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A synthetic peptide mimics troponin I function in the calcium-dependent regulation of muscle contraction

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A new technique for treating skinned cardiac muscle fibers has been developed in which troponin I is extracted, giving rise to unregulated fibers. The effect of the 12-residue troponin I peptide on these fibers indicates that this region of troponin I is solely responsible for muscle relaxation (inhibition of force). Furthermore, troponin I peptide–troponin C reconstituted fibers are stable through several contraction–relaxation cycles indicating the peptide can switch binding sites between actin and troponin C. The troponin I peptide can substitute for the native protein as part of the calcium-sensitive molecular switch that controls muscle regulation.

Troponin I; Synthetic peptide; Muscle regulation; Protein–protein interaction

1. INTRODUCTION

The role of troponin I (TnI) as an inhibitor of the contractile ATPase in muscle is now well established (for review see [1]). It has been previously demonstrated that a 12-residue synthetic peptide of troponin I, TnI peptide 104–115 (Ac-G-K-F-K-R-P-P-L-R-R-V-R-amide), is able to substitute for its native protein (which is 15-fold larger) in the inhibition of the acto-S1-TM ATPase activity by binding to acin–tropomyosin [2,3]. In addition, this inhibitory peptide has been shown to bind to troponin C (TnC) in the presence of Ca^{2+} [4–10] which releases TnI peptide inhibition [7]. These results lead us to propose that this region of TnI switches between binding sites on actin and TnC in a Ca^{2+}-dependent manner. This hypothesis is based on biochemical studies done in solution using purified proteins. It has also been shown that the TnI peptide inhibits force development in skinned striated muscle fibers, at submaximal Ca^{2+} activation [10,11]. Skinned fibers are muscle fibers in which the cell membrane has been permeabilized and hence represents a structurally intact system. These fibers respond to changes in the Ca^{2+} concentration in much the same way as living muscle, contracting at high concentrations of Ca^{2+} and relaxing at low levels. These results are complicated to interpret because the peptide competes with the native TnI present in the fiber for its binding site on TnC and/or on actin thereby directly inhibiting the actin–myosin interaction. By utilizing a new method of treating skinned cardiac muscle fibers with vanadate which permits the reversible extraction of TnI as well as some TnC and TnT [12,13], we now have obtained evidence for the direct interaction of TnI peptides with actin and a Ca^{2+}-dependent interaction with TnC. The TnI-depleted fibers have enabled us to further investigate the ability of the TnI peptide to substitute for TnI as part of the regulatory switch which controls muscle contraction.

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Abbreviations: ATP, adenosine triphosphate; DTE, dithioerythritol; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol (bis(β-aminoethylether) *N,N,N′,N′*,tetraacetic acid; NADH, nicotinamide adenine dinucleotide; NaN_3, sodium azide; S1, myosin subfragment 1; TM, tropomyosin; Tn, troponin; TnI, troponin I; TnT, troponin T; TnC, troponin C.

2. MATERIALS AND METHODS

2.1. *Preparation of peptide and protein*

The peptides were synthesized by standard solid-phase peptide synthesis procedures. The proteins, TnT and TnC were purified from whole Tn. The peptides and proteins were purified by reversed-phase HPLC [6,14]. For the skinned fiber assays, the peptides were dissolved in 20 mM imidazole, pH 6.7. TnT was dissolved and dialyzed against 60 mM imidazole, 20 mM ATP, 20 mM creatine phosphate, 10 mM MaN_3, 10 mM EGTA, 25 mM $MgCl_2$, pH 6.7 (double concentrated relaxing solution). To ensure TnC was Ca^{2+}-free, it was dissolved in 20 mM imidazole, 2 mM EDTA, 6 M guanidine-HCl, 1 mM DTT, then dialyzed extensively against 20 mM imidazole, pH 6.7. The concentrations of peptide stock solutions were determined by amino acid analysis and protein solutions by the Bradford assay.

2.2. *TnI-depleted cardiac skinned muscle fibers*

Porcine skinned cardiac fibers were mounted with fast-setting glue on an isometric force transducer (AME 80, SensoNor, Horten, Norway) and a glass rod attached to a micromanipulator [15]. When monitoring simultaneous changes in the ATPase activity and force the method described by Güth and Wojciechowski [16] and Strauss et al. [13] was used. Danz-labelled skeletal TnC was obtained from Dr. J.

