Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 786 (2003) 287–296
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Expression and purification of human cardiac troponin subunits and their functional incorporation into isolated cardiac mouse myofibrils

Martina Krüger*, Gabriele Pfitzer, Robert Stehle

Institute of Vegetative Physiology, University of Cologne, Robert-Koch-Strasse 39, D-50931 Köln, Germany

Abstract

The three subunits of the human cardiac troponin complex (hcTnC, hcTnI, hcTnT) were overexpressed in *E. coli*, purified and reconstituted to form the hcTn complex. This complex was then incorporated into subcellular bundles of mouse cardiac myofibrils whereby the native mcTn complex was replaced. On thus exchanged myofibrils, isometric force kinetics following sudden changes in free Ca^{2+} concentration were measured using atomic force cantilevers. Following the exchange, the myofibrillar force remained fully Ca^{2+} regulated, i.e. myofibrils were completely relaxed at pCa 7.5 and developed the same maximum Ca^{2+}-activated isometric force upon increasing the pCa to 4.5 as unexchanged myofibrils. The replacement of endogenous mcTn by wild-type hcTn neither altered the kinetics of Ca^{2+}-induced force development of the mouse myofibrils nor the kinetics of force relaxation induced by the sudden, complete removal of Ca^{2+}. Preparations of functional Tn reconstituted myofibrils provide a promising model to study the role of Tn in kinetic mechanisms of cardiac myofibrillar contraction and relaxation.

Keywords: Expression; Purification; Myofibrils; Troponin; Calcium-regulatory proteins

1. Introduction

In cardiac muscle the thin filament proteins tropomyosin and Tn regulate the Ca^{2+}-dependent cyclic interaction of the force-generating myosin heads with actin. Tn is a heterotrimer consisting of troponin C (TnC), troponin I (TnI), and troponin T (TnT). TnC is the Ca^{2+}-binding subunit, TnI inhibits the interaction between myosin and actin and TnT integrates the complex into the thin filament through its strong binding to tropomyosin [1]. In order to study the functional role of Tn in the regulation of muscle contraction, different protocols have been developed in skinned cardiac and skeletal fibres to exchange endogenous Tn for species- and muscle-specific isoforms, recombinant modified or mutant Tn subunits. For instance, incubation with orthovanadate resulted in extraction of endogenous TnI and TnC from chemically skinned cardiac preparations, which were then reconstituted with exogenous subunits [2]. Other ways to exchange Tn in muscle fibres are based on first replacing the endogenous Tn complex by an excess of either TnT [3] or a mixture of TnI and TnT [4] and thereafter completing the reconstitution by adding either TnI and TnC or TnC, respectively. Incubation of skeletal muscle fibres with an excess of the whole Tn complex also yields quantitative replacement of the endogenous Tn complex [5].

*Corresponding author. Tel.: +49-221-478-6951; fax: +49-221-478-6965.
E-mail address: martina.krueger@uni-koeln.de (M. Krüger).

1570-0232/02/$ – see front matter
doi:10.1016/S1570-0232(02)00763-8

A particular advantage of this protocol is that the muscle has its complement of the three Tn subunits during the whole exchange procedure. The structural integrity and the contractile properties of skinned skeletal fibres in which the endogenous Tn complex was replaced by fluorescently labelled skeletal Tn complex with this protocol were not affected [5–7].

These approaches gave important insights regarding the structural requirements of the Tn complex for the regulation of the actomyosin interaction in cardiac muscle under steady-state conditions [8–15]. However, due to diffusion limitations of these multicellular preparations, information regarding the kinetics of contraction or relaxation is sparse and could only be obtained by the use of so-called caged compounds, which release Ca^{2+} or Ca^{2+} chelators upon flash photolysis of the biologically inert precursor compound. This approach has limitations, however, e.g. incomplete relaxation because of the low Ca^{2+} affinity of the currently available Ca^{2+} chelators.

In contrast, myofibrils or myofibrillar bundles having a diameter of a few micrometres completely equilibrate with the surrounding solution within 1 ms and are, hence, ideally suited for studying transient kinetics invoked by either rapid mixing methods or rapid solution change. Rapid flow-quench and fluorescence stopped-flow techniques, which are usually restricted to proteins in solution, have been successfully applied to myofibrillar suspensions to investigate the transient kinetics of myofibrillar ATPase [16,17]. In spite of their small size, myofibrils consist of contractile sarcomeres containing the structurally organised complete assembly of contractile and regulatory proteins. Thus, using EGTA-buffered solutions, the free Ca^{2+} concentration can be rapidly raised or lowered by a microflow-based solution change technique to well-defined concentrations without the need for caged compounds to invoke Ca^{2+}-induced force kinetics of isometrically installed myofibrils [7]. In addition, the small size of these specimens will also facilitate diffusion of proteins into the myofilament lattice which may improve the homogeneity of binding of exogenous protein in exchange studies.

