Ca^{2+} activation of troponin C-extracted vertebrate striated fast-twitch muscle fibers

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To characterize the tension control in vertebrate striated muscle fibers, and to obtain insights into the cross-bridge mechanisms, Ca^{2+} activation on troponin C (TnC)-extracted skinned fibers was studied in standard (180 mM, physiological) and low (20–41 mM) ionic strength solutions. By tension measurement, TnC-extracted fibers had nearly lost their Ca^{2+} sensitivity in the standard ionic strength solutions, but surprisingly the fiber still exhibited significant tension on activation with Ca^{2+} in low ionic strength. Also, the presence of weak bridges (zero-force bridges) was inferred by stiffness measurements in Ca^{2+}-free low ionic strength solution, and were found even after TnC extraction. The possibility is discussed that dual regulation by Ca^{2+} is present in the vertebrate muscle. One mechanism activates the thin filaments. The second may directly control the kinetic step for the transition between the weak and strong bridges, in the cross-bridge cycle in the fiber, and in this way may act as an additional Ca^{2+} switch.

Dual regulation *Myosin-linked control* *Troponin C* *Calcium switch* *Muscle contraction* *Cross-bridge mechanism* *Weak bridge* *Strong bridge*

1. INTRODUCTION

Calcium controls tension development in vertebrate striated muscle as an 'on-off' switch of the cross-bridges [1,2]. The switching mechanism is thought to be on thin filaments and is initiated when calcium binds to troponin C (TnC) [3], although in other systems, such as invertebrate smooth and striated muscles, as well as vertebrate smooth muscles, the Ca^{2+} regulation is either solely myosin-linked [4,5] or may involve both actin- and myosin-linked processes [6]. Two recent studies suggested a secondary Ca^{2+} sensitivity of vertebrate skeletal muscle myosin ATPase as well, but the results were controversial and the effect was on modifications of the actomyosin ATPase by a factor of at most two over the basal level [7,8]. In the present study we find conditions (low ionic strength) where significant Ca^{2+} regulation for tension control was still present in vertebrate fibers whose TnC had been maximally extracted. These results raise the possibility of an alternate regulatory mechanism in the cross-bridge cycle during tension development in vertebrate skeletal muscle [9].

2. MATERIALS AND METHODS

Psoas muscle fibers from adult 9-month-old Syrian hamsters (Strain RB; University of Toronto) were utilized for the majority of the studies and skinned either mechanically or chemically. The fast-twitch fibers were selected (see below). To extract TnC, the fibers were transferred from the standard (180–190 mM ionic strength) relaxing solution (20 mM imidazole, 80 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 20 mM creatine phosphate (CrP), 250 units/ml creatine

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phosphokinase (CrPK) and 5 mM EGTA) to a rigor solution (20 mM imidazole, 165 mM KCl, 2.5 mM EGTA, 2.5 mM EDTA, pH 7.0) at 4°C for 5 min. Next the temperature was raised to 30°C and the fiber transferred to the extracting solution (10 mM imidazole, 5 mM EDTA, pH 7.2) for 5–120 min [10]. TnC can be fully extracted by this treatment as indicated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of normal and extracted single fibers (fig.1). In a few cases temperature during extraction was kept at 4°C [11], but it took as much as 4–8 h for 'full' extraction, and the extraction in this case was maximally about 80% as judged by the tension response to p*Ca* 4. All experiments on hamsters were repeated on rabbit fibers, and the results were similar in the two cases.

The standard activating solution was the same as the relaxing solution above (180–190 mM ionic strength) except that EGTA was replaced with Ca-EGTA so that $-\log [Ca^{2+}]$ or p*Ca* was 4. Calculations for the solution composition were made as before [12]. The low ionic strength solution (41 mM) in the experiments contained: 10 mM imidazole, 5.6 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA or Ca-EGTA, 5 mM CrP and 250 units/ml CrPK. pH was adjusted to 7.00 at room temperature.

2.1. *Mechanical set-up*

The attachment procedures for the fibers and handling were similar to those described earlier [1,12]. The servo-motor and the transducers were also similar to before [13]. Solution chambers were thermoelectrically controlled as described by Gulati and Podolsky [14]. Temperature during the experiments was varied at different stages of the protocol, and this is indicated in the text.

