Expression of cardiac troponin T with COOH-terminal truncation accelerates cross-bridge interaction kinetics in mouse myocardium

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Submitted 23 February 2004; accepted in final form 25 May 2004

Stelzer, Julian E., Jitandarkumar R. Patel, M. Charlotte Olsson, Daniel P. Fitzsimons, Leslie A. Leinwand, and Richard L. Moss. Expression of cardiac troponin T with COOH-terminal truncation accelerates cross-bridge interaction kinetics in mouse myocardium. Am J Physiol Heart Circ Physiol 287: H1756–H1761, 2004. First published May 27, 2004; 10.1152/ajpheart.00172.2004—Transgenic mice expressing an allele of cardiac troponin T (cTnT) with a COOH-terminal truncation (cTnTtrunc) exhibit severe diastolic and mild systolic dysfunction. We tested the hypothesis that contractile dysfunction in myocardium expressing low levels of cTnTtrunc (i.e., <5%) is due to slowed cross-bridge kinetics and reduced thin filament activation as a consequence of reduced cross-bridge binding. We measured the Ca2+ sensitivity of force development [pCa for half-maximal tension generation (pCa50)] and the rate constant of force redevelopment (kF) in cTnTtrunc and wild-type (WT) skinned myocardium both in the absence and in the presence of a strong-binding, non-force-generating derivative of myosin subfragment-1 (NEM-S1). Compared with WT mice, cTnTtrunc mice exhibited greater pCa50, reduced steepness of the force-pCa relationship [Hill coefficient (nH)], and faster kF at submaximal Ca2+ concentration ([Ca2+]i), i.e., reduced activation dependence of kF. Treatment with NEM-S1 elicited similar increases in pCa50 and similar reductions in nH in WT and cTnTtrunc myocardium but elicited greater increases in kF at submaximal activation in cTnTtrunc myocardium. Contrary to our initial hypothesis, cTnTtrunc appears to enhance thin filament activation in myocardium, which is manifested as significant increases in Ca2+-activated force and the rate of cross-bridge attachment at submaximal [Ca2+]i. Although these mechanisms would not be expected to depress systolic function per se in cTnTtrunc hearts, they would account for slowed rates of myocardial relaxation during early diastole.

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usage were conducted under strict guidelines established by animal use committees at the University of Colorado and the University of Wisconsin.

**Skinned myocardial preparations.** The methods used for preparing skinned myocardium for mechanical measurements under isometric conditions were described previously (13). After intraperitoneal injection of 5,000 U heparin/kg body wt, WT and cTnTtrunc mice (8–12 mo old) were anesthetized with inhaled isoflurane. Their hearts were excised, and right and left ventricles were dissected in Ringer solution (in mM: 118 NaCl, 4.8 KCl, 2 NaH2PO4, 1.2 MgCl2, 25 HEPES, 11 glucose, and 0.5 CaCl2; pH 7.4, 22°C). Both ventricles were then rapidly frozen in liquid nitrogen, a step that was essential for obtaining high-quality preparations (13). To prepare skinned myocardium, pieces of frozen ventricle were thawed and homogenized for ~5 s in ice-cold relaxing solution (in mM: 100 KCl, 20 imidazole, 7 MgCl2, 2 EGTA, and 4 ATP; pH 7.0) with a Polytron homogenizer, which yielded multilocular preparations of 100–250 μm × 600–900 μm. The homogenate was centrifuged at 120 g for 1 min, and the resulting pellet was washed with fresh relaxing solution and resuspended in relaxing solution containing 250 μg/ml saponin and 1% Triton X-100. After 30 min, the skinned preparations were washed three times with fresh relaxing solution and were dispersed in ~50 ml of relaxing solution in a glass petri dish. The dish was kept on ice at all times except during the selection of individual preparations for mechanical experiments.

**Experimental solutions, apparatus, and protocol.** Solution compositions were calculated with the computer program of Fabiato (4) and stability constants (corrected to pH 7.0 and 15°C) listed by Godt and Lindley (7). All solutions contained (in mM) 100 Na, 50 bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 14.5 creatine phosphate, and 5 DTT. In addition, 1) pCa 9.0 solution contained (in mM) 7 EGTA, 0.02 CaCl2, 5.42 MgCl2, and 4.74 ATP; 2) pCa 4.5 solution contained (in mM) 7 EGTA, 0.01 CaCl2, 5.26 MgCl2, and 4.81 ATP; and 3) preactivating solution contained (in mM) 0.07 EGTA, 4.52 MgCl2, and 4.74 ATP. The ionic strength of all solutions was adjusted to 180 mM with potassium propionate. A range of solutions containing different free Ca2+ concentrations ([Ca2+]i) were prepared by mixing appropriate volumes of solutions of pCa 9.0 and pCa 4.5.