Recently, it has been shown that exogenous fluorescently labelled Tn complex binds to skeletal myofibrils at specific locations in the sarcomeres [18]. The purpose of the present study was to establish a similar method to exchange the Tn complex in cardiac myofibrils and, furthermore, to confirm their contractile function after the exchange.

In recent years, hcTn has gained an important role in investigations of inherent human heart diseases. Linkage studies and candidate-gene approaches have demonstrated that about half the patients suffering from familiar hypertrophic cardiomyopathy (HCM) have mutations in a protein of the sarcomere (for reviews, see Refs. [19,20]). So far, nine mutations in the cTnI gene [20,21], several mutations in cTnT (for a review, see Ref. [22]) and just recently the first mutation in cTnC [23] can be linked to HCM. Due to their particular relevance in studying the mechanisms of heart diseases such as HCM, we exchanged the cTn complex formed by the three isolated recombinant human wild-type subunits, hcTnC, hcTnT and hcTnI. To the best of our knowledge, this is the first report of the force kinetics of the contraction–relaxation cycle in single cardiac myofibrils following exchange of endogenous Tn by exogenous Tn. The exchange protocol described here in principle allows the replacement of each individual native Tn subunit by a foreign wild-type or a mutant isoform. It therefore provides a general tool in basic and clinical research to study the effects of Tn subunit isoforms or Tn subunit mutations on molecular kinetic mechanisms of myocardial contraction and relaxation.

2. Materials and methods

2.1. Protein expression and purification of recombinant human cardiac troponin subunits

The human cardiac subunits were expressed in *E. coli* BL21(DE3) cells using constructs cloned into a pET 11c vector [24] with ampicillin resistance (TnC, TnT) or a pET 9a vector with kanamycin resistance (TnI). TnI and TnC expressing cells were grown at 37 °C in LB medium (Gibco, Paisley, UK) containing 50 μg/ml ampicillin or kanamycin. An overnight culture was diluted 1:100 and induced at OD=0.6 with 0.4 m*M* IPTG for 4 h. Since the hcTnT cDNA contains rare arginin codons the synthesis of hcTnT was performed in BL21 codon plus RP (DE3) cells (Stratagene, Amsterdam). Cul-

ture and induction was performed according to the protocol given by the suppliers. Expression of all subunits was confirmed by SDS–PAGE as described in Ref. [25].

Bacterial pellets from 1 l TnI and TnT cultures were resuspended in 20 ml homogenisation buffer (25 m*M* TEA–HCl, 8 *M* urea, 1 m*M* DTT, 5 m*M* EDTA, 1 m*M* PMSF). Pellets containing TnC expressing bacteria were resuspended in the same buffer without urea. Cells were lysed by ultrasonification at 10 s intervals for 2 min at 0 °C. The lysate was centrifuged at 15 000 *g* for 20 min at 4 °C. The resulting supernatants were dialysed overnight against the buffer used in the first chromatographic step.

TnI was purified using a modified protocol previously reported by Jha et al. [26]. The TnI-containing lysate was dialysed overnight against buffer A (25 m*M* TEA–HCl pH 7.5, 8 *M* urea, 2 m*M* EDTA, 1 m*M* DTT) and then loaded on a CM Sepharose FF column (XK16/20, Amersham Biosciences, Freiburg, Germany). TnI was eluted with a linearly increasing NaCl gradient in buffer A. TnI eluted at conductivity values of 12–15 mS. Isolated TnI fractions were dialysed against 1 m*M* HCl and lyophilised prior to storage at −80 °C.

TnC was purified according to Ref. [27]. TnC-containing lysates were dialysed against buffer B (50 m*M* Tris–HCl pH 7.5, 5 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 1 m*M* DTT, 50 m*M* NaCl) and then loaded onto a Phenyl-Sepharose column (XK 16/20, Amersham Biosciences) equilibrated in the same buffer. The column was then washed with buffer C (50 m*M* Tris–HCl pH 7.5, 0.1 m*M* $CaCl_2$, 1 m*M* DTT, 1 m*M* NaCl). TnC was eluted with buffer D (50 m*M* Tris–HCl pH 7.5, 1 m*M* EDTA, 1 m*M* DTT). Purified TnC was dialysed against 1 m*M* HCl and lyophilised prior to storage at −80 °C.

TnT was isolated based on a method described in Ref. [28] with the following modifications. Protein solution was dialysed against buffer E (50 m*M* sodium citrate pH 6.0, 6 *M* urea, 1 m*M* EDTA, 0.1 m*M* DTT) and then loaded on a CM Sepharose column (XK16/20, Amersham Biosciences). TnT was eluted by applying a linearly increasing gradient from 0 to 20% 0.5 *M* NaCl in buffer E. TnT-containing fractions were collected and dialysed against buffer F (50 m*M* Tris–HCl pH 8.0, 6 *M* urea, 1 m*M* EDTA, 0.1 m*M* DTT) before being loaded on an equilibrated DEAE Sepharose column (XK 16/20, Amersham Biosciences). TnT was again eluted with a linearly increasing NaCl gradient (0.5 *M* NaCl in buffer F) from 0 to 40% and then dialysed against 1 m*M* ammonium hydrogencarbonate and lyophilised prior to storage at −80 °C.

