Functional Analysis of Troponin I Regulatory Domains in the Intact Myofilament of Adult Single Cardiac Myocytes*

(Received for publication, March 29, 1999, and in revised form, June 1, 1999)

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Tropinin I is the putative molecular switch for Ca\textsuperscript{2+}-activated contraction within the myofilament of striated muscles. To gain insight into functional troponin I domain(s) in the context of the intact myofilament, adenovirus-mediated gene transfer was used to replace endogenous cardiac troponin I within the myofilaments of adult cardiac myocytes with the slow skeletal isoform or a chimera of the slow skeletal and cardiac isoforms. Efficient expression and myofilament incorporation were observed in myocytes with each exogenous troponin I protein without detected changes in the stoichiometry of other contractile proteins and/or sarcomere architecture. Contractile function studies in single, permeabilized myocytes expressing exogenous troponin I provided support for the presence of a Ca\textsuperscript{2+}-sensitive regulatory domain in the carboxyl terminus of troponin I and a second, newly defined Ca\textsuperscript{2+}-sensitive domain residing in the amino terminus of troponin I. Additional experiments demonstrated that the isoform-specific, acidic pH-induced contractile dysfunction in myocytes appears to lie in the carboxyl terminus of troponin I. Functional results obtained from adult cardiac myocytes expressing the chimera or isoforms of troponin I now define multiple troponin I regulatory domains operating in the intact myofilament and provide new insight into the Ca\textsuperscript{2+}-sensitive properties of troponin I during contraction.

TnI acts as a molecular switch during Ca\textsuperscript{2+}-activated contraction and relaxation (1). In relaxed muscle, when cellular Ca\textsuperscript{2+} concentrations are low, TnI binds strongly to actin and inhibits strong binding between the myosin cross-bridge and actin. The binding of Ca\textsuperscript{2+} to TnC upon Ca\textsuperscript{2+} release from the sarcoplasmic reticulum, induces conformational changes within TnI. These changes increase TnI-TnC binding and decrease TnI-actin interactions, such that force-generating crossbridges can form (2, 3). While the molecular switch function of TnI is known, the function of individual TnI domains in the context of intact myofilaments is not well characterized.

Gene transfer of exogenous contractile protein isoforms or mutant proteins can be used to study the function of individual contractile proteins within the intact myofilament (4, 5). Rapid and efficient exogenous gene expression is observed in adult cardiac myocytes with recombinant adenovirus-mediated gene transfer (4, 6), an approach that overcomes the inefficient protein expression obtained in adult cardiac myocytes treated with traditional DNA transfection techniques (6). Earlier studies established the validity of this gene delivery approach by showing that adult myocytes retain their highly differentiated phenotype over the culture period necessary for expression and myofilament incorporation of the exogenous contractile protein (6).

Adenovirus-mediated myofilament gene delivery into adult myocytes provides a new opportunity to understand contractile protein function within the intact myofilaments of cardiac myocytes. Virtually complete exchange of ssTnI for cTnI in the myofilaments was previously observed 5–7 days after viral delivery of ssTnI cDNA to adult rat cardiac myocytes maintained in primary culture (4). Sarcomere architecture and the isoform expression and stoichiometry of other contractile proteins were maintained in these myocytes. Functional studies on permeabilized myocytes demonstrated that ssTnI expression increased tension at intermediate Ca\textsuperscript{2+} levels, relative to control myocytes or myocytes receiving a recombinant adenovirus with an endogenous Tn expression cassette (4). Acidosis, which can affect in vivo cardiac performance as it develops during several pathophysiological states (7), greatly decreases myofilament Ca\textsuperscript{2+} sensitivity in cardiac myocytes (4, 8). This myofilament response to acidosis was notably blunted in myocytes expressing ssTnI (4). Collectively, tension measurements in myocytes expressing ssTnI provided the first direct evidence that TnI isoforms contribute to the different myofilament Ca\textsuperscript{2+} and pH sensitivities of tension in neonatal and adult myocardin, which express ssTnI and cTnI, respectively (4). The ability to measure contractile function after a specific, exogenous TnI is rapidly and efficiently incorporated into the myofilament of adult cardiac myocytes makes this a powerful system for comparing functional domains in TnI isoforms within the intact myofilament.