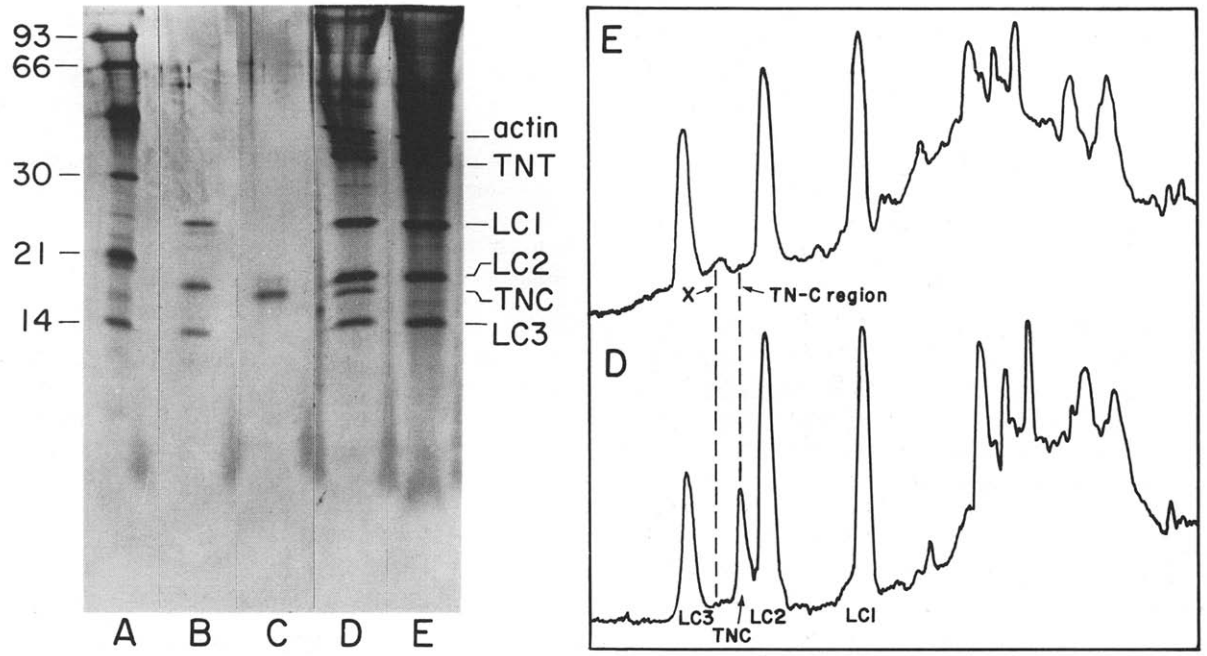

Fig.1. Silver stained 15% SDS-PAGE of control and TnC-extracted rabbit psoas fibers. Lanes: A, molecular mass (kDa) standards; B, purified rabbit myosin light chains; C, purified rabbit troponin C; D, control fiber; E, extracted fiber (2 h extraction period). Lanes D and E were overloaded (i) to facilitate TnC detection (in control), which has a general tendency to stain lighter than other proteins [10,11], and (ii) to substantiate the removal of TnC in the extracted fiber. At least 95% of the TnC was extracted (normalized to actin; note that loading of lane E was 26% more than lane D). The band below TnC (marked 'x' in the gel scan) was somewhat darker, and if this represented degraded TnC and the density corresponded to the amount of protein, this band still represents less than 15% of the original TnC. Thus most of the TnC is washed out of the extracted fiber. Note also the presence of the 3 light-chain (LC) bands in lanes D and E. The gels were scanned with the use of LKB laser densitometer.

2.2. *Selection of fast-twitch fibers*

The first set of activations of the fibers was (i) in a solution with p*Sr* 5 (p*Sr*: $-\log [Sr^{2+}]$) and then (ii) with p*Ca* 4, to select the fast-type fibers. Fast fibers are less sensitive to Sr^{2+} activation [15–17] and give nearly zero force in p*Sr* 5. In contrast, slow-twitch fibers produced nearly equal force in p*Sr* 5 and p*Ca* 4, and such fibers were excluded from the present study. An additional check on the fiber type was made by imposing a series of large length steps producing slacks during the plateau of force in p*Ca* 4, to measure V_{max} of shortening [1]. V_{max} of the fast fibers ranged between 4.5 and 9.0 L_o/s at 20°C.

