Volume 310, number 3, 229–234 FEBS 11604 October 1992

Troponin replacement in permeabilized cardiac muscle

Reversible extraction of troponin I by incubation with vanadate

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Received 11 August 1992

Calcium-dependent regulation of tension and ATPase activity in permeabilized porcine ventricular muscle was lost after incubation with 10 mM vanadate. After transfer from vanadate to a vanadate-free, low-Ca^{2+} solution (pCa >8), the permeabilized muscle produced 84.8% ± 20.1% (± S.D., n=98) of the isometric force elicited by high Ca^{2+} (pCa ~4.5) prior to incubation with vanadate. Transfer back to a high Ca^{2+} solution elicited no additional force (83.2% ± 18.7% of control force). SDS-PAGE and immunoblot analysis of fibers and solutions demonstrated substantial extraction (>90%) of Troponin I (TnI). Calcium dependence was restored after incubation with solutions containing either whole cardiac troponin or a combination of TnI and troponin C subunits. This reversible extraction of troponin directly demonstrates the role of TnI in the regulation of striated muscle contractility and permits specific substitution of the native TnI with exogenously supplied protein.

Troponin; Troponin I; Vanadate; Contractility; Muscle regulation

1. INTRODUCTION

A calcium-induced change in the interaction among the subunits of the troponin/tropomyosin complex relieves the inhibition of the actomyosin ATPase in striated muscle at low calcium concentrations [1]. The functional consequences of structural changes in the proteins comprising this regulatory complex is of interest generally to the study of calcium-dependent regulation, as well as of specific interest to the study of muscle regulation.

To complement the information already derived from biochemical studies using isolated proteins [2,3], it has become necessary to relate the structure of troponin to the regulation of striated muscle *contraction* in a structurally intact system. Yet, it is often difficult to interpret the role of changes in protein structure in vivo due to a lack of rigorous control of the highly complex system. Permeabilized, or 'skinned', fibers have often offered the possibility of bridging the gap between solution biochemistry and the in vivo condition.

Previous work in skinned skeletal muscle has demonstrated that the normal calcium dependence of contraction is dependent upon an intact troponi/tropomyosin complex [4]. The more classical of these studies used EDTA to selectively extract and replace troponin C (TnC) to demonstrate that the structure of TnC affected the characteristics of the regulatory system [4–6]. Troponin I (TnI), another of the subunits of troponin involved in the signal transduction between an increase in Ca^{2+} and the activation of actin, is a potential target of structural alterations which may result in a change in the calcium responsiveness of the system [7]. Some isoform changes and covalent modifications (e.g. phosphorylation) of TnI have been correlated with changes in the behavior of the calcium-dependent regulation of cardiac muscle in vivo [8,9]. For example, the calcium responsiveness of perinatal heart tissue containing predominantly one isoform of TnI is affected less by a decrease in pH than cardiac muscle from adults containing a different isoform [9]. Unfortunately, TnI, as well as troponin T (TnT) and tropomyosin, have not been quite so amenable to selective substitution in skinned fibers as TnC, making direct structure–function studies difficult. Some recent studies in skeletal muscle have approached the problem through partial extraction of TnI through incubation with excess TnT [10] or digestion of the troponin complex with limited proteolytic degradation [11].

We demonstrate here that incubation with a high concentration of sodium vanadate of short duration eliminates the calcium dependence of skinned *cardiac* muscle tissue through the extraction of >90% of the

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Abbreviations: TnI, troponin; TnC, troponin C; Tnt, troponin T; DOC-TCA, deoxycholate trichloroacetic acid; ATP, adenosine triphosphate; EDTA, ethylene-diamine tetraacetic acid; EGTA, ethylene glycol (bis(β-aminoethyl ether) *N,N,N′,N′* tetraacetic acid; DTE, dithioerythritol; NADH, nicotinamide adenine dinucleotide; UV, ultraviolet; HRP, horseradish peroxidase.

normal TnI content, while retaining up to 100% of the original force-generating capacity of the fiber. Moreover, calcium regulation could be partially restored by incubation with a solution containing troponin, thereby substituting some of the native troponin subunits with exogenously supplied protein.