2.2. Reconstitution of recombinant hcTn complex

The purified hcTn subunits were reconstituted to a complex using a previously reported method [28]. 1 mg/ml of each of the subunits was dissolved in a buffer containing 25 m*M* MOPS pH 7.0, 0.2 *M* NaCl, 0.5 *M* $CaCl_2$, 1 m*M* DTT and 8 *M* urea. After incubation on ice for 1 h, proteins were dialysed against the same buffer without urea, but with 1.0 *M* NaCl. NaCl was slowly reduced to a final concentration of 0.2 *M* by repeated dialysis. Formation of the hcTn complex was verified by gel filtration on a Sephacryl S-200 column as described for purification of skeletal Tn complex (see below). The complex was stored at −20 °C in 50% glycerol for up to 2 weeks.

2.3. Purification of native skeletal Tn complex

Skeletal muscles from an adult rabbit were dissected, homogenised and isolated by fractionated ammonium sulphate precipitation [29]. The precipitate was then dialysed against gel filtration buffer (20 m*M* Tris–HCl pH 7.5, 0.3 *M* NaCl, 10 m*M* DTT) and further purified on a Sephacryl S-200 column (XK 16/40, Amersham Biosciences). The isolated sTn complex was extensively dialysed against 1 m*M* HCl, lyophilised and stored at −80 °C.

2.4. Isolation of cardiac myofibrils

Adult mice (mouse strain: HIM of 1) were sacrificed by cervical dislocation. Papillary muscles were dissected from mouse left ventricle and skinned with 1% Triton X-100 in rigor buffer (132 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, 10 m*M* Tris pH 7.1, 5 m*M* EGTA, 1 m*M* NaAzid and an inhibitor cocktail) for 6–12 h and stored in the rigor buffer without Triton X-100 at 4–8 °C for up to 1 week. Myofibril suspensions were prepared immediately

before experiments by homogenising skinned papillary muscles in a blender (Ultra Turrax) for 10 s at 0 °C.

2.5. Exchange of Tn complex in mouse cardiac myofibrils

Replacement of native Tn by recombinant hcTn was performed based on exchange of the whole complex, first described by Brenner et al. [5]. Reconstituted hcTn complex or rabbit sTn complex was dialysed against rigor buffer (see above), centrifuged at 13 000 *g* for 10 min and filtered through a polypropylene mesh (30 μm pore openings) to remove aggregates. Myofibril suspension freshly prepared in the same buffer was mixed with an excess of Tn complex. The solution was then incubated at room temperature for 30 or 60 min with slow shaking. To remove excess Tn, myofibrils were washed several times by slow centrifugation in rigor buffer (3 min at 300 *g*, 10 °C).

2.6. SDS–PAGE and Western blot analysis

SDS–PAGE analysis of proteins was carried out using a Mighty Small II electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, USA). The protein samples were suspended in 100 μl of 1× sample buffer containing 50 m*M* Tris–HCl (pH 6.8), 4 *M* urea, 1% SDS, 15% glycerol, and 0.01% bromophenol blue (25), boiled for 2 min at 95 °C, and loaded on 12.5% SDS–PAGE [30]. Protein content was determined by the Bradford method with BSA as standard [31]. Gels were stained with Coomassie Brilliant Blue R-250 (Roth, Karlsruhe, Germany).

For Western blot analysis, protein components were separated on SDS–PAGE, transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by standard tank transfer Western blot and probed by a monoclonal antibody against cTnI (clone 6F9, Dunn Labortechnik, Asbach, Germany) which also recognises sTnI and a monoclonal anti-TnT antibody (JLT12, Sigma–Aldrich, Taufkirchen, Germany). The membranes were incubated with anti-mouse IgG-HRP (Sigma–Aldrich) and enzymatic activity was detected using an ECL kit (Amersham Biosciences).

2.7. Fluorescent labelling of Tn

The whole complex was first reduced at 30 °C in a buffer containing 100 m*M* KCl, 20 m*M* phosphate buffer, pH 6.5, 1 m*M* EDTA and 2 m*M* DTT and then exhaustively dialysed in 30 m*M* KCl, 10 m*M* imidazole pH 6.5, 1 m*M* $MgCl_2$, 1 m*M* EGTA and 0.1 m*M* DTT at 4 °C to remove DTT. A five-fold molar ratio of rhodamine dye (tetramethylrhodamine-5-iodacetamide dihydroiodide, 5-TMRIA, Molecular Probes, Eugene, OR, USA) to protein was added and the solution incubated for 4 h at 4 °C in the dark. The reaction was stopped with an excess of DTT. To remove unbound label the solution was dialysed extensively against rigor buffer (see Section 2.4).