Skinned preparations with well-defined edges and no free ends evident in the middle region were transferred from the petri dish to a stainless steel experimental chamber (11) containing relaxing solution. The ends of a preparation were attached to the arms of a motor (model 312B, Aurora Scientific) and a force transducer (model 403; Aurora Scientific) as described previously (11). The chamber assembly was then placed on the stage of an inverted microscope (Olympus) fitted with a ×40 objective and a CCTV camera (model WV-600; Panasonic). Light from a halogen lamp was passed through a cut-off filter (transmission >620 nm) and was used to illuminate the skinned preparations. Bitmap images of the preparations were acquired with an AIP 4X2X graphics card and associated software (ATI Technologies) and were used to assess mean sarcomere length (SL) during the course of each experiment. Changes in force and motor position were sampled (16-bit resolution, DAP5216a; Microstar Laboratories, Bellevue, WA) at 2.0 kHz with SLControl software developed in our laboratory (http://www.slicontrol.com) and saved to computer files for later analysis. Changes in force were also recorded on a chart recorder with a slow time base.

At the start of each experiment, the preparation was stretched to a mean SL of ~2.20 μm for measurements of steady-state Ca2+-activated force and the rate constant of force redevelopment (ktr) after a release and restretch maneuver (2). The force recorded in a solution of pCa 9.0 was taken as resting force and then subtracted from the total force in activating solutions to yield the Ca2+-activated force (P).

The values of ktr reported here were determined as the rate constant of force redevelopment after the release and restretch of Ca2+-activated preparations (Fig. 1). Mechanical measurements of steady-state Ca2+-activated force and the rate of force redevelopment were repeated after 15-min incubations of the preparations in pCa 9.0 solution containing either 3 μM or 6 μM non-force-generating derivative of myosin subfragment-1 (NEM-S1) (5). At the end of each experiment, the preparation was cut free, placed in SDS sample buffer, and stored at ~8°C until subsequent analysis of contractile protein content analysis by 12% SDS-PAGE (5). Gels were stained with silver and scanned with a densitometer (Molecular Analyst; Bio-Rad).

**Data analysis.** Cross-sectional areas of skinned preparations were calculated by assuming that the preparations were cylindrical and by measuring width of the mounted preparation. Submaximal Ca2+-activated force (P) was expressed as a fraction of the maximum Ca2+-activated force (Pm) generated at pCa 4.5, i.e., P/Pm. Force-pCa data were fitted with the Hill equation: P/Pm = [Ca2+]i^n/k where k is the [Ca2+]i required for half-maximal activation. Rate constants of force redevelopment (ktr) were determined by linear transformation of the half-time of force recovery ktr = −ln (0.5 × (t/2)^-1) (5, 13).

All data are reported as means ± SE. Comparisons between WT and cTnTtrunc data in the absence of NEM-S1 were made with an unpaired t-test and, when appropriate (nonparametric data), with the Wilcoxon Mann-Whitney test. To evaluate the effects of NEM-S1 on WT or cTnTtrunc myocardium, ANOVA with a Tukey post hoc test was used or, when appropriate, the Wilcoxon Mann-Whitney test. Significance level was set at P < 0.05.
RESULTS

Mechanical properties of skinned WT and TnT trunc myocardium. TnT trunc and WT myocardium generated comparable resting forces and maximum Ca\(^{2+}\)-activated forces (Table 1). In both cases, the force-pCa relationship was sigmoidal and was well fit with the Hill equation (Fig. 2). In the absence of NEM-S1, mean pCa\(_{50}\) was 5.81 ± 0.01 in WT myocardium and 5.92 ± 0.02 in TnT trunc myocardium, indicating that the Ca\(^{2+}\) sensitivity of force was greater in TnT trunc myocardium (ΔpCa\(_{50}\) = 0.11 ± 0.01; P < 0.01). Furthermore, the steepness of the force-pCa relationship was markedly reduced in TnT trunc compared with WT myocardium (n\(_H\) was 3.6 ± 0.1 in TnT trunc vs. 4.3 ± 0.2 in WT; P < 0.001), indicating that low levels of TnT trunc expression reduce the apparent cooperativity of force development.