Potter, University of Miami. Changes in fluorescence of Danz-labelled TnC was monitored as described by Güth and Potter [17]. The porcine skinned cardiac fibers were relaxed by immersion into relaxing solution (30 mM imidazole, 10 mM ATP, 10 mM creatine phosphate, 5 mM NaN_3, 5 mM EGTA, 12.5 mM $MgCl_2$ and 380 U·ml^{-1} creatine kinase, pH 6.7, ionic strength 100 mM). The contracting solution was analogous to the relaxing solution except EGTA was replaced by a Ca^{2+}-EGTA buffer to give a pCa of 4.5 (5 mM $CaCl_2$). The concentration of Ca^{2+} in the buffers was determined from the ratio of EGTA to Ca^{2+}-EGTA, essentially according to Fabiato and Fabiato [18], except that the apparent dissociation constant of 1.6 μM for Ca^{2+}-EGTA buffer at pH 6.7 and 20°C was used [19]. After contraction, the fibers were incubated in 10 mM vanadate (Na_3VO_4) in relaxing solution for 10 min [12,13]. The vanadate inhibited force. Vanadate was washed from the fibers using relaxing solution until maximum force was achieved. The fibers were tested for Ca^{2+}-insensitivity by transferring between fresh relaxing, contracting and relaxing solutions. Only the fibers which displayed no increase in force in the presence of Ca^{2+} were used in these experiments.

2.3. *Reconstitution of TnI-depleted fibers*

Vanadate-treated fibers were incubated at relaxing solution containing TnI peptide until maximum inhibition occurred (30–45 min). The TnI peptide-loaded fibers were tested for the absence of Ca^{2+} sensitivity (as above). The fibers were then incubated in relaxing solution containing TnC (20–30 min). Excess (unbound) TnC was washed from the fiber by immersion in relaxing solution. Incubation of the vanadate-treated fibers with 30 μM TnT (15–30 min) subsequent to the TnI peptide-TnC reconstitution does not overly change the Ca^{2+}-dependent characteristics of the fibers (Fig. 4), however, it did increase the consistency of the reconstituted fibers displaying full Ca^{2+}-sensitivity (contraction followed by relaxation). Therefore, an initial TnT incubation has now been adopted as the standard reconstitution protocol.

2.4. *SDS-gel electrophoresis*

Protein samples and individual fibers were homogenized in a SDS (2%) urea (6 M) sample buffer. The proteins were separated by SDS-PAGE (15% acrylamide–0.4% bis-acrylamide) on duplicate gels [13]. One gel was stained for total protein content using Coomassie blue, while the other was electroblotted to nitrocellulose for immunoanalysis. This blot was probed for TnI content with a monoclonal TnI antibody obtained from Dr. Dhoots, University of London, and horseradish peroxidase-labelled secondary antibody (Jackson Immunolab, Dianova GmbH). Under these conditions, TnC is not clearly resolved from LC_2.

3. RESULTS AND DISCUSSION

We have confirmed that extraction of TnI and partial extraction of TnC and troponin T (TnT) from the cardiac skinned fibers (cross-striated muscle) occur after treatment with 10 mM vanadate [12,13], giving rise to unregulated fibers. Fig. 1 shows SDS-PAGE gels and the corresponding immunoblot using an antibody against TnI for a control fiber (a fiber not treated with vanadate), a vanadate-treated TnI-depleted fiber, and vanadate-treated fiber reconstituted with skeletal TnT, TnI peptide, and skeletal TnC. It is clear from the immunoblot that there is no significant amount of TnI remaining in the vanadate-treated fibers compared to the control fiber. SDS gel electrophoresis showed that the vanadate-treated fibers contain substantial quantities of endogenous TM and TnT ([13] and Fig. 1). However, exogenously supplied TnT is required for consistent and functional TnI peptide-TnC reconstitution indicating some TnT may be extracted during vanadate-treatment (discussed later). Since TnC does not stain well by Coomassie blue and migrates very near a light chain of myosin (LC_2), it is difficult to determine the amount of TnC retained in the vanadate-treated fibers. However, because incubation with TnC restores full Ca^{2+} sensitivity of TnI reconstituted fibers [13] and TnI peptide reconstituted fibers (Fig. 3), it may be concluded that a significant amount of TnC has been extracted.

