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An Improved Method for Exchanging Troponin Subunits in Detergent Skinned Rat Cardiac Fiber Bundles

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We describe a method for the removal of endogenous troponin (Tn) complex from bundles of detergent-treated cardiac fibers. After 70 min treatment with cTnT-cTnI most of the endogenous Tn complex was removed from fiber bundles. Complete reconstitution of the Tn complex was achieved by reconstituting with cardiac troponin C (cTnC) in fully relaxing conditions. Ca^{2+}-dependent maximum force of the fibers treated with cTnT-cTnI or cTnT-$cTnI_{33–211}$, which was used to aid in the visualization of the troponin exchange, decreased to 85–90% of the force developed by fibers before the treatment. SDS-PAGE analysis of the cTnT-$cTnI_{33–211}$ and the $cTnT_{77–289}$-$cTnI_{33–211}$ treated fiber bundles demonstrated that 70–80% of the endogenous Tn subunits were removed. After reconstitution with cTnC, approximately 80–85% of the Ca^{2+}-regulated force was restored in cTnT-cTnI/$cTnI_{33–211}$ treated fibers. Our results demonstrate that by minimizing the prolonged exposure of skinned cardiac fiber bundles to rigor conditions, successful exchange of all three subunits of the Tn complex can be accomplished with minimal loss of function.

Switching striated myofilament activity from relaxed to active states involves complex steric, allosteric and cooperative processes (1). In the relaxed state the thin filament proteins, troponin (Tn) and tropomyosin (Tm), impede the reaction between actin and myosin cross-bridges. Ca^{2+} binding to troponin C (TnC) triggers release from the troponin I (TnI) induced inhibition and alters thin filament protein-protein interactions leading to force generation. To aid in our understanding of Tn mediated regulation of muscle contraction, studies have been done in which naturally occurring variants or modified thick and thin filament proteins have been isolated and reassembled stoichiometrically to obtain functional soluble preparations (2). While providing useful data on molecular signaling, these preparations lack a force generating myofilament lattice.

Thus, approaches have been developed for replacing thin filament components in detergent treated muscle preparations that retain the intact native myofilament lattice. One useful approach is to remove the native TnC by treatment with metal chelators such as EDTA and CDTA (for a review see (3)). This approach has provided useful information, which has advanced our understanding of the role of TnC in the regulatory process. Treatment of fiber bundles with proteases (4) and vanadate (5) have been used substitute the endogenous Tn complex or the cTnI-cTnC in detergent skinned muscle preparations. These procedures have the disadvantage of being nonspecific or causing irreversible loss of complete relaxation and maximum contractile force. Alternative methods for selective substitution of Tn subunits in thin filaments include transgenic approaches employing targeted expression of proteins and the adenoviral vector transfer of DNA encoding thin filament proteins into adult cardiac myocytes in primary culture. These versatile techniques have made it possible to study the function of virtually any protein in cells that retain the native structure. However, in both transgenic (6, 7) and adenoviral transfection (8) approaches it is sometimes difficult to express higher than 5–10% of the protein of interest.

To substitute higher amounts of Tn in intact myofilaments, a novel method was proposed (9) in which exogenous skeletal TnT (sTnT), in large excess, was added to muscle preparations and skinned fiber bundles under special conditions. By mass action, the ex-

Abbreviations used: s, fast skeletal; c, cardiac; TnC, troponin C; TnI, troponin I; TnT, troponin T; Tm, tropomyosin; EDTA, ethylenediamine-tetraacetic acid; EGTA, ethylene glycol bis(β-amino ethyl ether)-*N*,*N*,*N*′,*N*′-tetra acetic acid; MOPS, (3-[*N*-morpholino] propane-sulfonic acid); MES, 2-[*N*-morpholino]ethanesulfonic acid; Tris, tris (hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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ogenous sTnT displaced most of the Tn complex. Various isoforms or otherwise modified skeletal TnI (sTnI) and skeletal TnC (sTnC) subunits could then be used to reconstitute the myofilament preparations. We have found this method to be particularly useful in studies on sets of mutant cardiac troponin subunits, when applied to cardiac myofibrillar preparations of the order of 5–10 μm in diameter. However, when we applied the method to skinned cardiac fiber bundles, we encountered problems that resulted in poor recovery of maximum force generating capability in approximately 50% of the fibers following a long extraction/reconstitution episode in which the fiber bundles developed Ca^{2+}-independent force.

