Myofilament Calcium Sensitivity and Cardiac Disease
Insights From Troponin I Isoforms and Mutants

Margaret V. Westfall, Andrea R. Borton, Faris P. Albayya, Joseph M. Metzger

Abstract—The heightened Ca\(^{2+}\) sensitivity of force found with hypertrophic cardiomyopathy (HCM)–associated mutant cardiac troponin I (cTnIR145G; R146G in rodents) has been postulated to be an underlying cause of hypertrophic growth and premature sudden death in humans and in animal models of the disease. Expression of slow skeletal TnI (ssTnI), a TnI isoform naturally expressed in developing heart, also increases myofilament Ca\(^{2+}\) sensitivity, yet its expression in transgenic mouse hearts is not associated with overt cardiac disease. Gene transfer of TnI isoforms or mutants into adult cardiac myocytes is used here to ascertain if expression levels or functional differences between HCM TnI and ssTnI could help explain these divergent organ-level effects. Results showed significantly reduced myofilament incorporation of cTnIR146G compared with ssTnI or wild-type cTnI. Despite differences in myofilament incorporation, ssTnI and cTnIR146G expression each resulted in enhanced myofilament tension in response to submaximal Ca\(^{2+}\) under physiological ionic conditions. Myofilament expression of an analogous HCM mutation in ssTnI (ssTnIR115G) did not further increase myofilament Ca\(^{2+}\) sensitivity of tension compared with ssTnI. In contrast, there was a divergent response under acidic pH conditions, a condition associated with the myocardial ischemia that often accompanies hypertrophic cardiomyopathy. The acidic pH-induced decrease in myofilament Ca\(^{2+}\) sensitivity was significantly greater in myocytes expressing cTnIR146G and ssTnIR115G compared with ssTnI. These results suggest that differences in pH sensitivities between wild-type ssTnI and mutant TnI proteins may be one factor in helping explain the divergent organ and organismal outcomes in TnI HCM- and ssTnI-expressing mice. (Circ Res. 2002;91:525-531.)

Key Words: troponin I ■ myofilament proteins ■ hypertrophic cardiomyopathy ■ heart

Familial hypertrophic cardiomyopathy (HCM) is an autosomal dominant disorder resulting from diverse mutations within thick and thin filament contractile proteins.\(^1\,^2\) Mutations within individual contractile proteins have been shown to cause primary alterations in myofilament Ca\(^{2+}\) sensitivity of force production in myocytes and in the hearts from animal models expressing these mutations.\(^3\) These changes in myofilament Ca\(^{2+}\) sensitivity are postulated to be important for the development of the clinical and pathological manifestations of HCM.\(^2\) However, the mechanism whereby a primary change in myofilament Ca\(^{2+}\) sensitivity leads to the compensatory or dysfunctional structure/function responses observed in the human disease state is not well understood.

The thin filament regulatory protein troponin I (TnI) is known to directly influence the myofilament’s response to Ca\(^{2+}\) activation. The human cTnIR145G mutation,\(^4\) and the analogous cTnIR146G mutation in transgenic mouse hearts,\(^6\) increases myocardial Ca\(^{2+}\) sensitivity of tension. In transgenic mice, expression of cTnIR146G causes myocyte hypertrophy, myofibrillar disarray, fibrosis, and premature death, outcomes attributed to the cTnIR146G.\(^6\) For comparison, the slow skeletal troponin I (ssTnI) isoform, normally expressed only during early cardiac development,\(^7\,^8\) also increases myofilament Ca\(^{2+}\) sensitivity of tension,\(^4\) yet ssTnI transgenic mice have apparent normal cardiac morphology and life span.\(^9\) These findings suggest that other factors, together with heightened Ca\(^{2+}\) sensitivity, must be associated with the cTnIR146G mutation to cause these divergent organ-level outcomes between ssTnI and R146G transgenic mice.