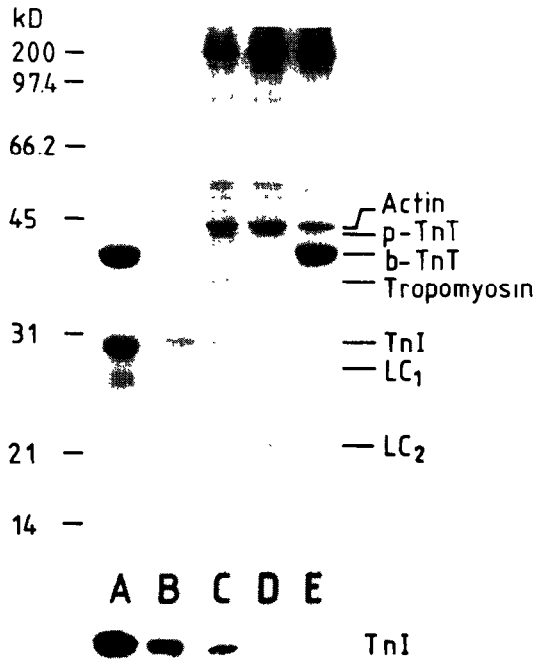

Fig. 1. SDS-gel electrophoresis (upper panel) and corresponding immunoblot (lower panel) of: troponin (lane A), bovine TnI (lane B), a control skinned porcine cardiac fiber (lane C), a vanadate-treated fiber (lane D), and a vanadate-treated porcine skinned fiber reconstituted with skeletal TnT, TnI peptide and skeletal TnC (lane E). The cardiac TnT endogenous to the fiber (labelled p-TnT) has a different mobility on the gel than the exogenously supplied skeletal TnT (labelled b-TnT). The peptide-reconstituted fiber (lane E) contained no TnI and was comparable to the vanadate-treated unregulated fiber (lane D).

Incubation with 10 mM vanadate relaxed the fibers completely but after transfer to relaxing solution (pCa 8.0) without vanadate, force slowly developed despite the continued absence of Ca^{2+} [12,13]. In fact, after removal of the vanadate, the skinned muscle fibers (TnI-depleted fibers) develop near maximum force which is similar to the force induced by Ca^{2+} (pCa ~ 4.5) in the fibers prior to vanadate-treatment. However, the vanadate-treated fibers, unlike the non-extracted fibers, remain contracted regardless of the Ca^{2+} concentration, i.e. the vanadate-treated fibres are Ca^{2+}-insensitive. Using these unregulated fibers we have determined the effects of the TnI inhibitory peptide on the contractile behavior of functionally unregulated muscle

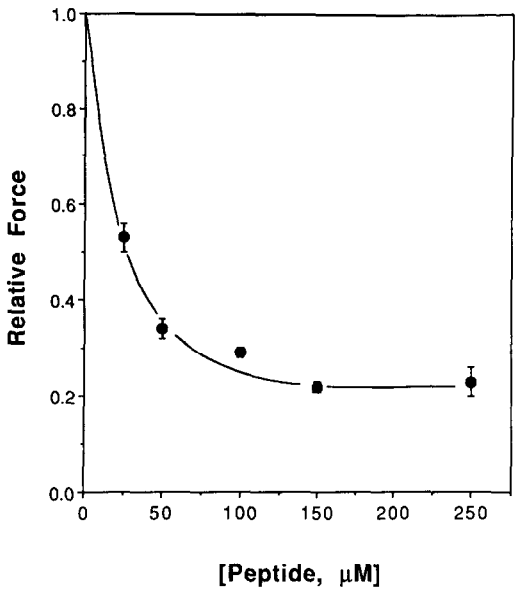

Fig. 2. Dose dependence of the skeletal TnI peptide inhibition of the vanadate-treated TnI-depleted fibers. The relative force obtained at each concentration of peptide was taken as a fraction of the force elicited in relaxing solution after vanadate-treatment and washout of the vanadate (mean ± S.D. of 4 experiments). Following vanadate treatment and wash out of the vanadate with relaxing solution, the fibers were incubated with increasing quantities of the TnI peptide (●) in relaxing solution.

and determined the ability of the peptide to function as part of the regulatory switch.