In the present study, a chimeric TnI protein containing the amino terminus of ssTnI and the carboxyl terminus of cTnI was constructed to establish the region(s) responsible for TnI isoform-specific differences in the Ca\textsuperscript{2+} threshold, Ca\textsuperscript{2+} sensitivity, and cooperativity of the myofilament within the context of the adult cardiac myocyte. Earlier biochemical studies provided fundamental knowledge about TnI interactions with other contractile proteins and concluded that the inhibitory function of TnI is localized within the carboxyl terminus (9, 10). However, until now, the TnI domain(s) conferring isoform specificity and the influence of different TnI domains on the relationship between tension and Ca\textsuperscript{2+} at physiological as well as acidic pH have not been examined experimentally. Experi-

* This work was supported by grants from the National Institutes of Health (to J. M. M.) and from the American Heart Association (to J. M. M. and M. V. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: TnI, troponin I; Ad, adenovirus serotype 5; Ab, antibody; cTnI, cardiac troponin I; CMV, cytomegalovirus; HEK, human embryonic kidney; mAb, monoclonal Ab; m.o.i., multiplicity of infection; ssTnI, slow skeletal troponin I; Tn, tropomyosin; TnC, troponin C.
Sac tagenized at nucleotides 385 and 390 to introduce a respectively. To begin construction of the chimeric TnI, cTnI was muf

ments with the TnI chimera now demonstrate that both the carboxyl and amino portions of TnI confer isoform specificity and influence myofilament Ca^{2+} sensitivity. Furthermore, experiments with myocytes expressing the TnI chimera now demonstrate that a domain in the carboxyl terminus of TnI plays an important role in the myofilament response to acidic pH. Taken together, these results provide new knowledge about the functional TnI domains in the context of the intact myofilament and furnish unique information about the function(s) of TnI isoforms and their individual roles as myofilament regulatory proteins.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis Strategy**

Full-length wild-type ssTnI and cTnI cDNAs (11) were used to generate a Tnl chimera, and the alignment of the three cDNAs relative to one another is shown in Fig. 1. The ssTnI and cTnI cDNAs were subcloned into the pGEM-3Z vector at the BamHI and EcoRI sites, respectively. To begin construction of the chimeric TnI, cTnI was mutagenized at nucleotides 385 and 390 to introduce a SacI site at position 390 (cTnI-SacI) using the QuikChange site-directed mutagenesis kit (Stratagene; La Jolla, CA) with two oligonucleotide primers (nucleotide

**TABLE I**

| mAb   | Treatment group | Treatment group |
|-------|-----------------|-----------------|
| cTnI  | +               | +               |
| ssTnI | +               | +               |
| TnI   | +               | +               |
| MAB 1691 | +         | +               |
| 2F6.6 | +               | -               |

**Primary Cultures of Rat Ventricular Myocytes**

Ventricular myocytes were isolated from adult female rats as described by Westfall et al. (14). An aliquot of Ca^{2+}-tolerant myocytes (2 × 10^6 myocytes) was then plated on a laminin-coated coverslip and incubated at 37°C in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin for 2 h. After gentle aspiration, cells were incubated with recombinant adenovirus in Dulbecco’s modified Eagle’s medium plus penicillin/streptomycin for 1 h followed by the addition of 2 ml of Dulbecco’s modified Eagle’s medium plus penicillin/streptomycin. Serum-free medium was changed the day after adding virus and then every 2–3 days for up to 8 days of culture.

**Analysis of Protein Composition by Gel Electrophoresis and Western Blots**

**Gel Electrophoresis**—Approximately 10 ventricular myocytes were collected on the tip of a glass micropipet and transferred to microcentrifuge tubes containing 10 μl of sample buffer for analysis by gel electrophoresis. Fiber segments of soleus muscles were collected as described previously (16). Sonicated samples were separated on SDS-polyacrylamide gels prepared with 3.5% acrylamide in the stacking gel and 12% acrylamide in the separating gel as described earlier in detail (17). Gels were then fixed in glutaraldehyde, silver-stained as described by Giulian et al. (18), scanned with an Arcus II laser densitometer (AGFA-Gevaert NV, Mortsel, Belgium), and analyzed with Multi-Analy

**Western Blot Analysis**—Protein expression in cultured ventricular myocytes and HEK 293 cells was detected by collecting cells in sample buffer 3–8 days after plating, separating proteins by gel electrophoresis as described above, and then transblotting onto polyvinylidene difluoride membrane for 2000 V-h as described previously in detail (17).