In work presented here we describe a new and improved method in which the skinned rat cardiac fiber bundles remained relaxed through much of the extraction/reconstitution procedure. Skinned fiber bundles were treated with a mixture of cTnT-cTnI to displace the endogenous Tn. Using this method we obtain a high percent success rate of extraction of the endogenous Tn in cardiac fiber preparations in which nearly all of the Ca^{2+}-dependence of myofilament activation is restored after reconstitution with cTnC.

METHODS

Expression and purification of proteins. Recombinant human cTnC (10), mouse cTnI (11) and mouse $cTnI_{33-211}$ (11) were purified, as previously described. Rat cTnT was purified by a modified procedure of Chandra *et al.* (12). Wild type cTnT was expressed in BL21(DE3) cells using the pSBETa expression system (Boehringer Mannheim). After growing BL21(DE3) cells overnight in Luria Broth containing 30 mg/ml kanamycin, cells were centrifuged and sonicated in 50 mM Tris (pH 8.0), 6 M urea, 1.0 M KCl, 5 mM EDTA, 0.2 mM PMSF, 1 mM benzamidine-HCl and 1 mM DTT. The insoluble fraction was separated by centrifugation and the supernatant fraction was subjected to $(NH_4)_2SO_4$ fractionation (30%, 43%, and 60%). The pellet from the 60% $(NH_4)_2SO_4$ cut was dissolved in 50 mM Tris (pH 8.0), 6 M urea, 0.1 M KCl, 5 mM EDTA, 0.2 mM PMSF, 1 mM benzamidine-HCl and 1 mM DTT and purified by chromatography on a DEAE-sepharose column. cTnT was eluted with a 0.1–0.4 M KCl gradient. Purified cTnT from the DEAE-sepharose column was extensively dialyzed against 0.1% formic acid and 10 mM β-mercaptoethanol. After dialysis, cTnT was lyophilized and stored at −80°C.

Preparation of detergent skinned fiber bundles. Left ventricular papillary muscle fiber bundles from male Sprague-Dawley rat hearts were used for skinned fiber experiments. After dissection, the fiber bundles, approximately 200 μm in width and 2.5–3 mm in lengths, were extracted overnight in a high relaxing solution (HR, pCa 9.0) containing 20 mM MOPS (pH 7.0), 53.3 mM KCl, 10 mM EGTA, 0.025 mM $CaCl_2$, 5.35 mM ATP (5 mM $MgATP^{2-}$), 6.81 mM $MgCl_2$ (1 mM free Mg^{2+}), 12 mM creatine phosphate, 10 IU/ml creatine kinase, 1.0 mM DTT and 1% Triton X-100 at 4°C. A cocktail of protease inhibitors was included in all buffers (pepstatin 1 μg/ml, leupeptin 5 μg/ml and 0.2 mM PMSF). A computer program was used to calculate the amount of KCl, ATP, $CaCl_2$ and $MgCl_2$ required to establish the HR and activating solution (pCa 4.5) at a constant ionic strength of 150 mM.