3. RESULTS

Fig.2A shows the altered tension response to p*Ca* 4 (standard activating solution) of the extracted fiber from hamster and the recovery to near normal tension level when the fiber was incubated with 2–6 mg/ml purified TnC from rabbit fast skeletal muscle (incubation time 20–30 min at 20°C). The near absence of tension in fig.2A ('minus-TnC') is additional evidence (fig.1, lane C) that residual TnC was close to zero after extraction. Near normal level of tension in the reconstituted fiber (3rd trace, fig.2A) indicates that cross-bridge properties for force development were preserved despite the extraction treatment.

Table 1 shows that the rigor stiffness was also unchanged after TnC extraction, which gave another indication of the preservation of the contractile components involved in cross-bridge interaction between actin and myosin.

Fig.2B compares the Ca activations of TnC-extracted fibers at two different ionic strengths, standard 180 mM (HIS) and low 41 mM (LIS). At standard ionic strength, the tension response of the extracted fiber (1st trace, fig.2B) was close to zero independent of free calcium level (like the middle trace, fig.2A). In low ionic strength (4 mM free Mg^{2+}) the TnC-extracted fiber remained relaxed at low Ca^{2+} (p*Ca* 6–7) but when the free calcium concentration was raised to p*Ca* 4 in low ionic strength we observed tension development that was close to the force (P_o) of the unextracted fiber in standard ionic strength with p*Ca* 4. Such response to Ca was seen in fibers whether they were extracted at high

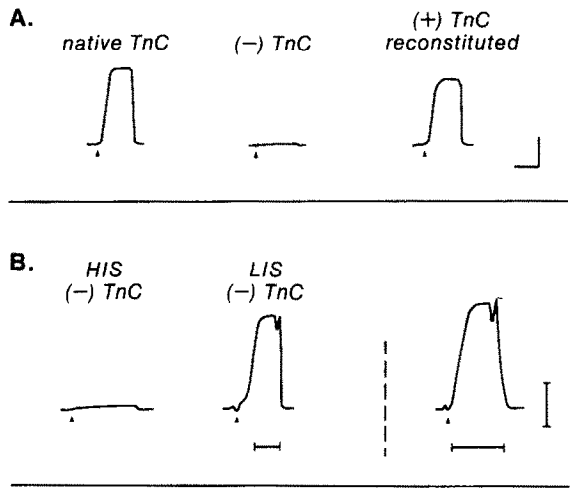

Fig.2. (A) Effect of troponin C extraction and reconstitution on tension response to p*Ca* 4 activation. First trace is on the native fiber from hamster; middle trace, after the complete TnC extraction (50 min extraction period); last trace, after the same fiber was reconstituted with purified rabbit psoas TnC. The skinned fiber was tied between a force transducer and a servo-motor as described before [1]. Sarcomere length was adjusted at 2.5 μm by laser diffraction, in the relaxing (HIS) solution. The transfer to p*Ca* 4 solution was made at the arrowhead. (B) Compares the typical tension responses of the TnC-extracted fiber from hamster to Ca activation in standard (HIS) and low ionic strength (LIS) solutions. The force in the LIS solution is similar to the force with Ca activation in HIS solution on the same fiber with native TnC (compare middle trace with last trace). The LIS response was somewhat variable from fiber to fiber, ranging between 0.5 and 1.0 P_o (P_o, force of the unextracted fiber in HIS and p*Ca* 4). Temperature, 20°C, but similar results were found for activation at 4°C in LIS; vertical bar, 50 kN/m^2; horizontal bar, 10 s.

Table 1

Fiber stiffness in rigor

	Native TnC	(−) TnC
Rigor stiffness (P_o/nm per h)	0.19 ± 0.09 (5)	0.20 ± 0.07 (5)

Stiffness of the hamster fibers was measured by stretching the fiber by 0.5% L_o in 750 μs. Temperature, 5°C. P_o was measured with p*Ca* 4 prior to TnC extraction

Table 2

Relaxed fiber stiffness in low ionic strength (LIS)

	Native	(−) TNC
Stiffness (fraction of rigor stiffness) [N = 5]	0.46 ± 0.10	0.49 ± 0.09

The ionic strength was 20 mM in these experiments and the solution was the same as in [19]

(30°C) or low (4°C) temperature (middle trace, fig.2B).