Inmunodetection was carried out as described by Westfall et al. (17) on blots fixed in glutaraldehyde. TnI isoform/chimera composition was determined using a 1:500 dilution of the anti-TnI mAb, MAB 1691 (Chemicon Inc., Temecula, CA), which recognizes all striated muscle isoforms from rat. TnI chimera expression also was examined by Western blot with the TnI-specific mAbs, TI-1 (1.500) and 2F6.6 (1.500). The 2F6.6 mAb recognizes the amino terminus of cTnI (19), which is not present in the TnI chimera, and as expected, TnI chimera expression was not recognized by the 2F6.6 mAb but was detected with the TI-1 mAb (Table I). Troponin T and Tm expression were detected with the JLT-12 (1:200; Sigma) and TM311 (1:106; Sigma) mAbs, respectively.

**Indirect Immunohistochemistry in Single Cardiac Myocytes**—Indirect immunofluorescence with a dual mAb protocol was used to evaluate the extent of thin filament remodeling within single cardiac myocytes expressing ectopic ssTnI or chimeric TnI. Incubation conditions were as described in earlier studies (17). The primary mAbs used to evaluate TnI isoform composition were 1) cardiac specific TI-1 mAB (20) or 2F6.6 (1:1000; Ref. 19) and 2) either TI-4 mAB or MAB1691 (1:1000; Chemicon), which are mAbs recognizing all forms of TnI including the TnI chimera. Goat anti-mouse IgG antibodies conjugated to Texas Red or fluorescein isothiocyanate were used to detect cTnI-specific and TnI binding, respectively (17). Immunofluorescence was examined on a Leitz Aristoplan microscope, and representative cells were photographed on a Noran OZ laser scanning confocal microscope.

**Measurement of Ca^{2+}-activated Tension in Single Cardiac Myocytes at pH 7.0 and 6.2**

**Solutions and Preparation of Samples for Mechanical Studies**—Complete descriptions of the experimental chamber and attachment procedure for mounting single cardiac myocytes and soleus fibers have been
reported elsewhere (16). Details of the solutions, permeabilization, and tension measurement protocols are briefly described below. Relaxing and activating solutions used for experiments contained 7 mM EGTA, 20 mM imidazole, 1 mM free Mg\(^{2+}\), 14.5 mM creatine phosphate, and 4 mM MgATP with sufficient KCl to yield a total ionic strength of 180 mM. Solution pH was adjusted to 7.00 (or 6.20 for acidic pH experiments) with KOH/HCl. The relaxing solution had a pCa of 9.0, and maximal activation was achieved with a pCa of 4.0. The final concentrations of each metal, ligand, and metal-ligand complex were calculated with a computer program (21), employing the stability constants of Godt and Lindley (22).

Cultured cardiac myocytes permeabilized by brief treatment with 0.2% Triton X-100 were attached to a force transducer (model 403A; Cambridge Technology Inc., Watertown, MA) and a high performance moving coil galvanometer (model 6350; Cambridge Technology) at a sarcomere length set to 2.1 \(\mu\)m. The experimental temperature was set at 15 \(^\circ\)C to allow comparison with earlier work (4) and because preparation viability decreases and sarcomere length nonuniformity increases more rapidly at higher temperatures. Permeabilized slow soleus fibers were mounted at a sarcomere length of 2.50–2.60 \(\mu\)m (16).