Preparation of extraction solution containing cTnT-cTnI. For exchange experiments, an extraction solution containing a mixture of cTnT and cTnI was prepared as follows: cTnT (1.5 mg/ml, W/V) and cTnI (1.0 mg/ml, W/V) were initially dissolved in 50 mM Tris-HCl (pH 8.0), 6 M urea, 1.0 M KCl, 10 mM DTT and 0.2 μM PMSF, and then dialyzed overnight against the extraction buffer containing 20 mM MOPS (pH 6.5), 250 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 1.0 mM DTT at 4°C (for example, 4 ml vs 4 liters). Any insoluble protein was removed by a 30 min centrifugation (table top) at 5000 rpm. After centrifugation, protease inhibitors (see above) were added to the supernatant containing cTnT-cTnI. We used freshly prepared cTnT-cTnI samples for each exchange experiment.

Preparation of cTnC solution for reconstitution. In the protocol for reconstitution with cTnC, the fiber bundles were treated with a solution containing approximately 4 mg/ml (W/V) of cTnC dissolved in HR containing protease inhibitors. The pH of the solution was adjusted to 7.0. The cTnC solution was stored at −70°C for repeated use.

cTnT-cTnI treatment of detergent skinned rat cardiac fiber bundles. The endogenous troponin was replaced by exogenous Tn by first treating the fibers with exogenous cTnT-cTnI followed by cTnC reconstitution. After the detergent skinned fiber bundle was mounted between a micro manipulator and a force transducer, sarcomere length was adjusted to 2.3 μm. Before treatment with cTnT-cTnI, the initial maximum isometric force was measured in activating solution (pCa 4.5) containing 20 mM MOPS (pH 7.0), 33.8 mM KCl, 10 mM EGTA, 9.96 mM $CaCl_2$, 5.39 mM ATP, 6.47 mM $MgCl_2$, 12 mM creatine phosphate, 10 IU/ml creatine kinase, 1.0 mM DTT. The fiber bundle was relaxed in HR solution. The Tn exchange experiment was carried out in the extraction solution (2 ml) containing either cTnT-cTnI or cTnT-$cTnI_{33-211}$ or $cTnT_{77-289}$-$cTnI_{33-211}$ for approximately 70 min at room temperature with constant stirring. After cTnT-cTnI treatment, the fiber bundle was washed in 2 ml of extraction buffer (without cTnT-cTnI) for 10 min, and 2 ml of HR for 10 min with constant stirring. To determine the extent of endogenous Tn removed, Ca^{2+}-activated residual force was measured in pCa 4.5 solution. A substantial decrease (85–90%) in Ca^{2+}-activated maximum isometric force indicated that most of the endogenous Tn was removed. Next, the cTnT-cTnI treated fiber bundle was relaxed in HR solution. The cTnT-cTnI treated fiber bundles were reconstituted with cTnC (4 mg/ml in HR, pH 7.0) for approximately 90–120 min at room temperature with constant stirring. After cTnC reconstitution, Ca^{2+}-activated maximal force was measured in pCa 4.5 solution.

Polyacrylamide gel electrophoresis. We analyzed cardiac myofilament proteins from skinned fiber bundles on a 12.5% SDS-Polyacrylamide gels. Sample preparation and electrophoresis were as described previously (12). Non SDS alkaline urea-PAGE was performed using a 4% stacking gel and 8% separating gels containing 6 M urea (13). Non-SDS alkaline urea gels (14, 15) were used to detect changes in cTnC content before and after treating the fiber bundles with cTnT-cTnI. To determine the amount of endogenous Tn subunits removed, Coomassie stained gels were scanned with a densitometer (Molecular Dynamics) and area under the peaks for various myofilament proteins were quantified using the Image-QuaNT, Image Analysis Software.

Data analysis. Data from the normalized pCa-force measurements were fitted to the Hill equation by using a nonlinear least-square regression procedure to obtain the pCa_{50} (−log of free Ca^{2+}-concentration required for half maximal activation) and the Hill coefficient (n). Statistical differences were analyzed by an unpaired *t*-test with the criteria for significance set at $p < 0.05$. Data are expressed as mean ± S.E.

