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Effect of troponin C on the cooperativity in Ca^{2+} activation of cardiac muscle

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This study describes the effects of exchanging native cardiac troponin C (CTnC) from the right ventricular muscle of Syrian hamster for purified skeletal (S) TnC from fast twitch muscles in triggering cardiac contraction. Ca^{2+} sensitivity of the myocardium became decreased with STnC to 62% of the original value with CTnC. Furthermore, the slope of the p*Ca*-force curve of cardiac muscle was found to be increased with STnC. The results show that the TnC moiety, as part of the switching mechanism during activation, also regulates thin-filament cooperativity in muscle. Modifications in both the Ca^{2+} sensitivity and cooperativity are associated with alterations in the primary structure of TnC.

Troponin C; Structure-function relationship; Cooperativity; Regulation; Amino acid sequence; Mutagenesis

1. INTRODUCTION

Despite significant dissimilarities in the primary structures of troponin Cs of cardiac and skeletal fast-twitch muscles, contraction of both muscles appears to be similarly triggered by Ca^{2+} binding to the low-affinity sites on TnC. The amount of tension made by skinned fibers is determined by free Ca^{2+} concentration in the activating solution [1]. In skeletal muscle, each fiber type has a characteristic sensitivity as determined from its p*Ca*-force relationship [2], but the characteristic differences expected between skeletal and cardiac muscles from their TnC types are not well documented. Furthermore, the slope of the p*Ca*-force relation in mid p*Ca* range provides additional information into the cooperative mechanism during activation, but whether TnC itself has a direct role in regulating cooperativity in cardiac muscle is not known.

To study these we presently utilize a recent technique by which TnC is extracted from a skinned cardiac muscle preparation and replaced with purified TnC from fast-twitch type skeletal muscle [3,4]. Comparisons of the sequences of STnC and cardiac CTnC have indicated, among other mutations, two key amino acid replacements in the loop portion of an EF-hand motif at the N-terminus portion of the CTnC molecule. These replacements are responsible for difference in the Ca^{2+} binding abilities of STnC and CTnC (both molecules contain 4 EF-hand motifs, but CTnC binds 3 Ca^{2+} and STnC binds 4) [5,6] that causes the loss of one of two putative trigger sites. In efforts to characterize functional implications of structural modifications in regulatory proteins, we presently compare p*Ca*-force relations on the same trabeculae with either CTnC or STnC. Despite overall similarity in the triggering mechanisms of cardiac and skeletal muscles, the results show that Ca^{2+} sensitivity and cooperativity in the activation mechanism in myocardium are influenced by certain alterations in the primary structure of TnC.

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2. MATERIALS AND METHODS

2.1. *Tissue preparation and TnC extraction*

Skinned trabeculae from the right ventricle of freshly excised

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hamster hearts were used (6–12-month-old Syrian hamsters of either sex). The separated heart was continuously oxygenated in Krebs solution. After clearing the blood, the right ventricle was exposed and the bathing solution was changed to cold (4°C) relaxing solution (see below) containing 0.5% lubrol-X. Search was made for a suitable trabecula between the septum and the outer wall. After attachment to the force transducer, sarcomere length of the preparation was adjusted at 2.2 μm using laser diffraction [3,7].

Relaxing solution contained 100 mM K-propionate, 20 mM imidazole, 5.6 mM $MgCl_2$, 5 mM ATP, 5 mM EGTA, 20 mM creatine phosphate and 250 units/ml creatine phosphokinase. EGTA to Ca-EGTA ratio was varied to achieve the appropriate level of free Ca^{2+} in the activating solutions. The pH of relaxing and activating solutions was 7.00 ± 0.01 and ionic strength was maintained at 190 mM.

TnC extraction of the attached preparation was made by incubation in 5 mM EDTA, 10 mM imidazole, pH 7.2, extracting solution for up to 30 min at 30°C. About 20–30% residual TnC was retained to avoid the possible complications arising from extended extraction. TnC levels in the experimental preparations were checked with SDS gels. Reconstitution was accomplished by incubating the preparations for 30 min at 15°C in a solution with 0.5 to 2 mg/ml purified TnC (CTnC or STnC).

2.2. *Purification of TnC*

Purified STnC was made from rabbit back muscles, and CTnC from rabbit or bovine hearts, by a method slightly modified from Szynkiewicz et al. [8].

2.3. *SDS-PAGE*

SDS-polyacrylamide slab gels (10–15%) were run routinely on trabeculae saved at the end of the experiments. Samples were dissolved in a buffer containing 0.1% SDS following the BioRad protocol (BioRad Bulletin no. 1081) with an additional essential step that the sample was briefly ultrasonicated with an intense source (Branson Sonifier, model 200, with a doublestep microtip). The sample buffer also contained either 1 mM EGTA or 1 mM $CaCl_2$. Also the gel slabs were exposed to 5% glutaraldehyde for a few hours prior to silver staining. STnC band was best resolved from the cardiac LC2 band with EGTA in 12% gel. CTnC band was resolved from LC2 in 15% gels whether or not EGTA was added. Only the 12% runs (with EGTA) are shown in fig.1, but all dta (10–15% gels) were included in table 1.

