicken cardiac TnC. Rabbit muscle parvalbumin and bovine brain calmodulin were purchased from Sigma, and the synthesized form of oncomodulin, an analog of parvalbumin found naturally in neoplastic tissue, was supplied by Dr M. Henzle (University of New Mexico).

3. RESULTS

3.1. *Effect of salt concentration on efficacy of cardiac TnC*

We studied the effect of 100 mM salt on regulation of fast fiber by cardiac TnC. Cardiac TnC down-regulates Ca^{2+}-activated force development in standard (190 mM) salt solution, as shown in fig.1A. Typical force responses to p*Ca*4 activation are shown for a native (unextracted), TnC-extracted, and a cardiac TnC-loaded fiber. The gels (fig.1B) of three separate fiber segments of the fast muscle indicate typically 30% residual TnC after extraction and full reconstitution with cardiac TnC (and skeletal TnC). This shows that down-regulation was not due to the inability of cardiac TnC to combine in fast fiber.

Fig.2 shows that the efficacy of loaded cardiac TnC was greatly improved when assayed in a solution of lower salt concentration. Force response was better than 70% in 100 mM salt solution in these experiments.

3.2. *Effect of cardiac TnC exchange on Sr^{2+} sensitivity*

Fig.3 shows the p*Sr*-force relationships of psoas fibers containing skeletal or cardiac TnC. Experiments were performed in 100 mM salt solutions. Fibers loaded with cardiac TnC are found to be 4–5-fold more sensitive than native or skeletal TnC-loaded fibers. This result is similar to the physiological difference in sensitivities between skinned heart and skeletal muscles, as indicated in the inset to fig.3.

3.3. *Uptake of Ca^{2+}-binding proteins by fiber*

To gain new insights into the structure/function relationships of the Ca^{2+}-binding sites on TnC, a variety of proteins were used. Parvalbumin and oncomodulin were selected, since they lack part of the structure in the N-terminus of TnC (missing approx. 50 starting residues), and because the global structure of parvalbumin is ellipsoidal. *Limulus* TnC was selected, despite the presence of all 4 EF hands (and thereby presumably having the dumb-bell shaped structure), because it appears to have two active Ca^{2+}-specific sites [18]: one each in the N- and C-termini (sites 2 and 4; site 1 is inactive, and the status of site 3 is somewhat uncertain).

The results obtained on the force response and uptake of proteins (measured using SDS gels, not shown) by fibers are listed in table 1. In this series we also included calmodulin for control studies. Calmodulin has all four sites active, but becomes immediately desorbed from the TnC sites on ex-

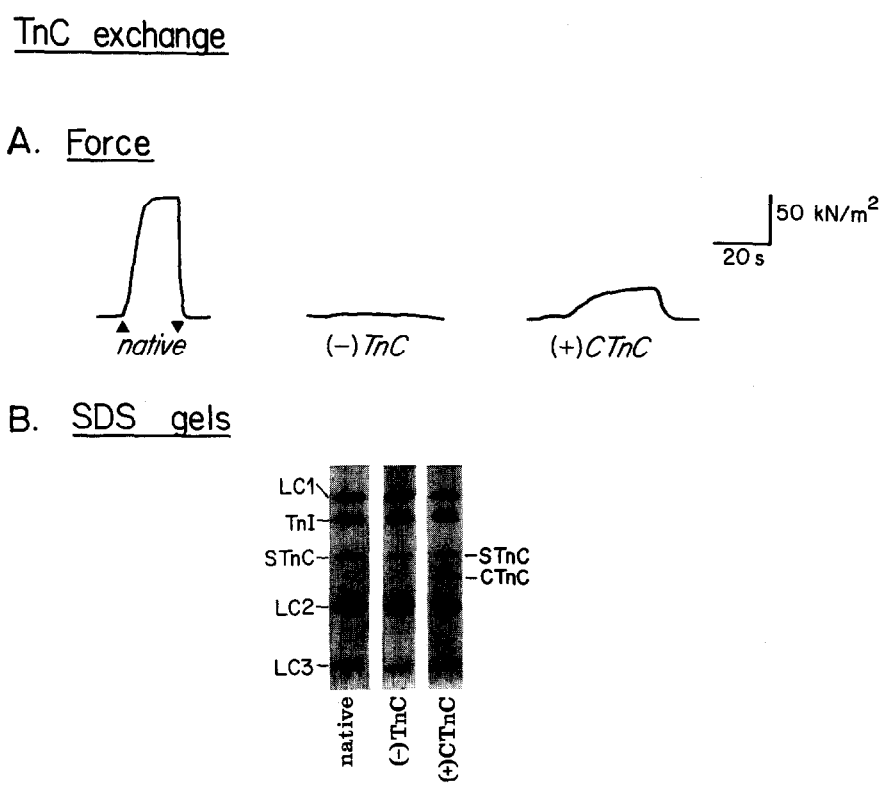

Fig.1. Down-regulation of fast-twitch fibers in standard salt (190 mM) with cardiac TnC. (A) Typical rabbit psoas fiber from which about 70% of the original skeletal TnC was extracted gave 25% force response after loading with cardiac TnC. (B) Gel showing the typical recombination of cardiac TnC in rabbit fast fibers. Uptake by both rabbit and hamster fibers was comparable to the amount of extracted TnC.

posure of the fiber to EGTA-relaxing solution [17]. Data concerning cardiac and skeletal TnCs, as well as those for two recombinant mutants of cardiac TnC, are also included for comparison.