The k\(_r\) after rapid release and restretch of myocardium is frequently used to estimate the kinetics of cross-bridge cycling in contracting muscle. Representative traces showing the Ca\(^{2+}\) dependence of the kinetics of force redevelopment in WT and TnT trunc myocardium are shown in Fig. 3. At pCa 4.5, the mean values of maximal k\(_r\) recorded in WT myocardium did not differ from those recorded in TnT trunc myocardium (Table 1). In both WT and TnT trunc myocardium, k\(_r\) varied with the level of Ca\(^{2+}\) activation (Fig. 4A), although expression of TnT trunc significantly reduced the Ca\(^{2+}\)-dependent increase in k\(_r\). The k\(_r\) increased more than fourfold in WT myocardium (from 4.10 ± 0.16 s\(^{-1}\) at pCa 6.0 to 17.21 ± 0.60 s\(^{-1}\) at pCa 4.5), which is consistent with the results of earlier studies (1, 6, 15, 25), but only threefold in TnT trunc myocardium (from 5.29 ± 0.22 s\(^{-1}\) at pCa 6.1 to 17.24 ± 0.50 s\(^{-1}\) at pCa 4.5).

The k\(_r\) was also plotted as a function of steady-state isometric force at each pCa to assess the variation in k\(_r\) with a variable that reflects the overall level of thin filament activation. In both WT and TnT trunc myocardium, k\(_r\) increased as a function of increasing isometric force, gradually at lower forces and more steeply at high forces (Fig. 4B). However, at low forces expression of TnT trunc resulted in values of k\(_r\) that were greater than WT values. These data suggest that expression of TnT trunc enhances the cooperativity of thin filament activation.

Effects of NEM-S1 on mechanical properties of skinned WT and TnT trunc myocardium. The effects of 3 and 6 μM NEM-S1 on force-pCa relationships in skinned wild-type and transgenic myocardium are summarized in Table 1. In both WT and TnT trunc myocardium, NEM-S1 increased both resting force and submaximal Ca\(^{2+}\)-activated forces in a concentration-dependent manner. Mean pCa\(_{50}\) increased significantly in both WT and TnT trunc myocardium after treatment with 3 μM NEM-S1, indicating an increase in the Ca\(^{2+}\) sensitivity of force. Qualitatively similar increases in mean pCa\(_{50}\) were observed after treatment with 6 μM NEM-S1. In addition, treatment with 3 or 6 μM NEM-S1 significantly reduced the steepness of the force-pCa relationships in both WT and TnT trunc myocardium, an observation consistent with results reported previously in skinned ventricular myocardium (6).

Treatment with either 3 or 6 μM NEM-S1 had no appreciable effect on the kinetics of force redevelopment in maximally activated preparations. However, at low levels of Ca\(^{2+}\) activation, NEM-S1 had concentration-dependent effects on k\(_r\). In both WT and TnT trunc myocardium, treatment with 3 or 6 μM
NEM-S1 increased $k_{tr}$ at low levels of activation to values identical to $k_{tr}$ obtained in maximally activated preparations. At intermediate levels of activation, NEM-S1 increased $k_{tr}$ to greater than control values, but $k_{tr}$ was still less than maximal. Also, the $k_{tr}$ values obtained at intermediate levels of activation in the presence of NEM-S1 were significantly greater in TnT trunc myocardium than in WT myocardium.

**DISCUSSION**

**Summary.** Earlier functional studies on whole hearts isolated from cTnT trunc transgenic mice indicated that the mutation induced severe diastolic dysfunction and mild hypocontractile systolic dysfunction (19), whereas studies in reduced systems found that the cTnT trunc mutation increased the Ca$^{2+}$ sensitivity of force (10), reduced the inhibition of actin-activated myosin ATPase activity in regulated thin filaments, and reduced inhibition of sliding in an in vitro motility assay comprised of regulated thin filaments (14). Although the mechanisms underlying this range of phenotypes are likely to be complex, an hypothesis that is consistent with these findings is that cTnT trunc directly affects cross-bridge transitional rate constants such that the rate of force development is slowed by reductions in the apparent rate constants of both attachment and detachment of cross bridges from actin. In fact, our results showed that the opposite was the case: at submaximal activations cTnT trunc myocardium exhibited faster rates of force development (Fig. 4A), implying that the rates of cross-bridge attachment or detachment or both were accelerated compared with WT myocardium. Our results further show that at least some of these effects of the cTnT trunc mutation were a consequence of greater cross-bridge recruitment and increased thin filament activation due to the cTnT trunc mutation.

**Fig. 3.** Force records obtained during measurements of $k_{tr}$ in skinned wild-type and transgenic myocardium. The force records have been scaled to the peak steady-state force attained after the step change in muscle length to facilitate direct visualization of differences in rates of force recovery in skinned myocardium from wild-type (A) and TnT trunc (B) mice.