Measurement of Steady-state Isometric Tension-pCa Relationship—At each pCa, the preparation was rapidly (<0.5 ms) slackened after peak steady isometric tension developed to obtain the tension base line and then immersed in relaxing solution. Total tension is the difference between peak steady isometric tension developed to obtain the tension base line and then immersed in relaxing solution. Submaximal activations were bracketed by maximal activations at pCa 4.0. Curve fits were performed using the Marquardt-Levenberg nonlinear least squares fitting algorithm for the Hill equation:

\[ P = \frac{\text{[Ca}^{2+}]^{n_H}}{K_c + \text{[Ca}^{2+}]^{n_H}}, \]

where \(P\) is tension as a fraction of maximum tension, \([\text{Ca}^{2+}]\) is the free calcium concentration, \(K_c\) is the \(\text{Ca}^{2+}\) required for half-maximal activation, and Hill coefficient \((n_H)\) are measures of myofilament \(\text{Ca}^{2+}\) sensitivity and cooperativity, respectively.

Statistics
Values for each group are expressed as mean ± S.E. Significant differences between groups (\(p < 0.05\)) were tested with an analysis of variance and post hoc Student-Newman-Keuls multiple comparison test when needed.

RESULTS
Optimization of TnI Gene Transfer—The first series of experiments fully characterized the time course of exogenous TnI expression after adenovirus-mediated gene transfer of ssTnI into adult cardiac myocytes. Western blot analysis indicated nearly complete exchange in TnI isoflament expression as early as 5 days post-gene transfer (Fig. 2A), with a time course following a single exponential curve from day 2 to day 7 post-ssTnI gene transfer (Fig. 2B). These results are consistent with the published 3.2-day half-life for TnI turnover within the intact heart (23). Gene transfer of ssTnI was further optimized by varying the m.o.i. (plaque-forming units/rod-shaped myocyte) between 200 and 2000. Dose-dependent differences in ssTnI expression were observed at 5 days, with optimal isoflament exchange by 7 days with 400–2000 m.o.i. of AdCMVssTnI (results not shown). Optimal ssTnI expression in this m.o.i. range is in agreement with earlier studies using reporter genes, which showed that >90% of cells are infected and expressing reporter between 100 and 1000 plaque-forming units/myocyte (6, 24). High levels of ssTnI expression with 1000–2000 m.o.i. after 5 days indicate that TnI turnover time can be reduced, perhaps due to a new steady state between TnI synthesis and degradation. These changes in TnI isoflament expression were not associated with significant changes in the stoichiometry of other contractile proteins nor in total TnI content when comparing ssTnI- and cTnI-expressing myocytes (Table II). Tight regulation of contractile protein stoichiometry in transgenic mice expressing individual contractile proteins is attributed to post-transcriptional control mechanisms (25), and a similar regulatory mechanism is likely operating within myocytes maintained in primary culture.

The effect of increasing doses of recombinant adenovirus on ssTnI expression and myofilament incorporation within cardiac myocytes also was examined by immunohistochemistry using a pair of anti-TnI mAbs (4, 17). The mAb pair used for these experiments included the TI-1 mAb to follow disappearance of cTnI expression and the TI-4 mAb to show ssTnI protein in myocytes receiving AdCMVssTnI. Representative low power (Fig. 3, A and B) and high power (Fig. 3C) images show unchanged cTnI-specific labeling in control cells, while cTnI-specific staining is absent in myocytes following ssTnI gene transfer (2000 m.o.i.). A positive striated pattern of immunostaining was observed with TI-4 mAb in cTnI- and ssTnI-expressing myocytes, and these findings are evidence for expression and myofilament incorporation of exogenous TnI without changes in cell morphology. Similar results were obtained with 200–1000 m.o.i. of AdCMVssTnI (results not shown). Immunostaining with a second pair of mAbs (2F6.6 and MAB 1691) was in agreement with the TI-1/TI-4 mAb results (Fig. 3D, Table II). The number of rod-shaped, ssTnI-expressing cardiac myocytes no longer staining positive for cTnI with the 2F6.6 mAb increased from 48% at 4 days (\(n = 411\)) to 61% (\(n = 1148\)), 72% (\(n = 1056\)), and 81% (\(n = 1335\)) at 5, 6 (Fig. 3D), and 7 days in culture, respectively. Taken together, Western blot and immunohistochemical analysis showed marked ectopic TnI expression in the myofilaments of adult cardiac myocytes 5–7 days after treatment with 200–2000 m.o.i. of