Recent studies using X-ray diffraction and stiffness measurements [18–20] have found the presence of attached cross-bridges in low ionic strength solution even when tension was zero in normal fibers. These were termed 'weak bridges'. In this study we wanted to determine whether the presence of these weak bridges was influenced by TnC extraction. The results are shown in table 2. The fiber stiffness in low ionic strength in the absence of Ca^{2+} (zero tension) was found to be the same whether or not the fiber was subjected to the extraction treatment, which indicates that the weak bridges in this solution are unaffected by TnC.

4. DISCUSSION

The results in this study show that Ca sensitivity for tension development in vertebrate skinned fibers is present in low ionic strength despite the extraction of native TnC. A number of mechanisms are possible for this finding.

One rests on the postulate that calmodulin in the fiber substitutes for the lost TnC. This possibility arises because calmodulin has been found to replace TnC in the ATPase and the binding experiments with isolated contractile proteins [21,22], but the explanation may be questionable for the present fiber studies because (i) all the diffusible calmodulin would have washed out on skinning, and (ii) Ca sensitivity was seen in the TnC-extracted fibers only in low ionic strength. The fact that the Ca-sensitive tension in low ionic strength was stable over several contractions, rules out another possibility that the residual calmodulin (CaM) might bind to TnI in the fiber and remove the complex (CaM-TnI) out of the thin filament [22].

The second mechanism for the Ca sensitivity of the TnC-extracted fibers is the possibility of increased effectiveness of the residual TnC in low ionic strength, combined with a possible cooperative activation of the fiber by a few cross-bridges. Effectiveness of TnC could be increased by the weakened binding of tropomyosin to actin in low ionic strength [23]. To check this possibility we tried the effect of Mg, since tropomyosin binding is improved with increasing Mg [23,24]. But the tension in TnC-extracted fiber was found to be insensitive to Mg in the 20 μM–4 mM concentration range (Babu and Gulati, unpublished).

Another possibility is that the Ca sensitivity in TnC-extracted fibers in low ionic strength is on the thick filament, suggesting a dual regulation for tension control in the vertebrate muscle under these conditions. Myosin-linked modification of ATPase activity in myofibrils [7] and of purified actomyosin [8] was suggested previously as either altering the intrinsic enzymatic properties (a 2-fold effect), when there was no or poor thin filament control, or as a fine tuner of the calcium switch on troponin [25]. Also partial extractions of the LC_2 moiety of myosin had a specific effect upon the skeletal [25,26] and cardiac [27] actomyosin Mg-ATPase, and decreased the V_{max} of skinned fibers by a small factor [28]. In the present study thin filament control (TnC) was dissociated from thick filament control (LC's) by TnC extraction while maintaining full relaxation (with native TnI). In this way, the present results demonstrate for the first time the possibility of myosin-linked control over the entire tension range (0 to ~100% P_o), which may truly reflect the characteristics of an independent additional switch in vivo.

A potential role for the dual switching mechanism may be in separating the regulation of weak and strong cross-bridge attachments. Increasingly, it is becoming apparent that power stroke in the cross-bridge cycle follows the transition from a 'weakly' attached state to the 'strong' state [18,29,29a,30]. Since weak bridges are already present in low ionic strength, even in the absence of TnC (table 2), the present results raise the possibility that Ca^{2+} is necessary for the forward progression of the cross-bridge cycle for the weak state to the strong state, in vivo. Thus, the

simplest explanation for the second role of Ca^{2+} (first being turning on the thin filament) is that it acts directly on the cross-bridge during the kinetic cycle. Chalovich and Eisenberg [31] arrived at a similar conclusion with studies on isolated proteins, and the present results suggest that a myosin-linked Ca^{2+} switch is possible in the fiber too. Additional experiments are needed to critically test the various possibilities noted above, and the TnC-extracted fiber preparation in low ionic strength may be an important tool for further characterizing the dual switching mechanism proposed here in the vertebrate muscle.

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