The addition of the skeletal TnI peptide 104–115 to the TnI-depleted fibres inhibits the Ca^{2+}-insensitive force in a dose-dependent manner (Fig. 2). Maximum inhibition of 67% ± 2.8 ($n = 9$) occurs at a concentration of TnI peptide of approximately 100 μM. This is similar to the degree of inhibition obtained when the TnI protein is incorporated into the vanadate-treated fibers [13]. Neither the TnI peptide or protein can totally inhibit the Ca^{2+}-insensitive force. The inhibition by the TnI peptide may be specifically attributed to the structure of the peptide rather than to its net charge (6 positive charges), since a scrambled peptide in which the amino acid sequence has been altered (Ac-R-F-R-V-K-L-R-P-R-G-K-P-amide) was less effective than the native peptide. At 50 μM, the native TnI peptide induces a significant loss of force (65% in 10 min) while the scrambled peptide at the same concentration induced only a very small loss of force (10% in 10 min) (data not shown). In addition, single glycine analogs of the TnI peptide are much less potent than the native peptide in binding TnC and inhibiting the acto-S1 ATPase activity [5]. These results indicate that any change to the amino acid sequence of the native peptide reduces the biological activity.

The vanadate-treated fibers which were relaxed by the native TnI peptide (peptide-loaded fibers) developed little or no force upon addition of calcium, i.e. the peptide-loaded fibres demonstrated essentially no Ca^{2+} sensitivity. In fact, given sufficient time, the peptide could be washed from the vanadate-treated fibres in either the presence or absence of Ca^{2+} (data not shown). Washout of the peptide was monitored by the return of high level of force in the fiber that was Ca^{2+}-independent. This indicates that there is reversible binding of the peptide to the vanadate-treated fibers and that the TnI peptide by itself is not sufficient for imparting Ca^{2+} dependence of contraction of the muscle fiber.

After incorporation of skeletal or cardiac TnC into the peptide-loaded fibers, calcium-dependent force was restored (Fig. 3A). In fact, the reconstituted fibres (skeletal TnI peptide and TnC) were stable through several contraction-relaxation cycles. The Ca^{2+}-induced contraction of the reconstituted fiber was accompanied by a corresponding increase in the ATPase activity and the Ca^{2+}-dependent conformational changed in TnC as monitored by the qualitative change in the fluorescence of the Danz-labelled TnC (Fig. 3B). These results suggest that the TnI peptide is able to switch between its binding site on TnC and actin–TM depending on the concentration of Ca^{2+}. It is important to note that the contraction cycles of the peptide–TnC reconstituted

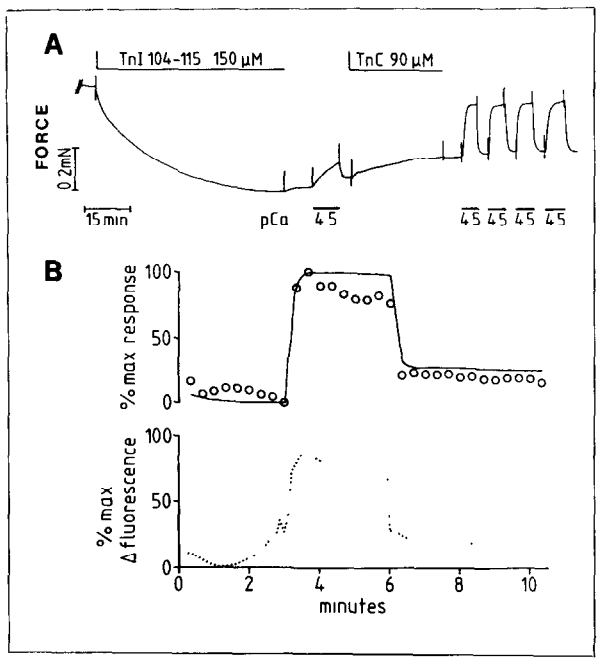

Fig. 3. Force tracing of the reconstitution of the peptide–TnC fiber and return of the Ca^{2+} sensitivity (panel A). The TnI-depleted fibres following vanadate treatment and washout (fully contracted) were incubated in relaxing solution containing 150 μM skeletal TnI peptide until maximum inhibition (> 90% in this experiment) occurred in approximately 30 min. The TnI peptide-loaded fibers where then incubated in relaxing solution containing 90 μM bovine cardiac TnC for 30 min. Panel B shows the qualitative changes in the fluorescence of a fiber reconstituted with peptide and Danz labelled skeletal TnC (dotted line) and simultaneous changes in the ATPase activity (○) and force (solid line) of a fiber reconstituted with peptide and skeletal TnC in the absence and presence of Ca^{2+}.