2.4. *Data analysis and statistics*

The fit to the p*Ca*-force relations was made with the Hill equation by the method of least squares. The p*K* and *nH* parameters obtained thereby represent the apparent Ca sensitivity and the slope of the curve, respectively. The test for the significance of the data was made by the Student's *t*-test, wherever appropriate.

3. RESULTS AND DISCUSSION

Fig.1 compares the p*Ca*-force relations on trabeculae of three different types: (a) native, (b) trabeculae subjected to extraction treatment and then reloaded with CTnC, and (c) extracted trabeculae loaded with STnC. The curve for STnC-loaded myocardium is seen to be shifted to the right by 0.14 unit, indicating that the presence of STnC reduced the Ca sensitivity. The CTnC-loaded preparations had the same sensitivity as native trabeculae, which shows that the extraction and loading procedures themselves had not modified the tissue. The curve for the STnC-loaded myocardium is also seen to be steeper: the Hill coefficient *nH* was increased by 58%.

The force recovery for maximal activations was similar on reconstitution with CTnC and STnC as shown in table 1. A typical gel run is shown in fig.1B and indicates a new band for STnC in the STnC-loaded preparation (lane 2). This band was missing in the CTnC-loaded trabecula (lane 1). The uptake of STnC was comparable to the amount of deleted CTnC (table 1).

From both biochemical and physiological studies involving activations with Sr^{2+}, it is well known that cardiac muscle is 5–10 times more sensitive than fast-twitch skeletal fibers [9,10] and this property is almost fully accounted for by the differences in their TnC moieties in the fibers [3]. The present finding that Ca^{2+} sensitivity of the myocardium is decreased after CTnC/STnC exchange, is consistent with the Sr^{2+} results although the difference in the Ca^{2+} sensitivities with STnC and CTnC is small. Such a difference between the Ca^{2+} sensitivities of skeletal and cardiac muscles has not

Table 1

TnC uptake and force recovery in myocardium

Tissue preparation	Force	TnC/LC1	
		cardiac	skeletal
Native	1.0	0.32 ± 0.05	-
(−) CTnC	0.97 ± 0.01	0.30 ± 0.04	-
(+) STnC	0.91 ± 0.04	0.07 ± 0.02	0.23 ± 0.03

Tension of the skinned myocardium was measured by activation with maximal free Ca^{2+} (p*Ca* 4). All force values were normalized to the maximal tension (P_o) of the native unextracted preparation. TnC contents were normalized to the LC1 band in the same lane on the SDS-PAGE slab. STnC staining was found to be slightly lighter than CTnC by this procedure, but the appropriate correction was determined and applied to the data shown. There were 8 preps in the native and 4 each in the other categories. The mean tension on activation at the end of extraction was 0.09 ± 0.02 P_o