With CBM1, where the inactive site (site 1) of cardiac TnC was selectively repaired by site-directed mutagenesis, to restore Ca^{2+} binding as in skeletal muscle TnC, but retaining the other differences, the force response of maximally activated fast fiber was below normal ([3]; table 1). The force response of cardiac muscle was full with CBM1 (not shown; see also [3]). In 100 mM salt, the efficacy of CBM1 was increased in the fast fiber (Gulati et al., unpublished data), similar to the results with cardiac TnC. With CBM2A, in which site 2 was inactivated so that only the Ca^{2+}-Mg^{2+} sites in the C-terminus portion were active, tension development was completely blocked in both cardiac and skeletal muscles. The results

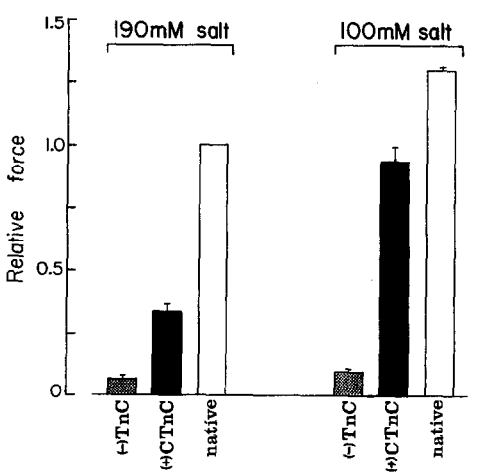

Fig.2. Recovered efficacy of cardiac TnC in reduced salt (100 mM). Total 14 fibers for 190 mM salt, and 10 fibers for 100 mM salt (4 rabbit fibers were included in each case).

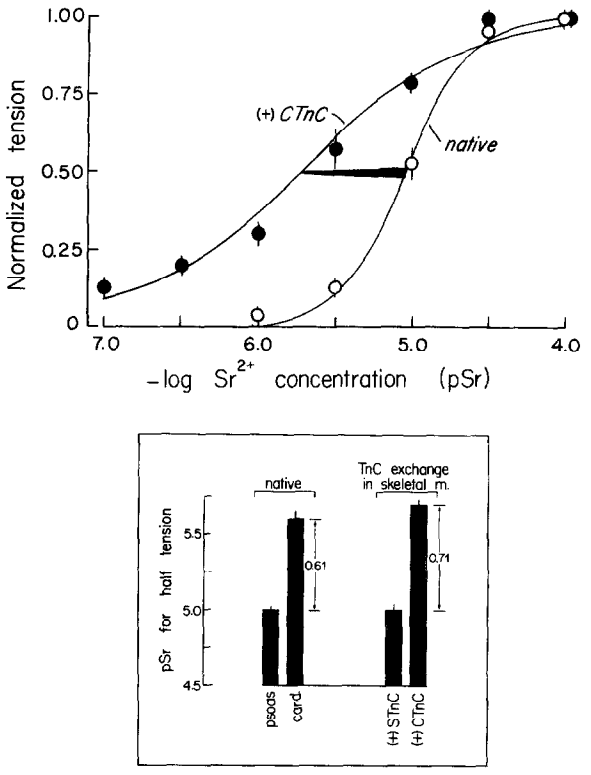

Fig.3. The p*Sr*-force relationship of cardiac TnC-loaded fiber characteristic of the TnC. The fibers are seen to become more sensitive to activation by Sr^{2+} on loading with cardiac TnC. Inset compares data (± SE; 4 hamster fibers in each case) on native and loaded fibers and cardiac muscle.

for CBM1, CBM2A, and calmodulin summarized in table 1 include the previous data on fast fibers [3,17].

TnC-extracted fiber challenged with parvalbumin produced no Ca^{2+}-activated force response in standard (1 mM free) or low (20 μM free) Mg^{2+}, nor, by using gels, was parvalbumin found to recombine with the TnC-denuded sites in the presence or absence of Ca^{2+}. Oncomodulin behaved similarly. In contrast, *Limulus* TnC with apparently the same total number of active sites as in parvalbumin recombined in the fiber (in the presence of Ca^{2+}) and also produced Ca^{2+}-activated force in low (40 mM) salt. The salt concentration required for the *Limulus* effect in fibers was similar to that used earlier for ATPase measurements [19]. Like calmodulin, *Limulus* TnC was found to be released in EGTA (Ca^{2+}-free) solution.