RESULTS AND DISCUSSION

Figure 1 illustrates the procedure we developed, which significantly improved the ability to substitute endogenous Tn complex of skinned cardiac fiber bundles with exogenous Tn components. The upper left

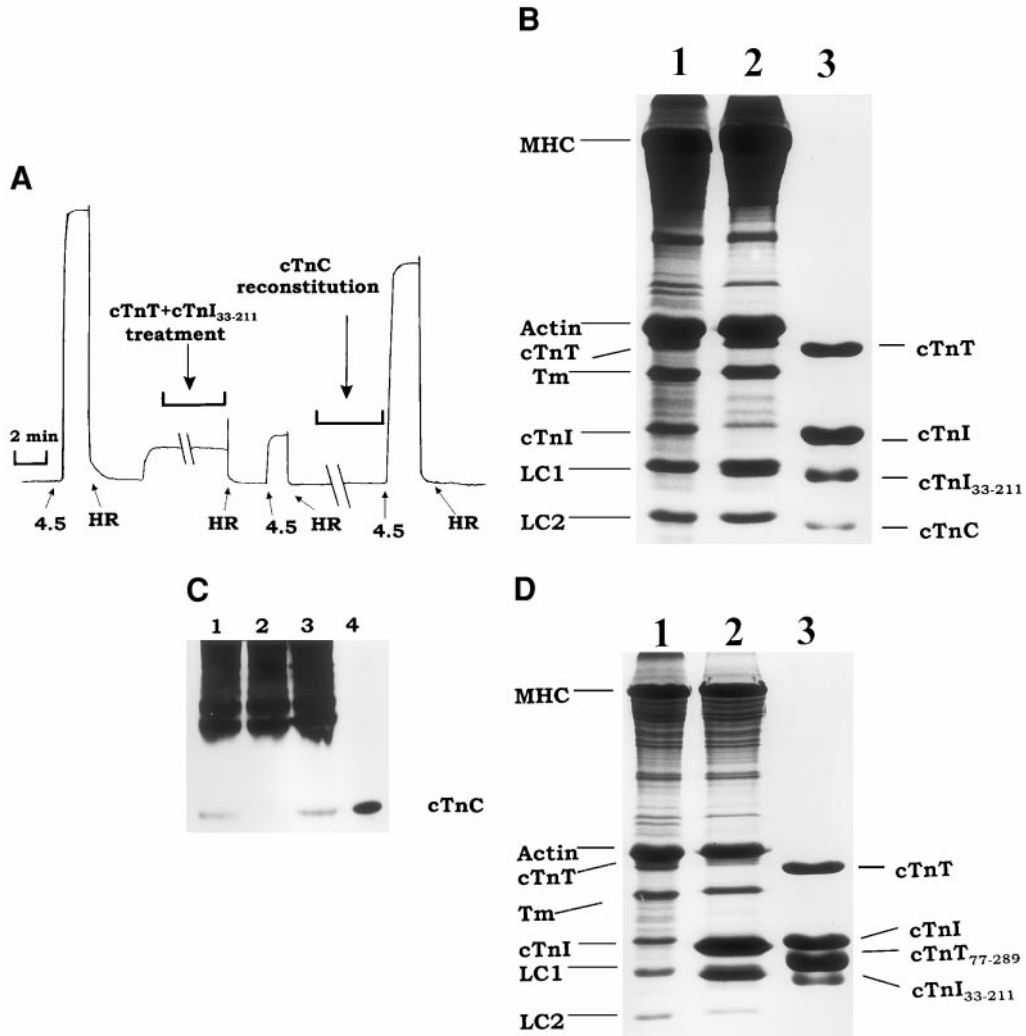

FIG. 1. Procedure for Tn exchange in Triton X-100 skinned rat cardiac fiber bundles. (A) Original force tracings from the cTnT-cTnI$_{33-211}$ treated and cTnC reconstituted experiment. Tn exchange was carried out as described in Methods. (B) Coomassie-stained SDS-PAGE (12.5%) analysis of the cTnT-cTnI$_{33-211}$ treated detergent skinned rat cardiac fiber bundles. Lane 1, control untreated fibers; lane 2, cTnT-cTnI$_{33-211}$ treated fibers; lane 3, protein standards. (C) Non-SDS alkaline urea PAGE of the cTnT-cTnI$_{33-211}$ treated and cTnC reconstituted fiber bundles. Lane 1, control untreated fibers; lane 2, cTnT-cTnI$_{33-211}$ treated fibers; lane 3, cTnT-cTnI$_{33-211}$ treated fibers + cTnC reconstituted fibers and lane 4 is pure standard cTnC. (D) Coomassie-stained SDS-PAGE (12.5%) analysis of the cTnT$_{77-289}$-cTnI$_{33-211}$ treated fiber bundles. Lane 1, control untreated fibers; lane 2, cTnT$_{77-289}$-cTnI$_{33-211}$ treated fibers; lane 3, protein standards.

panel (Fig. 1A) shows a record of changes in force during the protocol. In the example shown, we used a truncated mutant of cTnI, cTnI$_{33-211}$, in order to visualize the exchange of endogenous cTnI on SDS PAGE (Fig. 1B). Typically, an initial contraction was generated by switching from HR to pCa 4.5 contraction solution. This and all other subsequent procedures were done at room temperature. The fiber bundle was then soaked with constant stirring in extraction containing cTnT-cTnI$_{33-211}$, 20 mM MOPS (pH 6.5), 250 