**Fig. 4.** Effects of 3 μM NEM-S1 on the activation dependence of $k_{tr}$ in wild-type and transgenic myocardium. Force redevelopment after rapid release and restretch was measured in the absence (open symbols) and the presence (closed symbols) of 3 μM NEM-S1 in wild-type (circles) and TnT trunc (squares) myocardium. Data points are means, and the error bars are SE. A: Ca$^{2+}$ dependence of $k_{tr}$. B: $k_{tr}$ as a function of force (expressed as a fraction of maximal Ca$^{2+}$-activated force) measured at each pCa.
filament responsiveness to the activating effects of Ca²⁺ and strong-binding cross bridges.

Altered Ca²⁺ sensitivity of force and apparent cooperativity in cTnTtrunc myocardium. Previous studies reported that expression of cTnTtrunc reduced maximal Ca²⁺-activated force compared with controls (12, 24). Our results did not reproduce this effect, and because the maximal rates of force redevelopment (kₚ) did not differ between cTnTtrunc and WT myocardium, a reduction in maximal Ca²⁺-activated force is not likely to contribute to the changes in cross-bridge kinetics observed in cTnTtrunc myocardium at submaximal activations.

Our data also show that transgenic expression of small amounts (<5%) of cTnTtrunc produced dramatic effects on Ca²⁺ sensitivity of force and the apparent cooperativity of thin filament activation, as indicated by changes in nₜ. The pCa required at half-maximal activation (pCa₅₀) was shifted to lower [Ca²⁺] by 0.11 units (Fig. 2) in transgenic myocardium compared with WT myocardium, indicating that less Ca²⁺ is required to produce the same amount of force as controls. This finding agrees well with results of Montgomery et al. (10), who reported a 0.14-pCa unit shift in the tension-pCa relationship in the same cTnTtrunc mice compared with WT myocardium.

Another notable finding is that nₜ, an index of apparent cooperativity in the activation of force, was reduced in cTnTtrunc myocardium as evidenced by the shallower slope of the force-pCa relationship (Fig. 2). This phenomenon was observed earlier by other investigators (3) and may manifest a diminished capacity of the Tn-Tn complex to inhibit crossbridge binding. Impaired suppression of myosin binding at low levels of Ca²⁺ is consistent with slowed and incomplete diastolic relaxation and may indirectly give rise to diminished systolic function in cTnTtrunc mice (19). It has been suggested that the TnT truncation destabilizes the blocked conformation of the thin filament regulatory strand, which enhances the transition from the blocked to the active state. Thus, in cTnTtrunc myocardium, there is greater likelihood that the thin filament will assume the “switched-on” state even at low [Ca²⁺] and thereby reduce cooperativity in myosin binding to actin (3, 23).

Accelerated cross-bridge kinetics in cTnTtrunc myocardium. Although cTnTtrunc had no effect on the maximal rate of force redevelopment compared with control, the mutation increased kᵣ, at submaximal [Ca²⁺] (Fig. 4). Such increases could be due to a direct effect of the mutation on cross-bridge cycling kinetics or an indirect effect of increased cross-bridge binding to the thin filament. Both mechanisms appear to be operational in cTnTtrunc myocardium, because some of the difference in rates between cTnTtrunc and WT disappeared when results were plotted against relative force as a measure of Ca²⁺ activation; however, at P/P₀, less than ~0.4, kᵣ was still faster in cTnTtrunc myocardium. The reduction in difference between cTnTtrunc and WT myocardium when kᵣ was plotted against force implies that some of the difference of the difference (Fig. 4A) is due to acceleration of kᵣ in cTnTtrunc myocardium secondary to the activating effects of greater numbers of cross bridges bound to the thin filament. The observation that some of the difference persists at low levels of activation suggests that cTnTtrunc also has direct effects to accelerate cycling kinetics. In this regard, it is important to reiterate that the acceleration of kᵣ at low levels of Ca²⁺-activation was observed despite low levels of cTnTtrunc expression (19). Incorporation of <5% cTnTtrunc into the thin filament was sufficient to elicit a significant increase in the rate of force redevelopment in the absence of NEM-S1 (Fig. 4B).

Earlier measurement of ATPase activity is consistent with these results. Montgomery et al. (10) reported that maximal ATPase cycling rates did not differ between WT and cTnTtrunc; however, the ATPase activity-pCa relationship was shifted to lower [Ca²⁺] in cTnTtrunc, indicating that cross-bridge cycling rates were increased at submaximal [Ca²⁺]. Reports of increased sliding speed of actin filaments in vitro motility assays (14, 22), decreased inhibition of ATPase activity in the absence of Ca²⁺ (18), and the accelerated kᵣ at low Ca²⁺ observed in the present study are consistent with the idea that cTnTtrunc attenuates inhibition of thin filaments at low Ca²⁺.