4. DISCUSSION

Our results show that the cardiac TnC induced down-regulation of maximal force development in fast fibers is balanced by the presence of moderately reduced salt. It is interesting that the substitution of skeletal TnC (with both trigger sites active) for cardiac TnC (with a single trigger site) in cardiac muscle produced the characteristic regulation

Table 1

Comparison of the Ca^{2+}-binding properties of the various modulators

Modulator	Molecular mass (kDa)	EF-hands (no.)	N-terminus Ca^{2+} sites	C-terminus Ca^{2+}-Mg^{2+} sites	Recombination	Tension
Skeletal TnC	18.0	4	2	2	+	1.0
Cardiac TnC	18.4	4	1	2	+	0.37 ± 0.03 (14) [0.72 ± 0.04]
Calmodulin	17.4	4	2	2[c]	+[b]	0.71 ± 0.04 (18)
Limulus TnC	18.0	4	1	1[c]	+[b]	[0.55 ± 0.07 (6)][a]
Oncomodulin	12.1	3	1	1	0	0 (4)
Parvalbumin	11.4	3	–	2	0	0 (4)
CBM2A	18.3	4	–	2	+	0 (6)
CBM1	18.2	4	2	2	+	0.36 ± 0.12 (6)

[a] Tension recovery in 40 mM salt
[b] Recombination observed only in the continued presence of calcium
[c] Sites: low-affinity Ca^{2+}-specific

Recombination was evaluated by SDS-PAGE on experimental fibers. Data for tension recovery were normalized to the value with skeletal TnC in standard salt, namely 0.92 ± 0.01 (35) (numbers within parentheses indicate sample size); values within square brackets indicate measurements made in 100 mM salt

without milieu adjustment [10,11]. These findings suggest that the incompetence of cardiac TnC in fast fiber is the result of either an incomplete signal with Ca^{2+} or a misfit of the protein with other subunits of skeletal troponin, in standard salt. Our recent studies with engineered cardiac TnC (CBM1), where site 1 binds Ca^{2+}, also suggested that diversity in the actions of skeletal and cardiac TnCs involved other residue differences [3]. We show now that reducing the salt concentration by a factor of half induces modifications in the fiber, that up-regulate the action of cardiac TnC, comparable to skeletal TnC.

Additionally, on the basis of the results with *Limulus* TnC, we suggest that, besides the compact dumb-bell shaped structure formed by 4 EF-hands, the presence of two active sites (site 2 in N-terminus, and site 4 in C-terminus of the dumb-bell) may be sufficient to perform the switching action with Ca^{2+} in low salt.

4.1. *Structural requirements of the Ca^{2+}-binding protein*

TnC-TnI interactions are essential during the activation of muscle and the modifications in these interactions following Ca^{2+} binding to TnC very likely initiate the signal that triggers contraction [20]. Three patches each of 11 residues (nos 50–60, 90–100, 126–136) have been identified on skeletal TnC for TnC-TnI interactions [20–22]. By examining the sequences, similar patches are seen to be present in all Ca^{2+}-binding proteins studied here, and a possible explanation for the inability of parvalbumin and oncomodulin to anchor in the fiber (table 1) is that the required patches are inaccessible to TnI due to the ellipsoidal structure of these proteins in the tertiary arrangement. Another important, related, finding is that reported recently by Xu and Hitchcock-DeGregori [23], that deletions of up to 3 residues from the central helix of the skeletal TnC (residues 91–93: Lys-Gly-Lys, corresponding to residues 88–90 above) appear to activate permanently the regulated actomyosin ATPase. In this case, by comparison with calmodulin, which lacks these residues, presumably the overall shape is retained in the engineered TnC, although the lobes of the dumb-bell are closer (1.5 Å per residue) and twisted (approx. 100° per residue) relative to each other. The effect on ATPase is thought to be direct due to the fact that the deletions are adjacent to a TnI-binding patch.

The results with fiber are of further interest, since oncomodulin, unlike parvalbumin, appears to be active in the phosphodiesterase assay in solution [24], suggesting that the presence of 4 EF-hand motifs (and presumably the dumb-bell structure) of the Ca^{2+}-binding protein is a stringent requirement for interaction with TnI in the sarcomere.

Additionally, the data in table 1 suggest that the activity of at least one of the two C-terminus sites (putative Ca^{2+}-Mg^{2+} sites in vertebrate TnC [25]) is essential for anchoring the protein in the fiber, however, more work is needed to ascertain the precise nature of the influence of these sites on the TnC-TnI interacting patches. Furthermore, the current data provide additional insights into the mechanism of the C-terminus sites. The results show that the Ca^{2+}-specific (as in calmodulin and *Limulus* TnC) or Ca^{2+}-Mg^{2+} type (as in vertebrate TnC and retained in the bacterially synthesized mutants), characteristic of the C-terminus sites, in a particular protein, is the determining factor as to whether or not the continuous presence of Ca^{2+} is needed for anchoring in the fiber.

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