2. MATERIALS AND METHODS

2.1. *Skinned fibers*

Skinned cardiac muscle fibers from the right ventricle of swine heart were prepared according to previously published procedures [12]. Briefly, trabeculae free from the endocardial wall were excised and permeabilized at 4°C for 24 h in a solution of 50% (v/v) glycerol, 1% Triton X-100, and (in mM): NaN_3 10, ATP 5, $MgCl_2$ 5, EGTA 4, DTE 2, and imidazole 20, pH 7.0. Skinned fiber bundles were stored at −20°C in a similar solution but without Triton X-100. Immediately before use, smaller fiber bundles, 0.1–0.2 mm in diameter and 5 mm in length, were dissected from these stored fiber bundles.

2.2. *Solutions*

'Contracting' and 'relaxing' solutions were made as double concentrates and diluted to normal strength just prior to use. Normal relaxing solution contained 380 U/ml creatine kinase and (in mM): imidazole, 30; ATP, 10; phosphocreatine, 10; $MgCl_2$, 10; NaN_3, 5; and EGTA, 5. The pH was adjusted to 6.7 with either KOH or HCl, as needed. Ionic strength was adjusted as necessary with either potassium acetate or potassium methane sulfonate, pH 6.7. Maximum calcium-activating solution (contracting solution) contained also 5 mM $CaCl_2$ (pCa ~4.5). Solutions used for ATPase measurements substituted phosphoenol pyruvate and NADH for phosphocreatine, and lactate dehydrogenase and pyruvate kinase for creatine kinase, as previously described [13]. In experiments in which solutions were analyzed for protein extracted during incubation, all enzymes were omitted (creatine kinase, lactate dehydrogenase, and pyruvate kinase) to avoid interference by the high concentrations of these proteins in the SDS-PAGE analysis. Intermediate levels of Ca^{2+} were obtained by mixing relaxing and contracting solutions and correcting for ionic strength differences. Ionic strength, Mg^{2+}, MgATP, and Ca^{2+} were calculated by a computer program provided by Dr. Mark Andrews, New York College of Osteopathic Medicine, and was based on the method of Fabiato and Fabiato [14].

2.3. *Force and ATPase measurements*

Contractile function was measured by two methods. For standard force measurements, fibers were mounted isometrically between an AE 801 strain gauge (SensoNor, 3191 Horten, Norway) and a rigid post attached to a micrometer for length adjustment [12]. In a second type of experiment, force and ATPase activity were measured simultaneously according to the procedure of Güth and Wojciechowski [13]. All measurements were carried out at 21°C. The ATPase assay used in the second type of apparatus measures decrease in NADH fluorescence with an UV excitation wavelength of 340 nm. Because UV irradiation in the presence of vanadate is reported to photolyse myosin [15], the excitation beam was extinguished during incubation with vanadate.

2.4. *Vanadate treatment*

An initial test contraction–relaxation cycle was performed on all fibers to ensure the calcium dependence of force in each preparation. Following this, fibers were again contracted maximally (pCa 4.5) to establish a control level of maximum isometric tension. After a plateau with regard to tension had been reached, fibers were incubated for 10 min in relaxing solution containing 10 mM sodium vanadate. Following incubation with vanadate, fibers were transferred to fresh relaxing solution to remove the vanadate.

2.5. *Gel electrophoresis and immunoblot of TnI content*

Individual fibers were homogenized in 20 μl of an SDS (2%)-urea (6 M) sample buffer and heated (~95°C) for 5 min prior to electrophoresis. Vertical slab gel SDS-PAGE (15% acrylamide, 0.4% bisacrylamide) of proteins was performed [16]; some gels were stained with Coomassie blue while others were transferred to nitrocellulose membranes (0.2 μm mesh) for immunoanalysis [17]. A 5% powdered milk, Tris-buffered saline solution was used to block non-specific protein binding sites. Blots were incubated sequentially with monoclonal anti-TnI antibody (obtained from Dr. T. Dhoot, University of London), monoclonal anti-LC1 antibody (obtained through Dr. I. Morano, Heidelberg), and HRP-labeled anti-mouse IgG antibody (obtained from Jackson Immunolab through DIANOVA GmbH). Because of differences in the amount of protein loaded, TnI content was quantified as a ratio of the density of the TnI band relative to the density of the LC1 band, similar to the procedure of Moss [11]. This ratio for vanadate-treated fibers was compared to the ratio for untreated, control fibers in order to quantitate the percentage of TnI extracted.