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 1.0 mM DTT. This solution provided conditions that ensured solubility of the cTnI-cTnT complex. During the extraction period, which lasted 70–80 min, the fiber bundle developed a relatively small rigor force. After extraction with cTnT-cTnI$_{33-211}$, we measured Ca^{2+}-activated force to determine the extent of endogenous Tn removal. As shown by the example in Fig. 1A, there was a 85–90% decrease in the ability of Ca^{2+} to activate force as compared to the Ca^{2+}-regulated fiber bundle containing the full complement of native cTnC. This extent of extraction of Tn, as measured by residual Ca^{2+}-activated force was similar whether we used cTnT-cTnI or cTnT-cTnI$_{33-211}$. After the treatment with cTnT-cTnI$_{33-211}$, the Ca^{2+}-dependent maximum force decreased to 14 ± 2% (n = 8) of the untreated control; after treatment with cTnT-cTnI, it decreased to 6 ± 1% (n = 8) of control (Fig. 2). Following this test contraction, which permitted an assessment of the Tn extraction during the course of the experiments, cTnC was reconstituted into

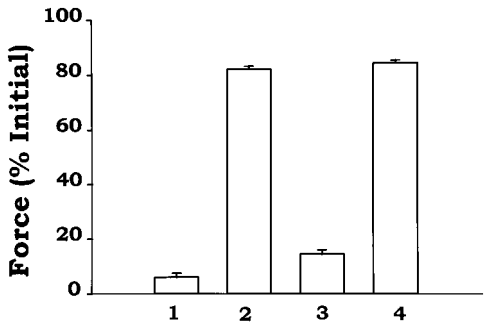

FIG. 2. Effect of $cTnI_{33-211}$ on Ca^{2+}-activated maximal isometric force in detergent skinned rat cardiac fiber bundles. cTnT-cTnI and cTnT-$cTnI_{33-211}$ treatment of the detergent skinned fiber bundle was as described in Methods. The maximum developed force was measured in pCa 4.5 solution. For direct comparison, Ca^{2+} activated maximum force generated by the fiber prior to the treatment was taken as 100%. 1, residual force after cTnT-cTnI treatment; 2, maximum restored force in cTnT-cTnI treated + cTnC reconstituted fiber; 3, residual force after cTnT-$cTnI_{33-211}$ treatment and 4, maximum restored force in cTnT-cTnI treated + cTnC reconstituted fiber.