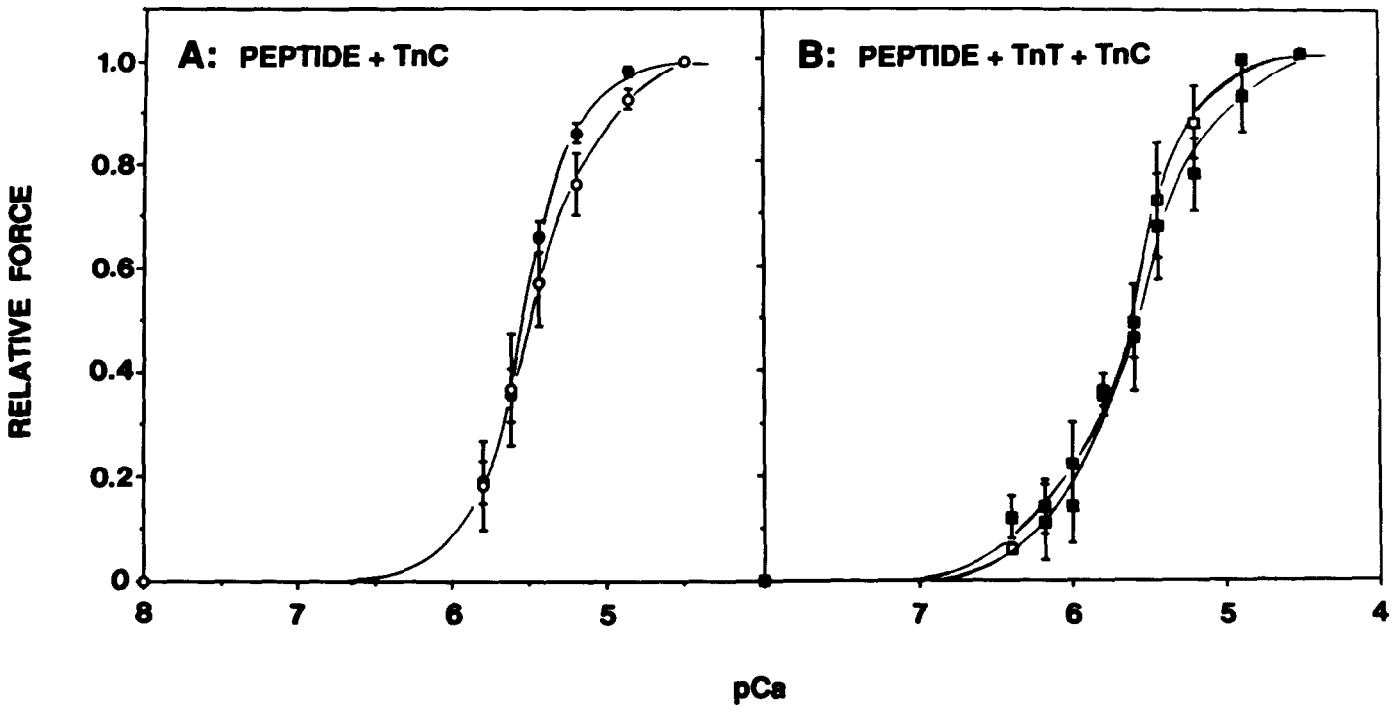

Fig. 4. pCa force relationship of cardiac skinned fibers before vanadate treatment and following vanadate treatment and reconstitution with either TnI peptide and skeletal TnC or the combination of TnI peptide and skeletal TnC and skeletal TnT. Panel A shows the control fibers (closed circles) and the TnI peptide–TnC reconstituted fibers (open circles, $n = 7$); panel B shows the control fiber (open squares) and the TnI peptide–TnC–TnT reconstituted fibers (closed squares, $n = 5$)). The error bars indicate the mean ± S.D. Concentration of peptide was 143 μM, TnC 100 μM and skeletal TnT if present was 30 μM. The vanadate-treated fibers were incubated in TnT or TnC for 20 min and incubated with the peptide until maximum inhibition of force had occurred (approximately 30–50 min).