2.6. *Source of troponin*

Whole cardiac troponin, as well as some purified cardiac TnI, was a gift from Dr. N. Beier, Merck Darmstadt, Germany. Additional purified troponin subunits were provided by Dr. R. Hodges, University of Alberta, Edmonton, Alberta, Canada. TnI was dialyzed against the double concentrated relaxing solution to provide sufficient ionic strength to dissolve the protein.

2.7. *Reconstitution of calcium dependence*

Following troponin extraction by incubation with vanadate, fibers were incubated with either whole troponin (2 mg/ml, ~18 μM) or with a sequential combination of purified TnI and TnC to restore calcium dependency. Because the degree to which calcium dependence was restored varied, the 'maximum calcium-dependent force' was defined as the difference between the force generated at pCa >8 and the force generated at pCa ~4.5. 'Maximum' tension is therefore the sum of the calcium-dependent force and the calcium-independent force.

2.8. *Statistics*

All values are means ± S.D., unless otherwise noted. Student's *t*-test, or a paired *t*-test where appropriate, was used to test significance. Significance was accepted at a P value of <0.05.

3. RESULTS

3.1. *Loss of calcium-dependent regulation*

Initially, all fibers demonstrated calcium dependence of isometric force, which increased when Ca^{2+} was raised from pCa >8 to pCa ~4.5. Incubation in 10 mM vanadate relaxed the fibers completely regardless of the concentration of calcium (Fig. 1). After transfer to normal relaxing solution without vanadate, force slowly developed despite the continued absence of Ca^{2+} (pCa >8), taking between 10 to 30 min to reach a stable plateau. Thus, it was demonstrated that calcium-dependent regulation of isometric force was lost. After the level of calcium-independent force had stabilized, fibers were transferred to a calcium-containing solution to demonstrate that no additional force (calcium-dependent force) is obtained with increased Ca^{2+} (Fig. 1). Partial loss of calcium-dependent regulation could be obtained at concentrations of vanadate as low as 1 mM and for as little as 2 min, however a 10 min incubation

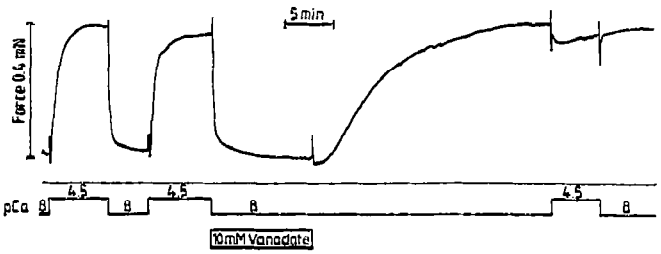

Fig. 1. Protocol for vanadate treatment of skinned cardiac muscle. pCa values of incubation solutions are indicated by numbers directly below the tracing. Duration of incubation with vanadate is indicated by the bar.

Table I

Isometric force and ATPase activity in vanadate-treated fibers

	Isometric force (%)	ATPase activity (%)
pCa > 8		
Average	67.5 ± 14.1	54.9 ± 17.0
Max.	101.6	117.7
Min.	36.1	30.0
pCa ~4.5		
Average	68.2 ± 15.0	61.0 ± 18.3
Max.	111.1	117.6
Min.	34.4	35.0
n	28	28

Values presented are relative to the initial control value obtained for reach fiber prior to vanadate treatment, as described in section 2.4. The average is the arithmetic mean ± S.D. for a group of 28 fibers. Max. and Min. are the maximal and minimum, respectively, of the range of values for this set of fibers.

of 10 mM vanadate was accepted as the optimal condition for both retention of the control maximum isometric tension (84.8% ± 20.5%, n=98) and elimination of calcium-dependent tension. In fact, force generation averaged less in the presence of calcium than in the absence (83.3% ± 18.71%), although this difference was not significant. Interestingly, while the results could be duplicated in bovine and rat cardiac tissue, the phenomena could not be demonstrated in glycerinated rabbit psoas muscle.