This phenomenon can also be interpreted in terms of a three-state model of thin filament activation (9) in which the thin filament exists in a blocked state (unable to bind myosin) in the absence of Ca²⁺ but rapidly transitions to a closed state on Ca²⁺ binding to TnC, with subsequent transition to an open state when cross bridges bind to actin. When Ca²⁺ binds to TnC, alterations in interactions among thin filament proteins are thought to release the thin filament from the blocked state and to promote the transition of weakly bound cross bridges to a strongly bound, force-generating state. The impairment in function caused by a low level of expression of TnTtrunc can be explained as an inability of those regions of the thin filament containing cTnTtrunc to completely assume the blocked state in the absence of Ca²⁺, which could result in incomplete relaxation. An increased number of strongly bound cross bridges at low levels of Ca²⁺-activation would also be expected to accelerate the rate of force redevelopment because of the greater activating affects of cross bridges already bound to the thin filament. This model implies that incomplete inactivation of cTnTtrunc thin filaments is most likely due to an incomplete return of Tm to the fully blocked position in the absence of Ca²⁺.

Effects of NEM-S1 on Ca²⁺ sensitivity of force and cooperativity in activation of force. In this study, NEM-S1 was used to assess the effects of strong-binding cross bridges on thin filament activation in WT and cTnTtrunc myocardium. As previously shown in rat myocardium (6), NEM-S1 slightly increases Ca²⁺ independent tension at pCa 9.0, a phenomenon that was similar in WT and cTnTtrunc myocardium. Furthermore, NEM-S1 markedly increased the Ca²⁺ sensitivity of force in both WT and cTnTtrunc myocardium, shifting the force-pCa relationships to lower [Ca²⁺] by similar amounts and having similar effects on WT and transgenic myocardium. Thus, although the cTnTtrunc mutation increased the Ca²⁺ sensitivity of force, it had no obvious effects on the cooperative activation of steady-state isometric force by strong-binding cross bridges.

Effects of NEM-S1 on rate constant of force redevelopment. Earlier studies from this laboratory (6) showed that NEM-S1 accelerates the rate of force development in rat skinned myocardium, presumably due to increased activation of the thin filament as a consequence of greater numbers of cross bridges bound to actin. In the present work, NEM-S1 increased kᵣ in both cTnTtrunc and WT myocardium but had greater effects in cTnTtrunc myocardium. In both WT and transgenic myocardium, the NEM-S1-induced acceleration of force development at submaximal [Ca²⁺] reduced the activation dependence of kᵣ. NEM-S1 had no effect on maximal kᵣ (measured at pCa 4.5).
and increased $k_c$ at very low [Ca$^{2+}$] to maximal values (Fig. 4). The difference in NEM-S1-induced effects on $k_c$ was observed at intermediate levels of activation, where $k_c$ was increased to values less than maximal with greater increases in $k_c$ in cTnTtrunc myocardium.

In vitro studies of thin filament proteins in the presence of TnTtrunc suggest that Tm is held less tightly by Tn in its inhibitory position (3), thus reducing steric hindrance of myosin binding to actin. Results from the present study showing reduced cooperativity of activation and accelerated cross-bridge kinetics during submaximal activation of TnTtrunc myocardium support this view. Likewise, the greater activating effects of NEM-S1 on cross-bridge kinetics in TnTtrunc myocardium can be explained in terms of increased likelihood of cross-bridge binding at low levels of activation.

**Functional significance of cTnTtrunc mutation.** The primary function of the thin filament regulatory strand is to inhibit myosin binding in the absence of Ca$^{2+}$ and to relieve this inhibition in the presence of Ca$^{2+}$. Several published reports show that incorporation of cTnTtrunc into the thin filament impairs the ability of the regulatory strand to completely inhibit myosin cross-bridge cycling at very low Ca$^{2+}$ or in its absence (3, 14, 18). Results from the present study support this view. Furthermore, incorporation of cTnTtrunc into the thin filament appears to accelerate cross-bridge cycling at submaximal Ca$^{2+}$ both by increasing the responsiveness of thin filaments to the activating effects of Ca$^{2+}$ and strong-binding cross bridges and by increasing the rate of cross-bridge interaction with actin. Together, our results could account for deficits in global ventricular function, including incomplete diastolic relaxation, which leads to decreased ventricular filling and a lower ejection fraction due to impaired systolic function (19).

**REFERENCES**