![Fig. 2. Characterization of ssTnI expression in adult cardiac myocytes treated with AdCMVssTnI. A, Western blot analysis demonstrating increasing ssTnI expression over time in adult myocytes after treatment with AdCMVssTnI and in control myocytes (C). The immunodetection was carried out using MAB 1691 anti-TnI mAb. Similar results had previously been observed using a TI-4 mAb (41). B, summary results of ssTnI expression over time in cardiac myocytes post-ssTnI gene transfer (~500 plaque-forming units/myocyte). Results indicate that an almost complete switch in TnI isoflament expression is observed within 6 days after gene transfer with AdCMVssTnI in 3–8 preparations of myocytes.](image-url)
adenovirus, without detectable changes in TnI content, contractile protein stoichiometry, or cell morphology. These findings provide further support for the conclusion that functional changes observed in myocytes expressing ssTnI result directly from ectopic TnI expression and myofilament incorporation in adult cardiac myocytes (4).

Expression and Myofilament Incorporation of the TnI Chimera in Cardiac Myocytes—TnI chimera expression was studied by Western blot analysis in adult cardiac myocytes cultured for up to 7 days. The TnI chimera protein migrated as predicted slightly faster than the fast skeletal troponin I (fsTnI) isoform present in psoas fibers (Fig. 4A), and expression increased from day 3 onward and reached a maximum by 6–7 days (Fig. 4, A and B). A similar expression pattern was observed in permeabilized myocytes (Fig. 4A), which provides indirect evidence for myofilament incorporation of the TnI chimera protein with-
Gene transfer, and myocytes in lanes I
soleus and psoas fibers, which express ssTnI and fast skeletal troponin collected 7 days after gene transfer. Shown for comparison are results with gene transfer in 2–5 preparations of myocytes.

Almost complete switch to TnI chimera expression within 6 days after treated with AdCMVcTnI or AdCMVTnI chimera. Results indicate an summary of TnI chimera expression over time in cardiac myocytes constructed using the amino-terminal 69 amino acids of ssTnI and the carboxyl-terminal 110 amino acids of cTnI. For permeabilized myocytes, the lane labeled TnI chimera contains myocytes collected 5 days post-gene transfer, and myocytes in lanes labeled cTnI or ssTnI were collected 7 days after gene transfer. Shown for comparison are results with soleus and psoas fibers, which express ssTnI and fast skeletal troponin I (fsTnI), respectively. TnI expression was detected with MAB 1691. B, summary of TnI chimera expression over time in cardiac myocytes treated with AdCMVcTnI or AdCMVTnI chimera. Results indicate an almost complete switch to TnI chimera expression within 6 days after gene transfer in 2–5 preparations of myocytes. However, Ca$^{2+}$-sensitive accumulation in the cytoplasm. As with ssTnI, dose-dependent TnI chimera expression was observed at 5 days with nearly complete replacement of cTnI by TnI chimera at 7 days with doses ranging from 400 to 2000 m.o.i. (results not shown). Gene transfer and expression of chimeric TnI in cardiac myocytes was not associated with changes in total TnI protein content (Table II). The stoichiometry of TnI expression relative to troponin T and Tm (Table II) and isoform expression of troponin T and Tm (results not shown) also were not different following TnI chimera gene transfer compared with control and AdCMVcTnI-treated myocytes. In addition, myosin (Fig. 5) and myosin light chain (results not shown) isoform expression were not changed by TnI chimera gene transfer. Thus, Western blot and SDS-polyacrylamide gel electrophoresis analysis collectively show that the TnI chimera is expressed in myocytes without detected changes in isoform expression or stoichiometry of other contractile proteins.