the fiber bundle by bathing the fibers in HR containing 4 mg/ml cTnC. After reconstitution with cTnC, the fiber bundle was fully relaxed in HR. Our observation that $cTnI_{33-211}$ retained full inhibitory activity in skinned fiber bundles is supported by previous work from our laboratory that demonstrated that both cTnI and $cTnI_{33-211}$ were equally effective in regulating the actin-myosin ATPase activity of both fully reconstituted preparations and isolated myofibrillar preparations (11, 16). After reconstitution with cTnC, the maximum Ca^{2+}-activated force at pCa 4.5 was greater than 80% of the initial force. For fiber bundles that were treated with cTnT-$cTnI_{33-211}$, 84 ± 1% (n = 8) of the initial force was recovered; for bundles treated with cTnT-cTnI, 82 ± 2% (n = 8) of the initial force was recovered after completion of the exchange protocol (Fig. 2).

We also assessed the efficacy of Tn removal by analytical PAGE of the skinned fiber bundles. Figure 1B shows an SDS-PAGE protein profile illustrating the extent of replacement of endogenous cTnI with $cTnI_{33-211}$, which migrates with a mobility faster than that of the full length cTnI. We estimated the extent of cTnI exchange by densitometric analysis of lanes 1 and 2 in the SDS-PAGE gel shown in Fig. 1B. After normalizing for actin or myosin light chain 2 content in the control lane, we estimated that at least 70–80% of cTnI was removed. The relative amount of cTnC in the untreated and cTnT-$cTnI_{33-211}$ fiber bundles was quantified using urea-PAGE gels in the absence of SDS (15, 17). In the presence of EGTA, cTnC migrates well ahead of other fiber bundle components as illustrated in Fig. 1C. Fiber bundles treated with cTnT-$cTnI_{33-211}$ (Fig. 1C, lane 2) contained little, if any cTnC. In order to visualize the extent of removal of endogenous cTnT, we treated fiber bundles with a truncated mutant of cTnT ($cTnT_{77-289}$) complexed with either cTnI or $cTnI_{33-211}$. Figure 1D shows the SDS-PAGE analysis of a preparation treated with $cTnT_{77-289}$-$cTnI_{33-211}$. Analysis of lanes 1 and 2 of Fig. 1D by quantitative densitometric analysis indicated that greater than 70% of the endogenous cTnT was removed. Therefore the results illustrated in Fig. 1 demonstrate that the endogenous Tn complex was removed as a whole and replaced by the exogenous recombinant components of the Tn complex.

Data shown in Fig. 3 compare the pCa-force relations for skinned fiber bundles extracted with either cTnT-$cTnI_{33-211}$ or with cTnT-cTnI and reconstituted with cTnC. The results shown in Fig. 3 indicate that there was a small decrease in Ca^{2+}-sensitivity of the myofilaments containing $cTnI_{33-211}$ as determined by the pCa_{50} (-log of the half-maximally activating molar Ca^{2+}) obtained from fitting the curves. The pCa_{50} value was 5.74 (±0.02) for the cTnT-cTnI treated fiber bundles and 5.64 (±0.01) for fibers treated and cTnT-$cTnI_{33-211}$. The Hill coefficient (n) values were n = 2.3 ± 0.1 fiber bundles containing cTnT-cTnI and 2.6 ± 0.1 for fibers containing cTnT-$cTnI_{33-211}$. The substitution of wild type cTnI into rat cardiac fiber bundles led to a small decrease in Ca^{2+}-sensitivity when compared to the untreated fibers (pCa_{50} value of 5.85 ± 0.01 and 5.74 ± 0.02 for the untreated and cTnT-cTnI treated fibers, respectively). It is possible that the reconstitution of recombinant mouse cTnI and human cTnC into rat cardiac fibers affected Ca^{2+}-sensitivity. However, both the reproducibility and the extent of maximum force recovered in the cTnT-cTnI treated and cTnT-$cTnI_{33-211}$ treated fibers demonstrate that the conditions used in our protocol have no significant effect the mechanical properties of the fibers.