In the present study, Ca\(^{2+}\)-activated tension under physiological ionic conditions is measured in adult myocytes expressing ssTnI or cTnIR146G, for comparison to results previously reported in papillary muscles from transgenic mice.\(^6\) In addition, cTnIR146G expression levels in transgenic mice lacking detectable pathology do show evidence of myocardial hypoxia/ischemia.\(^6\) This appears significant, because myocardial ischemia has been documented in asymptomatic patients with HCM.\(^10\) Given that hypoxia/ischemia-mediated cellular acidosis is known to directly depress myocardial contractile function,\(^11\,\,13\) we tested the hypothesis that under acidic pH conditions, ssTnI and cTnIR146G would have divergent effects on adult cardiac myocyte contractile function. To test this hypothesis, the influence of acidic conditions on myofilament tension is compared in adult...
ventricular myocytes expressing cTnI, ssTnI, mutant cTnI, or mutant ssTnI after gene transfer. Our results provide evidence that the apparent paradox of heightened Ca\(^{2+}\) sensitivity in myofilaments with mutant TnI or wild-type ssTnI is at least in part explained by the differential pH-dependent change in tension observed in myofilaments expressing mutant TnI proteins compared with wild-type ssTnI.

**Materials and Methods**

**Mutagenesis Strategy and Generation of Adenoviral Vectors**

The rat cTnIR146G and rat ssTnIR115G mutants (Figure 1A) were constructed using a pGEMZ vector containing cTnI and ssTnI cDNAs (kind gifts of A. Murphy, MD, Johns Hopkins University, Baltimore, MD), respectively. The Strategene Quick Change site-directed mutagenesis kit\(^{14}\) was used to mutagenize pGEM-3ZcTnI and pGEM-3ZssTnI with oligonucleotide primers (cTnI primer 1: CGGGCCACTCTCGGC-GAGTGAATTCCTCAGCAG; cTnI primer 2: CTGCCTGAT-TTCTCACTCGGCCGGAGTGGGCCGCC; ssTnI primer 1: CGTCCACC-CCTCGGCCGGGTCCGTGCCTC; primer 2: GAGACACGGACC-CGGCCAGGTTGGAGC; A FLAG epitope (DYKKDDDDK; Sigma) was engineered into the carboxyl-terminus of cTnI cDNA by PCR mutagenesis, as described by Michele et al.\(^{19}\) The 187-bp BamII fragment of cTnIFLAG containing the FLAG epitope was ligated to a 594-bp COOH fragment of cTnIR146G to form cTnIR146GFLAG. Sequenced mutant TnI cDNA was subcloned into a shuttle vector\(^{16}\) to form pAdcTnIR146G, pAdssTnIR115G, pAdcTnIFLAG, and pAdcTnIR146GFLAG.

Recombinant replication-deficient adenovirus vectors were constructed by cotransfection of the shuttle vector containing the cDNA of interest with pJM17 in HEK 293 cells.\(^{4}\) Large preparations of each plaque-purified virus were separated on a CsCl 2 gradient, stored in \(80\;\degree\)C, and verified by Southern blot analysis. Titers obtained for each virus were on the order of \(10^10\) plaque-forming units/mL.

**Primary Cultures of Rat Ventricular Myocytes**

Calcium-tolerant adult ventricular myocytes were isolated as described earlier.\(^{17}\) Details of the isolation procedure and viral incubations can be found in the expanded Materials and Methods section in the online data supplement at http://www.circresaha.org.

**Analysis of Protein Expression**

Control and virus-treated cardiac myocytes cultured for 4 to 6 days were collected from each coverslip in sample buffer. Permeabilized myocytes were prepared by adding ice-cold relaxing solution (pH 7.0; see below) containing 0.1% Triton X-100 (TX-100) to myocytes for 1 minute, rinsing cells in relaxing solution lacking TX-100, and then collecting in sample buffer. Protein expression in intact and permeabilized myocytes was analyzed by Western blotting, as described by Westfall et al.\(^{18}\) using a 1:500 dilution of MAB 1691 (Chemicon), a monoclonal antibody recognizing all TnI isoforms, including cTnIFLAG and cTnIR146GFLAG. Sig- nificant lower expression of cTnIR146GFLAG, relative to cTnI-FLAG, was detected at 500 and 1000 MOI of indicated virus. Western blots were analyzed by incubating blots with MAB 1691 (1:500), a monoclonal Ab recognizing all TnI isoforms, including cTnIFLAG and cTnIR146GFLAG.