Myofilament incorporation of chimeric TnI within cardiac myocytes was directly studied 4–7 days after adenovirus treatment using indirect immunofluorescence with the 2F6.6 and MAB 1691 anti-TnI pair of mAbs. A representative cardiac myocyte shown in Fig. 6, E and F, illustrates myofilament replacement of cTnI (Fig. 6E) by chimeric TnI (Fig. 6E) after 6 days in culture. Rod-shaped myocytes completely lacking cTnI progressively increased over time from 17% ($n = 548$) at 4 days, 42% ($n = 1087$) at 5 days, and 52% ($n = 1039$) at 6 days to 71% ($n = 1547$) by 7 days after treatment with AdCMVTnI chimera. Immunostaining with the cTnI-specific mAb, 2F6.6, remained 100% positive over the same time interval in control myocytes (Fig. 6B) and myocytes receiving AdCMVcTnI (Fig. 6D). These results, together with the Western blot and SDS-polyacrylamide gel electrophoresis results, indicate that there was expression and myofilament incorporation of the TnI chimera protein without detectable changes in sarcomere architecture, cell morphology, stoichiometry, and/or expression pattern of other key contractile proteins. The demonstration of specific and stoichiometric myofilament replacement of cTnI by the TnI chimera within cardiac myocytes now permits this protein to be used to address fundamental questions concerning TnI functional domains in the context of an intact myofilament.

Effect of TnI Chimera Expression on Contractile Function at Physiological pH—the functional significance of TnI chimera expression in adult cardiac myocytes was assessed by measuring tension over a range of Ca$^{2+}$ concentrations under physiological ionic conditions in permeabilized adult cardiac myocytes 5–7 days post-gene transfer. This approach was designed to test whether the carboxyl-terminal domain shared by cTnI and chimeric TnI (Fig. 1) primarily determines TnI function in myofilaments. Recombinant adenovirus alone did not significantly affect cardiac myocyte function, since gene transfer of cTnI into adult cardiac myocytes did not result in significant changes in maximum tension, myofilament Ca$^{2+}$ sensitivity, or cooperativity compared with control values (Fig. 7B). Maximum tension and cooperativity were also unchanged in myocytes expressing the TnI chimera compared with control and AdCMVcTnI-treated cardiac myocytes (Fig. 7, Table III). However, Ca$^{2+}$ sensitivity decreased significantly in myocytes expressing chimeric TnI compared with myocytes expressing cTnI (Fig. 7, A and B), with the most notable differences in tension observed at Ca$^{2+}$ levels near the $p_{Ca_{50}}$ (Fig. 7A). Upon further analysis of the tension-pCa relation, the threshold for Ca$^{2+}$ activation was not significantly changed when comparing myocytes expressing chimeric TnI and cTnI (Fig. 8). Taken together, the functional results demonstrate that the TnI chimera decreases myofilament Ca$^{2+}$ sensitivity compared with myocytes expressing cTnI, while the TnI chimera and cTnI similarly influence maximum tension, myofilament cooperativity, and the threshold for Ca$^{2+}$-activated tension. In contrast, TnI chimera expression affected Ca$^{2+}$-activated tension much differently from ssTnI, since expression of ssTnI in adult myocytes or soleus fibers caused a leftward shift in Ca$^{2+}$ sensitivity.
Immunolabeling was carried out using the MAB 1691 mAb in the left panels (A, C, and E) and 2F6.6 mAb in the right panels (B, D, and F). As with cardiac myocytes expressing ssTnI (see Fig. 3), immunolabeling with the 2F6.6 mAb was not detectable in myocytes expressing the TnI chimera (F). The striated immunolabeling pattern obtained with the MAB 1691 anti-TnI mAb (E) indicates chimeric TnI is expressed and incorporated into the myofilament. Bar, 25 μm.