An important feature of the Tn extraction/reconstitution protocol described here is that the fiber bundles remain relaxed during the lengthy cTnC reconsti-

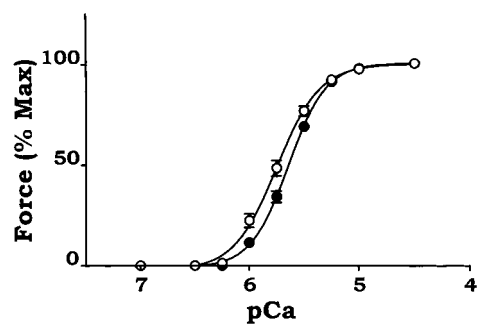

FIG. 3. Effect of $cTnI_{33-211}$ on Ca^{2+}-regulation of isometric force development in detergent skinned rat cardiac fiber bundles. The treatment of detergent skinned fibers with cTnT-cTnI and cTnT-$cTnI_{33-211}$ followed by reconstitution with cTnC was as described in Methods. Normalized pCa-force relation in: (○) cTnT-cTnI treated + cTnC reconstituted fibers; (●) cTnT-$cTnI_{33-211}$ treated + cTnC reconstituted fibers. Data are presented as mean ± S.E. for 8 determinations. Statistical differences were measured by an unpaired t-test with significance set at $p < 0.05$.

tution. In previous procedures (9, 12, 18), the cTnI-cTnC complex was reconstituted into the sTnT or cTnT treated fibers in a $MgATP^{2-}$ free solution at low ionic strength, which induced a rigor state throughout the 90–120 min reconstitution step. When transferred to relaxing solution containing $MgATP^{2-}$, although the fully reconstituted fibers were able to relax in the absence of Ca^{2+}, the restoration of Ca^{2+}-regulated maximum force remained unsatisfactory in many preparations. In our hands at least 30–40% of the cTnT treated fibers were unusable due the deterioration of force generating ability, which probably resulted from maintaining the fibers in rigor state for prolonged periods in $MgATP^{2-}$-free solutions. Our prediction was that the incorporation of cTnI into the myofilaments during the removal of endogenous Tn would maintain the fibers in relaxed state when transferred to $MgATP^{2-}$ containing relaxing buffers. As shown in Fig. 1A, the cTnT-$cTnI_{33-211}$ treated fiber bundle relaxed to normal levels when transferred to the relaxing buffer containing 5 mM $MgATP^{2-}$. Furthermore, the procedure described here demonstrated that most of the force generating capacity in the cTnT-cTnI treated fibers is retained after reconstitution with cTnC.

In summary the procedure described here has several important features: 1) Tn exchange protocol described here does not require pretreatment of skinned fiber bundles such as pre-skinning and glycerination at −20°C; 2) In addition, purified Tn subunits can be directly used without having to make either the Tn or the cTnI-cTnC complex; 3) the procedure used in this study differs from previous protocols in that the cTnT-cTnI treated fiber bundles were able to relax to normal levels in the $MgATP^{2-}$ containing relaxing buffer unlike the fibers that were treated with an excess of cTnT alone; 4) cTnC was dissolved in high relaxing buffer that ensured that the cTnT-cTnI treated fiber bundles remained completely relaxed through the length cTnC reconstitution procedure.

We think the procedure described in the present study provides a high throughput technique that will be useful in addressing the functional consequences of alterations in cardiac Tn subunits in the force generating myofilament lattice. For example various mutations in cTnT, cTnI have been implicated in the evolution of complications associated with familial hypertrophic cardiomyopathy (19). Furthermore, other complex changes in Tn subunits have been observed in failing human hearts, ischemia and reperfusion injury. These complex changes may include proteolytic clipping, re-expression of embryonic isoforms and alterations in the phosphorylation state of Tn subunits (20).

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