After a step change in length, i.e. a 'quick release' (<5% L_o), calcium-independent tension recovered to original levels, thereby demonstrating active shortening (data not shown). Simultaneous force and ATPase activity measurements on a subset of the fibers (28 of 98) demonstrated that an increase in calcium-independent isometric tension was paralleled by an increase of calcium-independent ATPase activity (Table I). We conclude from these data that actively cycling, energy-expending crossbridges underlie the increase in isometric tension, and not a type of rigor-like bridge.

When ATPase activity and tension were measured simultaneously, a small, though statistically insignificant (P > 0.2), increase was noted in the ATPase activity with an increase in the calcium concentration (~10% of calcium-independent ATPase) although the level of force did not increase (Table I). This small increase may be attributable to calcium-sensitive, crossbridge-independent ATPases, such as residual membrane calcium ATPase. Alternatively, this may represent some dissociation between the calcium regulation of ATPase activity of myosin and tension generation.

There was, however, a significant difference (P ≤ 0.005) between the two methods for measuring contractility with regard to the degree of force-generating capacity retained. Fibers in which isometric tension alone was measured in the basic apparatus (first method, see section 2.3) retained greater force-generating capacity (91.7% ± 18.4, n=70) than fibers tested for simultaneous force/ATPase measurements (67.5% ± 14.1, n=28). This difference may be attributable to photo-inactivation of myosin with UV irradiation in some fibers in the latter experiments which may have contained residual vanadate [15].

3.2. *TnI extraction*

SDS-PAGE analysis for protein content demonstrated that the vanadate-containing solution contained substantial amounts of TnI along with actin, and lesser amounts of tropomyosin and TnT (Fig. 2). No TnI and relatively little protein of any kind is found in control 'mock' incubation solutions in which vanadate was omitted, as is generally the case in skinned fiber experiments [4], demonstrating that protein is extracted from the fiber due to the presence of vanadate.

Additional SDS-PAGE analysis for total protein and immunoblot analysis for TnI were performed on control and vanadate-treated fibers (Fig. 2). The protein profile of the vanadate-treated fibers was significantly altered relative to the control fibers. Of particular interest was the near total disappearance of a band that had a mobility similar to that bovine TnI, yet the fibers still contained substantial amounts of myosin light chains and tropomyosin. Significant extraction of TnI was confirmed by immunoblot analysis; only 7% ± 4% (S.E.M., n=12) of TnI was retained (Fig. 2). In several cases, TnI could not be detected at all by immunoblot analysis.

3.3 *Reconstitution of calcium-dependent regulation*

Subsequent to extraction with vanadate, several TnI-depleted fibers (n=5) were incubated with solutions containing bovine troponin and tropomyosin (dissolved at an ionic strength, i.s., of 250 mM). Force was greatly inhibited in these fibers (Fig. 5) and remained so after return to regular relaxing solution (i.s. ~100 mM). Thus, a large portion of calcium-independent force was lost due to incubation with troponin (Fig. 3). In control experiments, the higher ionic strength itself inhibited