**DISCUSSION**

This study provides new insight into the function of TnI regulatory domains within the intact myofilament of adult cardiac myocytes. A comparison of Ca$^{2+}$-activated tension in adult cardiac myocytes expressing cTnI, ssTnI, or the TnI chimera yielded new evidence that both the carboxyl and amino portions of the TnI protein influence the myofilament response to Ca$^{2+}$. The carboxyl- and amino-terminal regions both appear to play a role in determining the myofilament contractile re-
Functional TnI Domains within the Intact Myofilament Defined by Isoforms and the Chimera—TnI chimera gene transfer and expression in adult cardiac myocytes was carried out in an effort to determine the regions of TnI that contribute to isoform-specific differences in Ca^{2+}-dependent contractile function. The results show that the TnI isoform-specific influence on Ca^{2+} sensitivity of myofilament tension is complex. The increase in Ca^{2+} sensitivity observed in myocytes expressing ssTnI relative to cTnI-containing control myocytes (Ref. 4; present study) along with the decrease in Ca^{2+} sensitivity measured in TnI chimera-expressing myocytes (Fig. 7, A and B) indicates that two regions of TnI influence myofilament Ca^{2+} sensitivity. These two regions of TnI are now incorporated into a working model of functional TnI domains (Fig. 10). An important domain influencing myofilament Ca^{2+} sensitivity lies in the carboxyl region of TnI and contributes to the different tension responses of cTnI- and ssTnI-expressing adult myocytes at submaximal Ca^{2+} concentrations. While localization of the TnI isoform-dependent effects on the Ca^{2+} sensitivity of tension has yet to be examined in more detail, TnI peptides/truncations have been used to examine TnI binding to other proteins and effects on actomyosin ATPase activity. In these biochemical studies, actin-Tm and TnC binding sites were localized in the carboxyl-terminal domain of TnI (9, 10, 31). Each TnI fragment/truncation also lacked at least some of the inhibitory properties of wild-type TnI in reconstitution assays, but it remains to be determined whether these peptides would similarly affect tension in the intact myofilament.

Studies presented here are the first to demonstrate that the amino portion of TnI influences submaximal Ca^{2+}-activated tension. This conclusion is based on the significant decrease in tension, relative to controls, observed at intermediate Ca^{2+} concentrations in TnI chimera-expressing myocytes (Fig. 7A), which contain the ssTnI amino acid sequence in this region. The decrease in submaximal Ca^{2+}-activated tension observed with the TnI chimera is concluded to be due to specific incorporation of this protein into the myofilament, because sarcomere architecture is maintained with TnI chimera expression (Fig. 6). This shift in the tension-Ca^{2+} relationship is likely to be physiologically important, since tension-Ca^{2+} shifts of lesser magnitude have been previously described in patients with hypertrophic cardiomyopathy (32). The submaximal tension response that is influenced by the amino terminus of TnI may involve long range interactions within TnI and/or interactions with other contractile proteins. In binding studies, amino-terminal fragments of TnI have been shown to interact in an antiparallel arrangement with TnC in a largely Ca^{2+}-independent manner and with troponin T (33–35). These interactions are thought to be functionally important, since actomyosin ATPase activity in the presence of amino-terminal TnI fragments is returned to a level comparable with the activity present with actin and myosin in the absence of Tm-Tn (31, 36). Alternatively, it is possible that conformational changes in the amino terminus of TnI could influence previously described carboxyl-terminal interactions with TnC and/or actin (34, 37). The relative importance of amino-terminal TnI interactions with other domains within TnI and/or with other troponin subunits in the intact myofilament requires further study.

TnI isoform expression additionally influenced the threshold and cooperativity of Ca^{2+}-activated tension, and this was first recognized when Ca^{2+}-activated tension was compared in cTnI- and ssTnI-expressing adult cardiac myocytes (4). In the present study, the threshold Ca^{2+} concentrations required for activation (Fig. 8) and myofilament cooperativity (Fig. 7) were similar for myocytes expressing cTnI and the TnI chimera and differed significantly from values observed in myocytes expressing...
ssTnI (present study). This finding is important, since cTnI and the TnI chimera share the same carboxyl-terminal 110 amino acids, while the ssTnI sequence differs in this region (Fig. 1).

Based on these results, it appears that TnI isoforms mediate their influence on Ca\textsuperscript{2+} threshold and myofilament cooperativity via the carboxyl region of TnI.

**Role of TnI in pH-induced Changes in Ca\textsuperscript{2+}-activated Tension of Cardiac Myocytes**—Alterations in Ca\textsuperscript{2+}-activated myofilament tension play a significant role in acidosis-induced changes in cardiac function during pathophysiological states, such as myocardial ischemia (7, 28). Gene transfer experiments demonstrated that TnI isoforms have a central role in the pH-sensitive response, since TnI isoform expression in adult myocytes influenced the acidosis-induced shift in myofilament Ca\textsuperscript{2+} sensitivity (Ref. 4; Fig. 9). The region(s) within TnI responsible for the isoform-specific acidosis-induced shift in myofilament Ca\textsuperscript{2+} sensitivity has until now remained undefined. The present results establish that acidic pH shifts myofilament Ca\textsuperscript{2+} sensitivity to the same extent in myocytes expressing cTnI or the TnI chimera (Fig. 9). Thus, pH sensitivity resides in the carboxyl portion of TnI, and this domain is now incorporated into Fig. 10.