2.8. Myofibrillar force measurements

For force measurements, myofibrils were mounted in the apparatus described in Refs. [10,32,33]. In brief, small myofibrillar bundles were attached at one end to a stiff micro-needle and at the other end to the tip of an atomic force cantilever which had been coated with a mixture of silicon adhesive and nitrocellulose. Rapid changes in Ca^{2+} concentration with an effective time of solution change of ≤10 ms were applied to the myofibrils using the micro-flow setup described in Refs. [32,33], according to Ref. [10], the technique developed by Poggesi and collaborators [7]. Force transients were detected by monitoring the deflection of the cantilever by laser light reflection and analysed as in Ref. [32]. The compositions of the relaxation solutions (pCa 7.5) and activation solutions (pCa 4.5) used in the experiments have been described previously [32]. Experiments were performed at 10 °C.

3. Results

3.1. Purification of Tn and its subunits

Human cardiac Tn subunits were purified from *E. coli* with yields usually ranging from 40 mg (hcTnT) up to 60 mg (hcTnI) isolated protein per litre of cultured bacteria. Fig. 1 demonstrates the purity of the isolated Tn subunits. TnT showed a smaller

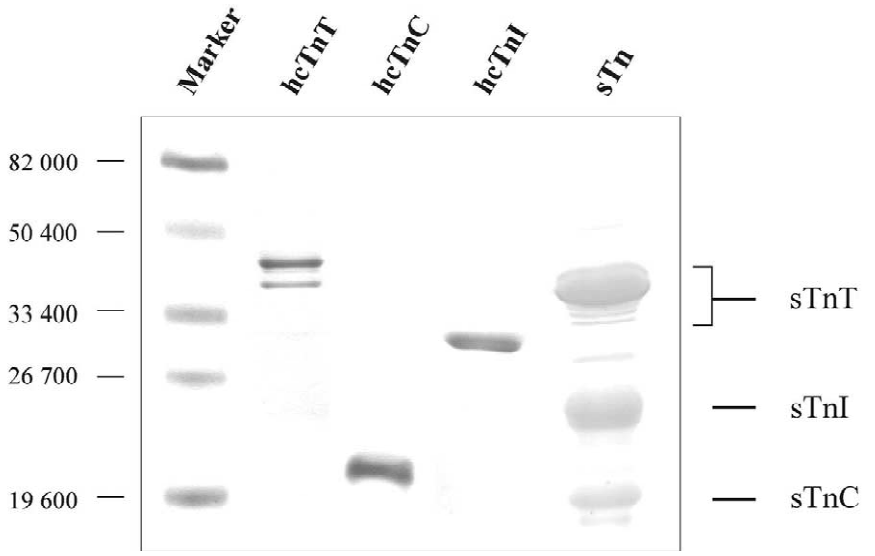

Fig. 1. Electrophoretic patterns of purified recombinant human cardiac Tn subunits expressed in *E. coli* and of skeletal Tn isolated from rabbit skeletal muscle obtained by SDS–PAGE (12.5% AA) stained with Coomassie Brilliant Blue.

degradation product. Further purification was not performed, since this degradation product was no longer detectable by SDS–PAGE after reconstitution of the subunits to the Tn complex (data not shown). The identity of the subunits was confirmed by Western blot analysis (data not shown). The protein pattern of the isolated sTn complex clearly demonstrates the different TnT isoforms existing in skeletal muscle. It also shows the difference in molecular masses between the tissue-specific isoforms of TnI and TnT.

3.2. Exchange of Tn complex in mouse cardiac myofibrils

Since it is difficult to differentiate hcTn from mcTn by Western blot analysis, the exchange protocol was first established with purified native rabbit skeletal Tn complex. Antibodies specific against TnI or TnT were used to distinguish these Tn subunits from other myofibrillar proteins. Fig. 2 shows a Western blot of myofibrils obtained 30 and 60 min after incubation with excess of rabbit skeletal Tn complex and of a control suspension of mouse cardiac myofibrils. Control samples were subjected to the same protocol except for the addition of exogenous Tn complex. After 30 min incubation with excess sTn complex, cTnI was only partly removed, whereas an additional protein band corresponding to sTnI was detected at M_r 28 000. After 60 min, endogenous cTnI was completely replaced by exogenous sTnI. The samples shown were also probed for TnT isoforms, resulting in the same time-dependent exchange behaviour and in complete removal of endogenous cTnT after 60 min incubation (data not shown).

To verify the incorporation of hcTn into the myofibrils, the same protocol was performed using reconstituted hcTn complex labelled with a rhodamine dye (5-TMRIA). Fig. 3 shows a microscopic image of mouse cardiac myofibrils exchanged with rhodamine-labelled hcTn complex. The image demonstrates that the sarcomeric striation of myofibrils is well preserved after the exchange. No structural modifications on the microscopic level, such as irreversible overcontraction of sarcomeres, were produced by the exchange protocol. In agreement with previous studies [5,18] we find that the labelled complex predominantly binds to the A-bands in the sarcomeres.