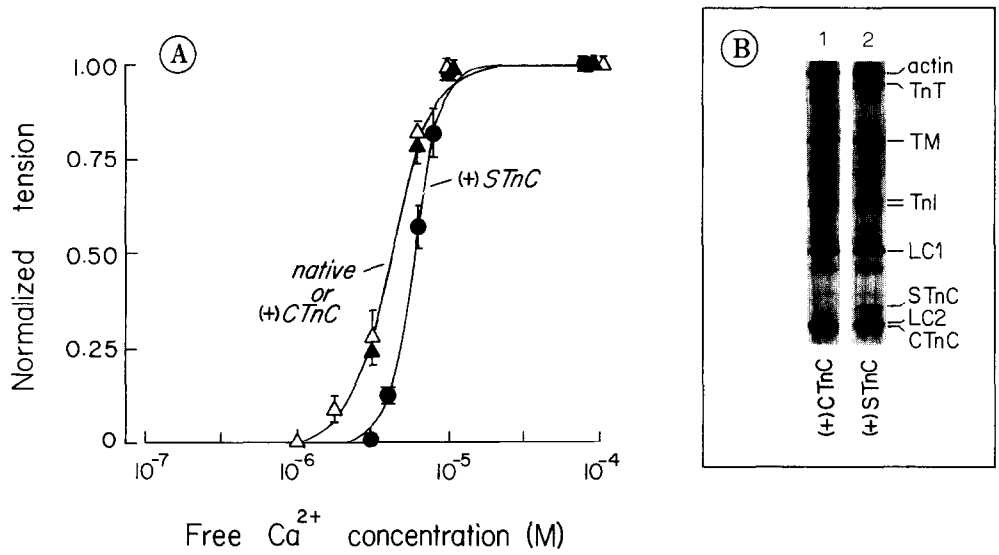

Fig.1. (A) Effect of STnC loading on the p*Ca*-force relationship of cardiac muscle. For these results, the TnC-extracted trabeculae were loaded with either CTnC (closed triangles) or STnC (closed circles). In each case results were compared with the native unextracted preparation (open triangles). All activations were made at 20°C. The p*K* value for the CTnC-loaded preparations was indistinguishable from native and the two sets of data points were combined to obtain the best fit: p*K* = 5.37, *nH* = 3.1. For STnC-loaded trabeculae, p*K* = 5.23 and *nH* = 4.9. (B) A typical SDS-PAGE run (12%; silver stained) from one CTnC-loaded (lane 1) and one STnC-loaded (lane 2) trabecula. EGTA was included in the sample buffers. Notice the STnC band in lane 2. The quantitation on the STnC-loaded trabeculae is included in table 1.

been well documented previously [11], but it is possible to explain this discrepancy because the earlier results comparing this parameter in the two systems were necessarily from separate preparations. The small difference in the Ca^{2+} sensitivities of skeletal and cardiac cells (unlike Sr^{2+}) could be masked by minor disparity in sarcomere lengths in the different preparations: the point is particularly notable since the length dependence of Ca^{2+} sensitivity is markedly different in the two tissues [4,12].

3.1. *Effect of TnC moiety on cooperativity*

The most significant finding of the present study is that Hill coefficient *nH* of the myocardium for Ca^{2+} activation (indicating the steepness of the p*Ca*-force relationship) was increased in the presence of STnC. In most current models of muscle activation, the value of Hill coefficient above unity implies cooperativity in the thin filament, and the increased value in the presence of STnC would, therefore, indicate a direct role for TnC in the cooperative mechanism. With isolated protein there is good experimental support for the idea that interaction between adjacent tropomyosins is also required to convert the thin filament into a cooperative unit [13–15]. This is supported also in fibers by recent studies showing that Hill coefficient decreased with partial extraction of TnC [16]. These and the results of the present study suggest, therefore, that during its action as a Ca^{2+} switch TnC also facilitates the interaction between tropomyosin molecules. The available evidence also suggests that TnC participation in thin-filament cooperativity occurs independently of intramolecular interactions between the Ca^{2+}-binding sites in TnC, because the binding of Ca^{2+} itself is found not to be highly cooperative and since cooperativity occurs in muscles whether TnC has a total of four Ca^{2+}-binding sites (as in skeletal muscle [1]), three sites (as in cardiac muscle [17,3]), or even a single site (in crayfish [18]). According to the presently observed difference with STnC and CTnC, it is possible, however, that cooperativity level is scaled (but not eliminated) by the number of Ca^{2+}-binding sites on TnC, and this is worth checking in future studies.

Cross-bridge attachment (numbers and the con-

figuration) has also been implicated in the overall Ca^{2+} sensitivity in muscle [19], but in an important study investigating the origin of cooperative mechanism using pure cardiac actin-troponin-tropomyosin, Tobacman [20] showed cooperativity to be intrinsic to the thin filament in relatively low salt (30 mM). He found that actomyosin ATPase activation by Ca^{2+} was cooperative even in the absence of cooperative actin-myosin binding, raising the possibility that any cross-bridge effect was important possibly only as one of the factors in muscle cooperativity. More recently Brenner [21] has suggested a rather novel interpretation for the p*Ca*-force relationship of skeletal fibers by arguing that a possible decrease in the attachment rate in the attachment-detachment kinetics in cross-bridge turnover could also influence the slope of the p*Ca*-force relation. But this could not be the primary mechanism for the present results on cardiac muscle, because to thus explain the increased myocardial cooperativity after exchanging CTnC with STnC would require that the TnC moiety also have a direct effect on myosin to influence properties of the detached bridge.

3.2. *Relation to other studies.*

The effect of StnC on the cooperativity in cardiac muscle is analogous to the observation with CTnC in fast-twitch skeletal fibers. But there was uncertainty in the earlier results, because CTnC in skeletal muscle shifted the p*Ca*-force curve towards higher p*Ca* values [22]. This would indicate decreased Ca sensitivity with CTnC, but this is opposite of the expected results with Sr^{2+} on skeletal and cardiac muscles. Part of the complication arises because CTnC was a poor substitute for STnC in skeletal fibers as is true under certain conditions [3,23]. It is also worth noting that the increased cooperativity in myocardium with STnC is more noticeable in experiments with Ca^{2+} than with Sr^{2+} [3], suggesting that the expected effect on this parameter with Sr^{2+} is small.

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