The detailed molecular basis for TnI isoform-specific variations in pH sensitivity remain to be determined. Fluorescence labeling studies indicate that pH affects the interaction between TnI and TnC (38). Acidosis-induced changes in TnC fluorescence are influenced by TnI isoforms (39) and may depend on charge differences between TnI isoforms. Interestingly, three charge differences between cTnI and ssTnI are found in the carboxyl terminus, with ssTnI containing the more basic residues (amino acids 157, 164, 166 in cTnI; see Ref. 11). More charge differences between these two TnI isoforms lie upstream and flank the inhibitory peptide region of TnI (amino acids 124, 127, and 130 in cTnI). Clearly, further studies are needed to determine whether the differential shift in Ca\textsuperscript{2+} sensitivity observed with cTnI and ssTnI depends on charge differences between the two isoforms.

**TnI and the Three-state Model of Thin Filament Regulation**—The varying abilities of the TnI isoforms and the TnI chimera to act as allosteric inhibitors of Ca\textsuperscript{2+}-activated tension...
within the intact myofilament are important, since they would be expected to influence the thin filament activation state. Results obtained with the TnI variants used in the present study are perhaps best understood using the three-state thin filament model of Geeves and co-workers (40, 41). The primary states in this model are the following: 1) a blocked state in which the thin filament prevents myosin interaction with actin; 2) a closed state in which the thin filament allows weak interactions between myosin and actin; and 3) an open thin filament state in which strong, force-generating interactions develop between myosin and actin.

The reduced threshold for Ca\(^{2+}\)-activated tension, reduced cooperativity, and increased Ca\(^{2+}\) sensitivity observed in ssTnI- versus cTnI-expressing cardiac myocytes (Ref. 4; Figs. 7 and 8) provides evidence that Ca\(^{2+}\) binding to TnC disinhibits the thin filament more readily in the presence of ssTnI than cTnI or the TnI chimera. An explanation for this finding could be that the ssTnI may increase the probability that functional units along the thin filament are in the closed rather than the blocked state compared with myofilaments containing cTnI. An increased proportion of thin filament units in the closed instead of blocked state may decrease the Ca\(^{2+}\) threshold and increase the Ca\(^{2+}\) sensitivity of tension generation (41), as was observed in myocytes expressing ssTnI.

The effects of cTnI versus the TnI chimera on Ca\(^{2+}\)-activated tension also can be understood in the context of the three state model of thin filament activation. The similar effects of cTnI and chimeric TnI on Ca\(^{2+}\)-activation threshold, and cooperativity, relative to ssTnI may indicate that the carboxyl terminus of cTnI maintains a higher proportion of actin-Tm in the blocked versus closed state in the absence of Ca\(^{2+}\). An additional group of amino acids in the amino terminus of cTnI could modulate this influence, such that more thin filament functional units are shifted to the closed state in myocytes expressing cTnI relative to myocytes expressing the TnI chimera. A disadvantage of this model is that it does not incorporate both cross-bridge and Ca\(^{2+}\)-mediated activation of the myofilament, and cross-bridge-mediated activation may contribute to the functional changes observed with the different TnI proteins (16, 42).

In summary, gene transfer of TnI isoforms and for the first time a unique TnI chimera into adult cardiac myocytes has provided new insight into the isoform-specific functional domains of TnI that influence Ca\(^{2+}\) sensitivity, cooperativity, and pH sensitivity within the intact myofilament. Future studies using TnI chimeras can now focus on developing a detailed map of key domains involved in determining the functional properties of TnI within the intact myofilament of cardiac myocytes.

Acknowledgments—We thank Anne Murphy for cTnI and ssTnI cDNAs, Christina Addison and Frank Graham for shuttle plasmids, and Jack Ladenson for TnI antibody 2F6.6. We also appreciate helpful comments from Philip Wahr and Daniel Michele on earlier versions of this manuscript.

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