In summary, these data indicate that the exchange procedure results in the complete removal of endogenous mcTn complex and in the incorporation of the recombinant hcTn complex without causing any

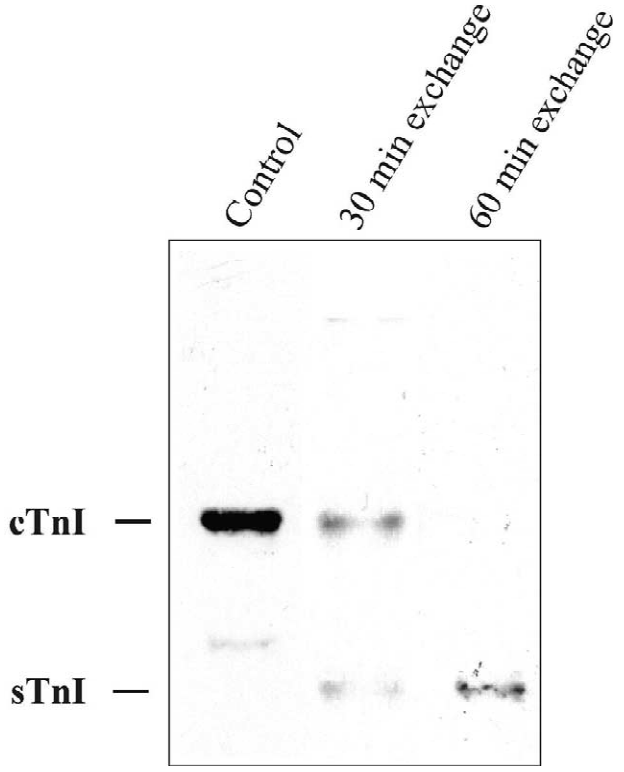

Fig. 2. Western blot analysis of time-dependent Tn exchange in mouse cardiac myofibrils. Samples were probed with mouse-anti-cTnI antibody. Control: myofibrils subjected to the exchange procedure in the absence of hcTn. Note the complete displacement of cTnI after 60 min exchange.

obvious structural damage, such as, for example, overcontraction of sarcomeres to the myofibrils.

3.3. Force activation and relaxation in mouse cardiac myofibrils exchanged with hcTn

Fig. 4 shows examples of force tracings from myofibrils from mouse heart (A) and hcTn exchanged mouse heart (B). Starting from relaxation at pCa 7.5, at times 0.5 s indicated in the left panel of Fig. 4, the myofibrils were maximally Ca^{2+} activated by rapidly increasing the [Ca^{2+}] to saturating levels (pCa 4.5). Following Ca^{2+} application, force developed mono-exponentially (rate constant k_{ACT}) to reach a plateau (maximum Ca^{2+}-activated force, F_{max}). When normalised to myofibrillar cross-sectional area (A), the hcTn exchanged mouse cardiac myofibrils developed the same maximum Ca^{2+}-activated force (F_{max}/A) as unexchanged mouse cardiac myofibrils (Table 1). Further, the exchange did not significantly increase the resting force per A ($F_{passive}/A$ in Table 1) determined by slackening the myofibrils in the relaxing solution of pCa 7.5 (at time 0.2–0.3 s in the force transients shown in the left panel of Fig. 4). This indicates that the myofibrils remained fully Ca^{2+} regulated after Tn exchange.

Myofibrillar relaxation kinetics are shown on enlarged time scales in the right panels of Fig. 4. After the rapid removal of Ca^{2+} (switching from pCa 4.5 to 7.5 at time 0) the force decays markedly biphasic, with an initial slow linear decay until time t_{LIN}, which was abruptly followed by a rapid exponential decay (rate constant k_{REL}). No significant difference in either t_{LIN} or k_{REL} could be detected between native myofibrils and myofibrils after replacement of native mcTn by exogenous hcTn (Table 1), demonstrating the capability of the reconstituted hcTn complex in the myofibrils to trigger

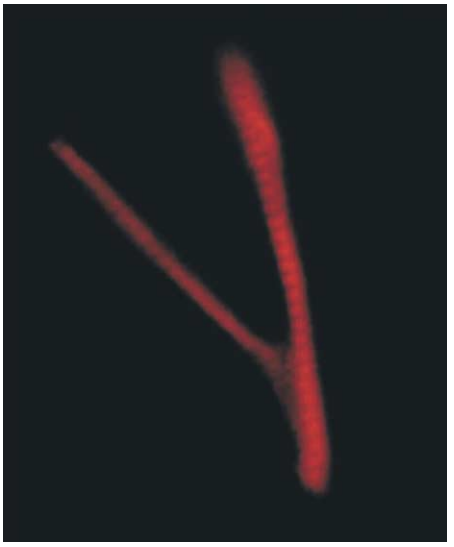

Fig. 3. Fluorescence image of myofibrils from mouse heart after 60 min incubation with 5′TMRIA-labeled hcTn. The periodic distribution in fluorescence along the myofibrils reflects the preferential binding of the fluorescently labelled complex to the A-bands in sarcomeres. Fluorescence microscopy was performed under excitation at 510–550 nm and emission at ≥590 nm.

force relaxation upon Ca^{2+} removal as fast and complete as the native Tn complex.