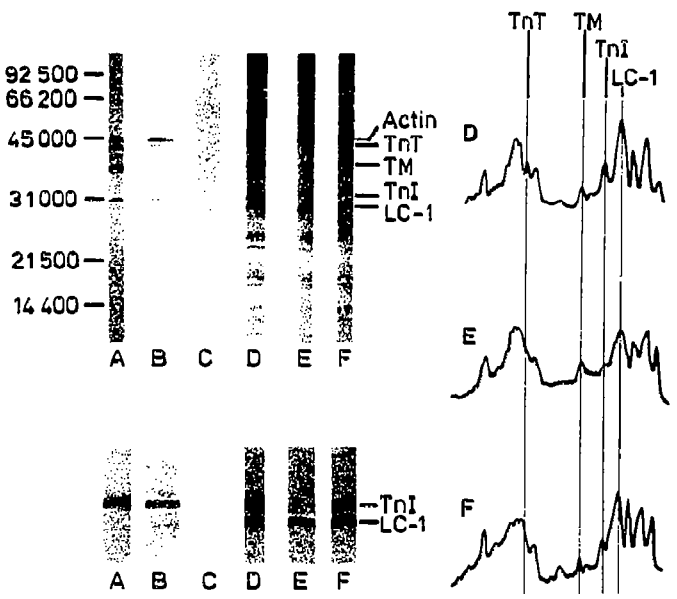

Fig. 2. SDS polyacrylamide gel protein analysis. Molecular weights are reported in Daltons. Lane A, crude troponin preparation containing bovine TnI, TnT and tropomyosin (TM). Lane B, DOC-TCA precipitable protein content of the vanadate-containing incubation solutions after incubation with skinned fibers. The vanadate-containing incubation solutions were pooled after incubation with between 6 and 10 fiber bundles. Lane C, DOC-TCA precipitable protein from control relaxing solution ('mock' extraction solution) demonstrating that no TnI is extracted in the absence of vanadate. Solutions were pooled after incubation with 10 fiber bundles. Lanes D,E,F, control skinned porcine cardiac fiber, vanadate-treated fiber, and vanadate-treated porcine skinned fiber, respectively, reconstituted with troponin. The lower panel shows an immunoblot which more clearly demonstrates the TnI and LC1 content of extraction solutions and individual fibers. The side panel shows the densitometric scans of the Coomassie blue-stained lanes, D, E and F. Note that the peak corresponding to TnI virtually disappears in the vanadate-treated fiber (lane E). This peak can again be observed in the troponin-reconstituted fiber (lane F).

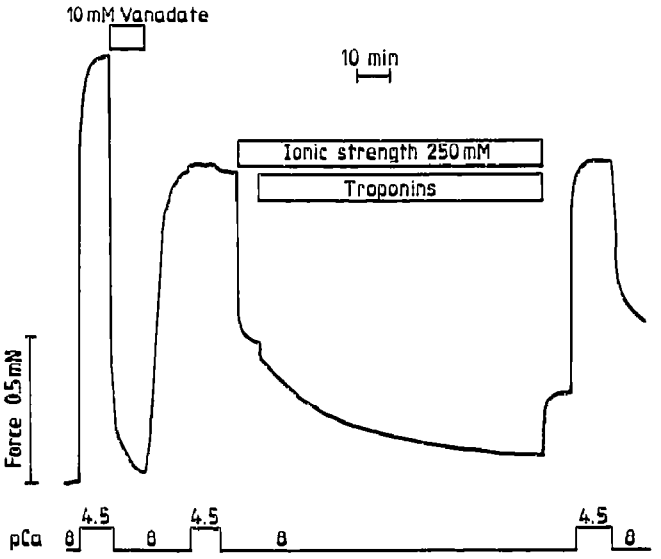

Fig. 3. Reconstitution of calcium-dependent regulation. Force tracing of a porcine cardiac fiber treated with vanadate and subsequently reconstituted with troponin. The fiber was incubated with whole bovine troponin/tropomyosin (Troponins, 2 mg/ml) for 90 min at high ionic strength (250 mM adjusted with potassium acetate).

force to some extent, as has been previously described for most skinned muscle [18], however, this effect can be reversed simply by returning the fiber to a solution with normal ionic strength (i.s. ~100 mM, data not shown).

Importantly, isometric tension could be increased to levels approaching the total maximum force observed initially after vanadate treatment by transferring the troponin-reconstituted fiber to contracting solution (pCa ~4.5), demonstrating that a large portion of the calcium dependence was functionally restored. This partial reconstitution of calcium regulation was associated with retention of troponin by the fibers, as demonstrated by SDS-PAGE analysis of the troponin-reconstituted fibers (Fig. 2).