fiber were done in relaxing and contracting solutions that contained neither TnI peptide nor TnC. During incubation with TnC, there is a small increase in force because of washout of the peptide (probably due to competition between TnC and actin for the peptide). Once in relaxing solution the reconstituted fibers (peptide plus TnC) were very stable and there was no washout of the peptide or TnC detected since there was no further increase in force with time in Ca^{2+}-free solutions. However, with increases in Ca^{2+}, the reconstituted fibers generated force. Essentially, the Ca^{2+}-dependent regulation of contraction in the fibers were restored. The maximum force developed upon addition of Ca^{2+} was approximately 87% of the force exhibited by the fiber following vanadate treatment. It should be noted that several fibers exhibited 100% recovery of the maximum force. As seen from the SDS-gel and corresponding TnI immunoblot (Fig. 1), TnI is extracted from the fiber upon vanadate treatment and is not present in the fibers reconstituted with the TnI peptide, TnT and TnC. Therefore, the contraction and relaxation exhibited by the reconstituted fiber must be due to the replacement of TnI by the TnI peptide. It is apparent that the TnI peptide is able to replace TnI in regulating the cycling of contraction and relaxation of the cardiac skinned muscle fiber.

The ability of the TnI peptide to substitute for TnI is further demonstrated by the similar relationship between Ca^{2+} and force development of the reconstituted fibres (skeletal TnI peptide plus TnC) compared to the control fibres containing endogenous Tn (Fig. 4A). The pCa_{50} values for the control and reconstituted fibers were 5.5 but there was a decrease (0.6) in the Hill coefficient from 2.5 to 1.9 for the control and reconstituted fibers, respectively. If TnT was also incorporated into the vanadate-treated fibers along with the TnI peptide and TnC (Fig. 4B), the effect on the Ca^{2+}–force relationship was similar to the fibers which were reconstituted with only peptide and TnC. The pCa_{50} values for the control and reconstituted fibers with TnT were 5.6 and there was a decrease (0.5) in the Hill coefficient from 1.9 to 1.4, respectively. Incubation of the vanadate-treated fibers with TnT subsequent to the TnC-peptide reconstitution did not overly change this characteristic of the fibers. In solution studies, TnT is not required for TnC (in the presence of Ca^{2+}) to release TnI or TnI peptide inhibition of the acto-S1-tropomyosin ATPase activity [7]. The advantage of incubating with TnT was that more of the reconstituted fibers displayed full Ca^{2+}-sensitivity (contraction followed by relaxation). Nine of the fourteen fibers reconstituted with TnI peptide–TnC were able to produce repeated Ca^{2+}-dependent contrac-

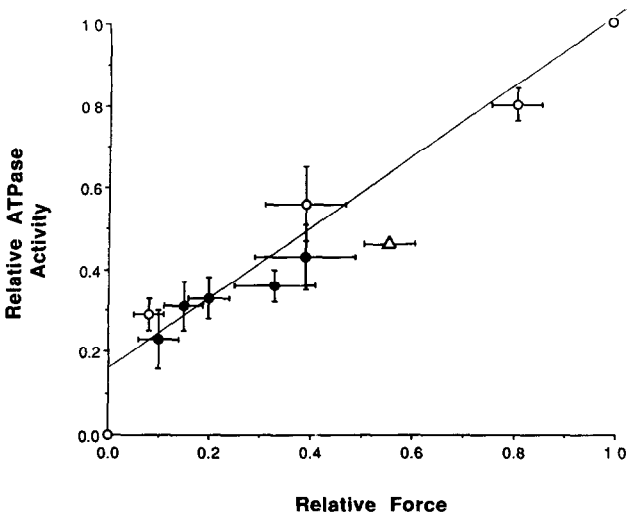

Fig. 5. Tension cost of a skinned cardiac fiber (○) compared to fibers reconstituted with the TnI peptide and skeletal TnT and TnC (●). The Ca^{2+}-dependent increase in force and ATPase of the skinned cardiac fiber prior to vanadate treatment and following reconstitution with TnI peptide (143 μM) and TnT (130 μM) and TnC (100 μM) was determined at the pCa values of 8.0, 6.0, 5.6, 5.2 and 4.3 (mean ± S.D., $n = 3$). (△) denotes the force/ATPase relationship of the TnI-depleted fibers following vanadate treatment and washout. The basal ATPase activity in relaxing solution was subtracted from the values obtained at the various Ca^{2+} concentrations.