4. Discussion

We have purified the subunits of hcTn overexpressed in *E. coli*, reconstituted them to a functional protein complex and incorporated the complex into subcellular bundles of myofibrils by a sensitive exchange procedure. Mouse cardiac myofibrils which had their endogenous mcTn complex replaced by hcTn by this procedure, retained their structural stability, their full capacity to regulate Ca^{2+}-dependent force development and showed no change in force kinetics induced by the rapid application and the rapid removal of the activator, Ca^{2+}, demonstrating the functionality of Tn reconstituted myofibrils and their suitability for studying the influence of Tn subunit structure on the kinetics of the contraction–relaxation cycle.

Systolic cardiac myofibrillar contraction is triggered by binding of Ca^{2+} to site II of cTnC. This binding induces complex conformational changes involving all regulatory proteins (cTnI, cTnT, tropomyosin), together turning the thin filament to an ''on'' state [14]. This is a prerequisite for the cyclic interaction of myosin cross-bridges and force generation. Reverse events take place during myofibrillar relaxation: dissociation of Ca^{2+} from cTnC turns the thin filament back to an ''off'' state. This results in detachment of cross-bridges, which causes the myofibrillar force to decay.

In this study we show that incorporation of the human isoform complex into mouse myofibrils does not significantly alter the kinetics of Ca^{2+}-induced force development and relaxation. Unfortunately, it is not known whether the on or off rates differ between Tn isoforms. Thus, we do not know whether the on and off rates of hcTn are slower than those of mcTn. It may be speculated from our results that, presumably, any functional Tn complex restoring the full Ca^{2+} regulatory capacity will not alter the kinetics of contraction or relaxation in the heart. This may be different if the interactions between individual subunits of the Tn complex are perturbed, e.g. by mutations, covalent modification or by exchange of individual subunits rather than the whole complex. These interesting questions are yet not clearly resolved, especially concerning the effects of Tn on the relaxation kinetics of cardiac myofilaments, which have so far only been studied using skinned multi-cellular preparations (e.g., skinned trabeculae) as a model. For example, the effects attributed to cTnI phosphorylation on trabecular relaxation kinetics reported in the literature differ greatly, ranging from no change [4], to a ≈50% increase [13] up to about a two-fold increase [15,34] in relaxation rates. One explanation for these variable results could arise from the difficulty in completely removing Ca^{2+} from skinned trabeculae. As diffusion in multicellular trabeculae is too slow to obtain kinetic information by solution changes, caged Ca^{2+} chelators are activated by flash photolysis to induce relaxation. However, the low Ca^{2+} affinities of caged Ca^{2+} chelators make it difficult to achieve complete relaxation and to control the final Ca^{2+} concentration after photolysis. Myofibrillar force relaxation kinetics, however, very strongly depend on the level of the final steady state reached after force decay. By applying the same solution