These experiments were repeated with purified individual troponin subunits. Incubation with bovine cardiac TnI alone slowly reduced the calcium-independent tension to between 5 and 30% of that obtained after vanadate treatment ($t_{\frac{1}{2}}$, time to 1/2 maximum inhibition, = 12.3 min., Fig. 4). Interestingly, upon transfer to calcium after incubation with just TnI, only a portion of the expected calcium-dependent force was generated. These fibers therefore qualitatively resembled partially TnC-extracted skeletal tissue from previous work [5,6]. Subsequent to this TnI incubation, fibers were incubated for an additional 30 min with purified bovine cardiac TnC. These TnI-, TnC-loaded fibers were then competent to generate substantially greater calcium-dependent tension, similar to the results obtained with total troponin (Fig. 3). TnC does not resolve well under these electrophoresis conditions, is not well stained by Coomassie blue, and migrates very near LC_2. It was therefore difficult to determine if TnC was retained by the fibers by SDS-PAGE analysis. However, because incubation with TnC enhances the restoration of calcium sensitivity, it may be concluded that a significant amount of TnC is also lost during vanadate treatment. Incubation with TnT or tropomyosin did not affect the restoration of calcium-dependent regulation, despite the fact that small amounts of both of these proteins could sometimes be detected in the vanadate extraction solution (Fig. 2). Incubation of the vanadate-treated fibers with TnC alone did not restore calcium dependence. We interpret this to mean that sequential incubation with TnI followed by TnC is essential and sufficient to substantially reconstitute the calcium-dependent regulation of vanadate-treated cardiac fibers.

In a set of four fibers, the force–pCa relationship was established by measuring force generation at various concentrations of calcium prior to incubation with va-

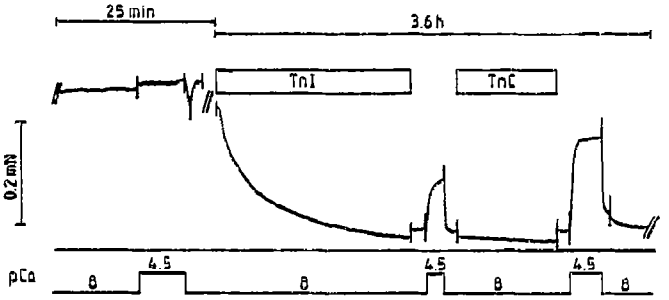

Fig. 4. Individual subunit replacement of troponin subunits. The tracing of the tension record is started after vanadate treatment and subsequent force redevelopment, as in Fig. 1, to conserve space. Sequential incubation of the calcium-unregulated fiber with TnI (3–5 mg/ml) and TnC (1.4 mg/ml), as indicated by bars, restored a substantial amount of the calcium-dependent regulation.

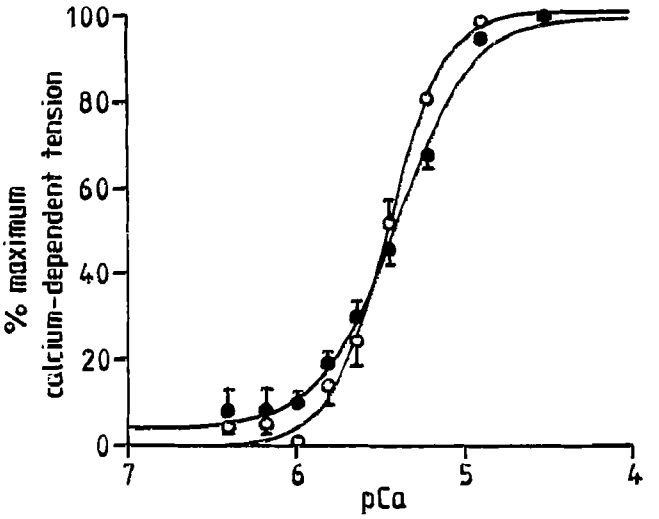

Fig. 5. Force–pCa relationship in reconstituted cardiac fibers. Force–pCa relationships were constructed prior to vanadate treatment (○) and following reconstitution (●). Fibers were reconstituted by sequential incubation with highly purified cardiac TnI (3–5 mg/ml) followed by purified cardiac TnC (1.4 mg/ml) from bovine cardiac muscle, similar to Fig. 4. Symbols represent means ± S.E.M. of tension relative to maximum dependent tension for four fibers, as described in the methods.

nadate. After reconstitution with a combination of TnI and TnC subunits, as in the experiment shown in Fig. 4, a second force–pCa relationship was determined. This set of fibers with a restored calcium regulatory system had a force–pCa relationship similar to that obtained prior to vanadate treatment (Fig. 5). Although the Hill coefficient was slightly reduced (from 2.4 in controls to 1.9 after reconstitution), the calcium sensitivity, as indicated by the pCa_{50} values, was essentially the same before extraction (pCa_{50} = 5.46) and after reconstitution (pCa_{50} = 5.41) of these fibers, indicating that an essentially normal calcium regulatory system had been reconstituted.