tions compared to approximately eleven of the twelve fibers reconstituted with TnT, TnI peptide and TnC. The reconstituted fibers that did not display full Ca^{2+} sensitivity (fibers contracted but then did not relax) were not used. It must be noted that full Ca^{2+} sensitivity is consistently reconstituted by addition of only TnI and TnC [13]. These results suggest either TnT is not required for Ca^{2+}-dependent contraction and relaxation of the muscle or that the amount of TnT extracted during vanadate treatment is variable. SDS gels of TnI peptide-TnC fibers (no TnT incubation) which display Ca^{2+} sensitivity, showed that there is still significant amounts of native TnT present (data not shown, [13]) while in the TnT–TnI peptide–TnC reconstituted fibers, there is excess TnT present (Fig. 1). It is possible that incubation with TnT followed by TnI peptide and TnC ensures that there is a full complement of 'regulatory complexes' in the fiber. TnT is not critical for TnI protein reconstituted fibers [13] most likely because TnI has multiple sites of interaction with TnC, and some are insensitive to Ca^{2+}. Therefore, under relaxing or contracting conditions, TnC would be bound to the thin filament via TnI. On the other hand, in the peptide reconstituted fibers in the absence of Ca^{2+} (relaxation), the peptide is bound to actin–TM while TnC is anchored to the thin filament only through its interaction with TnT. If TnT is not present, then TnC is dissociated from the filament and could be washed from the fiber. In order to keep TnC attached to the TnI peptide reconstituted thin filament during relaxation, TnT is required.

The relationship between the ATPase activity and force of cardiac muscle during Ca^{2+} activation of the fibers prior to vanadate treatment (control) and after vanadate-treatment and addition of peptide was also determined (Fig. 5). The Ca^{2+}-dependent ATPase of the native control and reconstituted fibers (TnT-TnI peptide-TnC) consists of two components, a Ca^{2+}-tension independent and tension dependent portion. The slope of the latter, the 'isometric tension cost' is identical in both the native and reconstituted fibers. This indicates that the 'tension cost' of the native and reconstituted fibers is similar, i.e. the same amount of energy (ATPase activity) is required to produce or maintain a certain amount of force for a given time. Since we assume the tension-dependent ATPase corresponds to the contractile ATPase, then the slope of the force-ATPase relationship may be taken as a reflection of the 'tension cost' and of the apparent crossbridge detachment rate constant 'g' [20]. The results suggest that the crossbridge detachment rate constant is similar in native skinned fibers and in skinned fibers reconstituted with the TnI peptide, TnT and TnC at different levels of Ca^{2+}.

4. CONCLUSION

The results of this study have shown that the TnI peptide is able to functionally substitute for the whole TnI protein in imparting calcium regulation of contraction in a semi-intact (skinned) cardiac muscle fiber. What makes this finding astonishing is that this 12-residue peptide can inhibit force development (relaxation) by binding to actin (residues 1–28) [21–23] and that in the presence of Ca^{2+} this inhibition is released by binding to TnC (contraction). We interpret this to mean that the TnI peptide must be switching binding sites from actin to TnC and vice versa depending on whether calcium is or is not present. Obviously, the peptide acts as a calcium-dependent molecular 'switch' and thus a true functional mimic of the TnI protein.

Since the peptide induces relaxation of skinned muscle fiber, this region of TnI and its interaction with actin is solely responsible for the inhibition of force development, possibly by inhibiting the formation of strongly bound crossbridges. This indicates that the interactions of the N-terminus of actin (residues 1–28), with myosin [23,24] must also be critically involved in both force generation and ATPase activity.

To our knowledge, this is the first report of a synthetic peptide which, by changing binding sites from one protein to another, may function as a molecular switch in a reconstituted complex cellular system such as that involved in muscle regulation. Clearly, these studies support the use of synthetic peptides to unravel complex protein-protein interactions in biological systems.

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