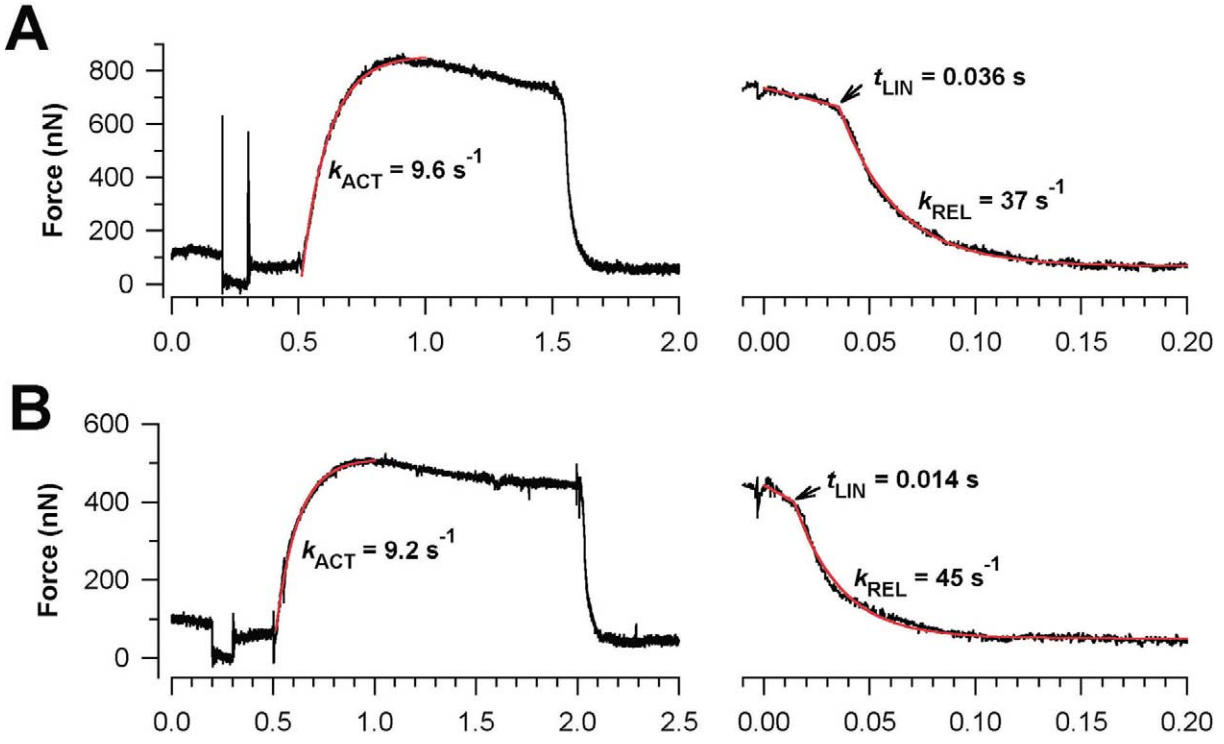

Fig. 4. Examples of force tracings showing activation and relaxation kinetics. The force transients (black line) were recorded from native cardiac myofibrils (unexchanged) (A) and from myofibrils exchanged with hcTn (B). The left panels show the full tracing, i.e. Ca^{2+}-induced force development and force decay following Ca^{2+} removal. The right panels show the relaxation kinetics on expanded time scales. Red lines indicate the best fit to the transient by a mono-exponential function (left panel) or by a function consisting of a linear and a single-exponential term (right panel) to obtain the kinetic parameters indicated. *x*-Axis indicates time in seconds.

change as used here for skeletal myofibrils, Tesi et al. [35,36] demonstrated that relaxation rates can decrease manifold when relaxation is only slightly incomplete, even when the pCa is reduced to levels at which myofibrils develop a final partial Ca^{2+}-activated force of as little as 10% of their maximum force.

The method of Tn exchange in cardiac myofibrils described here and the possibility of controlling the final [Ca^{2+}] may therefore provide an advantageous

Table 1
Effect of Tn exchange on cardiac myofibrillar force and force kinetics at 10 °C

Preparation	F_{max}/A (nN/μm^2)	$F_{passive}/A$ (nN/μm^2)	k_{ACT} (s^{-1})	t_{LIN} (s)	k_{REL} (s^{-1})
Native myofibrils	88±16	7±4	7.3±0.7	0.030±0.003	39±2
hcTn exchanged myofibrils	90±12	9±2	6.0±0.8	0.025±0004	37±3

Values are means±SEM of 10 (native) and seven (hcTn exchanged) myofibrils. F_{max}/A indicates the maximum Ca^{2+}-activated force at pCa 4.5 normalised to the cross-sectional area of myofibrillar bundles. The passive force per cross-sectional area ($F_{passive}/A$) was determined under relaxing conditions (pCa 7.5) at sarcomere lengths of 2.3 μm.

and sensitive approach for studying the effects of Tn structure on the regulation and kinetics of cardiac contraction.

5. Nomenclature

ATP	adenosine 5′-triphosphate
DTT	dithiothreitol
EGTA	ethylene glycol-*O*,*O*′-(bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid
IgG-HRP	immunoglobulin G–horseradish peroxidase
IPTG	isopropyl β-D-thiogalactopyranoside
k_{ACT}	rate constant of Ca^{2+}-induced force development
k_{REL}	rate constant of fast exponential relaxation phase
MOPS	3-(*N*-morpholino)-propanesulfonic acid
hβ-MHC	human β-myosin heavy chain
mα-MHC	mouse α-myosin heavy chain
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TEA	triethanolamine
t_{LIN}	duration of initial slow linear force relaxation
Tn	troponin
TnI	troponin I
TnT	troponin T
TnC	troponin T
hcTnI, hcTnT, hcTnC	human cardiac muscle isoforms of TnI, TnT and TnC
sTn	skeletal Tn
5-TMRIA	tetramethylrhodamine-5-iodacetamide dihydroiodide

Acknowledgements

We are grateful to Dr. Charles Redwood and Dr. Ian P. Trayer for supplying the vectors for the expression of hcTn subunits, and to Dr. Jennifer E. Van Eyk for the gift of purified sTn. This work was supported by the German Research Foundation DFG (SFB-612-A2) and a fellowship from the DFG Graduiertenkolleg (M. Krüger). R. Stehle is grateful to Köln Fortune (Faculty of Medicine, Cologne) for financial support.