4. DISCUSSION

Incubation of triton-skinned cardiac tissue with millimolar concentrations of vanadate promoted the loss of calcium dependence with regard to both force and ATPase activity without substantial loss of force-generating capacity. A high ATPase activity was associated with the calcium-independent force and the fibers were able to actively contract, taking up slack, thereby demonstrating that actively cycling crossbridges underlie tension generation. Importantly, total force-generating capacity is substantially maintained, suggesting that the contractile apparatus per se is relatively undisturbed. It has been previously shown that actomyosin from striated tissue prepared without the troponin subunits have an unregulated ATPase activity [1–3]. In an analogous way, we have verified that troponin extraction in cardiac tissue abolishes the regulation of contraction.

These data also demonstrate that TnI in particular is responsible for maintaining the muscle fiber in the relaxed state in the absence of calcium. Co-extraction of this protein with TnC resulted in unregulated force production, whereas previous work demonstrates that extraction of TnC alone only suppresses force generation capacity in the presence of calcium [5,6]. Importantly, the unregulated force can be inhibited by incubation with purified troponin I alone.

While TnI extraction could be directly demonstrated, it was concluded that partial TnC extraction was also promoted by this treatment from the fact that TnC significantly enhanced reconstitution of the regulatory system. In contrast, incubation with either tropomyosin or TnT did not enhance, and was apparently unnecessary for, reconstitution of the regulatory system. Moreover, SDS-PAGE analysis demonstrated that vanadate-treated fibers retained at least some tropomyosin. Post-extraction tropomyosin and TnT content was apparently sufficient for reconstitution of the regulatory system. It is concluded from these data that the loss of calcium-dependent force generation in these fibers is primarily via extraction of TnI and TnC.

Vanadate has been shown to interfere with the activity of a number of ATPases, including the myosin ATPase of both actomyosin preparations and skinned muscle fibers [19–21]. It is conceivable that a compound that affects myosin conformation could affect actin, tropomyosin, and troponin interactions so as to decrease the stable binding of one or more components; we have known for some time that they attachment of crossbridges are capable of affecting troponin function [22,23]. Alternatively, the mechanism for de-stabilizing the bound state of TnI and TnC may involve a different mechanism, possibly one specific for the polymeric, polyanionic structure of vanadate which exists at high concentrations [24]. The steep concentration dependence of vanadate concentration, from 1 to 10 mM, may argue

for a role for polymeric vanadate [24]. Despite the lack of molecular details regarding the mechanism of action, extraction of the calcium-dependent regulatory system occurs with vanadate treatment and is potentially valuable as a tool.

This novel procedure provides a highly reproducible means to isolate the structurally intact contractile machinery from the regulatory system of skinned cardiac tissue. These calcium-independent, or 'vanadated', fibers offer a means to study the contractile apparatus of striated muscle independent of the regulatory system. This model also makes possible studies of the function of structural changes in TnI by substitution with covalently modified cardiac TnI or different TnI isoforms. Until now, studies of the importance of TnI structure on the regulation of contractility have been limited to biochemical studies of purified proteins or correlation of changes in function with changes in structure in vivo [1–3,8,9]. Because it is now possible to substitute TnI (and TnC) in a structurally intact system, the function of changes in structure in terms of regulation of contraction in striated tissue may now be directly tested.

Acknowledgements: This material is based upon work supported in part by the Deutsche Forschungs Gemeinschaft (J.C.R.) and a grant from the North Atlantic Treaty Organization (1991–1992, J.D.S.). J.D.S. was a recipient of an A.v. Humboldt Fellowship (1990).

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