References

[1] I. Ohtsuki, K. Maruyama, S. Ebashi, Adv. Protein Chem. 38 (1986) 1.
[2] J.D. Strauss, C. Zeugner, J.E. Van Eyk, C. Bletz, M. Troschka, J.C. Ruegg, FEBS Lett. 310 (1992) 229.
[3] F. Shiraishi, M. Kambara, I. Ohtsuki, J. Biochem. (Tokyo) 111 (1992) 61.
[4] M. Chandra, J.J. Kim, R.J. Solaro, Biochem. Biophys. Res. Commun. 263 (1999) 219.
[5] B. Brenner, T. Kraft, L.C. Yu, J.M. Chalovich, Biophys. J. 77 (1999) 2677.
[6] B. Brenner, J.M. Chalovich, Biophys. J. 77 (1999) 2692.
[7] F. Colomo, S. Nencini, N. Piroddi, C. Poggesi, C. Tesi, Adv. Exp. Med. Biol. 453 (1998) 373.
[8] M. Chandra, D.E. Montgomery, J.J. Kim, R.J. Solaro, J. Mol. Cell. Cardiol. 31 (1999) 867.
[9] S. Morimoto, T. Goto, Biochem. Biophys. Res. Commun. 267 (2000) 912.
[10] S. Morimoto, Q.W. Lu, K. Harada, F. Takahashi-Yanaga, R. Minakami, M. Ohta, T. Sasaguri, I. Ohtsuki, Proc. Natl. Acad. Sci. USA 99 (2002) 913.
[11] H.M. Rarick, X.H. Tu, R.J. Solaro, A.F. Martin, J. Biol. Chem. 272 (1997) 26887.
[12] J.D. Strauss, J.E. Van Eyk, Z. Barth, L. Kluwe, R.J. Wiesner, K. Maeda, J.C. Ruegg, Pflugers Arch. 431 (1996) 853.
[13] R. Zhang, J. Zhao, A. Mandveno, J.D. Potter, Circ. Res. 76 (1995) 1028.
[14] R.J. Solaro, H.M. Rarick, Circ. Res. 83 (1998) 471.
[15] Y. Saeki, K. Takigiku, H. Iwamoto, S. Yasuda, H. Yamashita, S. Sugiura, H. Sugi, Jpn. J. Physiol. 51 (2001) 427.
[16] Y.Z. Ma, E.W. Taylor, Biophys. J. 66 (1994) 1542.
[17] R. Stehle, C. Lionne, F. Travers, T. Barman, Biochemistry 39 (2000) 7508.
[18] M. She, D. Trimble, L.C. Yu, J.M. Chalovich, J. Muscle Res. Cell Motil. 21 (2000) 737.
[19] G. Bonne, L. Carrier, P. Richard, B. Hainque, K. Schwartz, Circ. Res. 83 (1998) 580.
[20] C.S. Redwood, J.C. Moolman-Smook, H. Watkins, Cardiovasc. Res. 44 (1999) 20.
[21] A. Kimura, H. Harada, J.E. Park, H. Nishi, M. Satoh, M. Takahashi, S. Hiroi, T. Sasaoka, N. Ohbuchi, T. Nakamura, T. Koyanagi, T.H. Hwang, J.A. Choo, K.S. Chung, A. Hasegawa, R. Nagai, O. Okazaki, H. Nakamura, M. Matsuzaki, T. Sakamoto, H. Toshima, Y. Koga, T. Imaizumi, T. Sasazuki, Nat. Genet. 16 (1997) 379.

[22] L.S. Tobacman, D. Lin, C. Butters, C. Landis, N. Back, D. Pavlov, E. Homsher, J. Biol. Chem. 274 (1999) 28363.
[23] B. Hoffmann, H. Schmidt-Traub, A. Perrot, K.J. Osterziel, R. Gessner, Hum. Mutat. 17 (2001) 524.
[24] K. Elliott, H. Watkins, C.S. Redwood, J. Biol. Chem. 275 (2000) 22069.
[25] U.K. Laemmli, Nature 227 (1970) 680.
[26] P.K. Jha, Y. Du, Q.L. Wu, P.C. Leavis, S. Sarkar, Protein Expr. Purif. 5 (1994) 604.
[27] G.Q. Xu, S.E. Hitchcock-DeGregori, J. Biol. Chem. 263 (1988) 13962.
[28] J.D. Potter, Methods Enzymol. 85 (Pt B) (1982) 241.
[29] P.C. Chong, R.S. Hodges, J. Biol. Chem. 257 (1982) 2549.
[30] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbour, NY, 1989.
[31] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[32] K.M. Stehle, R. Scherer, P.K. Brixius, R.H.G. Schwinger, G. Pfitzer, Basic Res. Cardiol. 97 (Suppl. 1) (2002) 127.
[33] R. Stehle, M. Kruger, G. Pfitzer, Biophys. J. (in press).
[34] J.C. Kentish, D.T. McCloskey, J. Layland, S. Palmer, J.M. Leiden, A.F. Martin, R.J. Solaro, Circ. Res. 88 (2001) 1059.
[35] C. Tesi, F. Colomo, S. Nencini, N. Piroddi, Biophys. J. (in press).
[36] C. Tesi, F. Colomo, S. Nencini, N. Piroddi, C. Poggesi, J. Physiol. 516 (Pt 3) (1999) 847.