**Indirect Immunodetection of Mutant TnI Expression and Myofilament Incorporation**

Dual mAbs\(^{17,18}\) were used to determine the extent of filament remodeling resulting from mutant TnI expression within single cardiac myocytes in primary culture. To detect cTnIFLAG expression, the primary mAbs pairs used were the M2 anti-FLAG mAb (Sigma; 1:500) and MAB 1691 anti-TnI mAb (Chemicon; 1:500). Total replacement of cTnI with ssTnIR115G in myocytes was followed using the MAB 1691 and cTnI-specific TI-1 mAb,\(^{18}\) which does not recognize ssTnI. FLAG mAb and TI-1 mAb binding were each detected with a Texas Red–conjugated secondary goat anti-mouse Ab, whereas fluorescein isothiocyanate–conjugated goat anti-mouse Ab was used to detect MAB1691 binding. High-resolution images were obtained with a Noran OZ laser scanning
Measurement of Steady-State, Ca\(^{2+}\)-Activated Tension in Single Cardiac Myocytes at pH 7.0 and 6.2

Single rod-shaped cardiac myocytes, attached to micropipettes coated with silicone adhesive, were permeabilized in 0.2% Triton X-100 for 1 minute. Sarcomere length was set at 2.20 μm\(^{-1}\) and all tension measurements were performed at 15°C. Relaxing and activating solutions (pH 7.0 and pH 6.2) used for tension measurements contained 1 mmol/L free Mg\(^{2+}\), 4 mmol/L MgATP, 14.5 mmol/L creatine phosphate, 20 mmol/L imidazole, and sufficient KCl to yield an ionic strength of 180 mmol/L, as determined from the computer program by A. Fabiato.\(^{20}\) The pCa (−log[Ca\(^{2+}\)]) of relaxing solution was 9.0, whereas the pCa of maximal activation solution was 4.0. Steady-state, Ca\(^{2+}\)-activated isometric tension was measured at each pCa, as previously described in detail.\(^{19}\) Tension-pCa relationships were constructed by expressing tension (P) at various submaximal Ca\(^{2+}\) concentrations as a fraction of tension at maximal activation (P\(_{o}\) of each pCa 4.0). Every third activation was performed at pCa 4.0 to bracket submaximal Ca\(^{2+}\) activations for normalization of tension. The Marquardt-Levenberg nonlinear least squares fitting algorithm was used to derive values for the Hill coefficient (n\(_H\)) and Ca\(^{2+}\) required for half maximal activation (pCa\(_{1/2}\)) from the tension-pCa relationship, using the Hill equation (P = [Ca\(^{2+}\)]\(^n\)/([K\(^{+}\) + [Ca\(^{2+}\)]\(^n\)]), where P is the fraction of maximum tension (P\(_{o}\)), the pCa\(_{1/2}\) is used as an indicator of K, and n\(_H\) is the Hill coefficient.

Statistics

Values are expressed as mean±SEM. Grouped comparisons were performed using an analysis of variance and post hoc Student-Newman-Keuls multiple comparison test, with a value of P<0.05 considered significantly different.

Results

Expression and Myofilament Incorporation of cTnIR1446G

The extent of expression and myofilament incorporation of cTnIR145G in human myocardium\(^5\) and/or cTnIR1446G in transgenic mice\(^8\) is not known. Expression of cTnIR1446G in adult rat myocytes also could not be distinguished from endogenous cTnI by Western blot analysis (Figure 1B). Information about the degree of contractile protein expression and incorporation into the sarcomere is critical for subsequently establishing the specific effects of a particular isoform and/or mutation on the mechanical properties of myocytes. Thus, experiments initially focused on expression and myofilament incorporation of cTnIFLAG and cTnIR1446GFLAG in adult rat cardiac myocytes, to distinguish the epitope-tagged TnI proteins from endogenous cTnI. The differences in cTnIR145GFLAG replacement of cTnI remained unchanged 5 to 6 days after gene transfer of cTnIR1446G, cTnIFLAG, or cTnIR1446GFLAG compared with control values (see online Table, available in the data supplement at http://www.circresaha.org). Maintenance of contractile protein stoichiometry and isoform expression, and the lack of cytosolic accumulation, together support the idea that each delivered mutant is specifically incorporated into the contractile apparatus by stoichiometric replacement of endogenous cTnI.

Immunolabeling experiments were carried out to directly analyze myofilament incorporation of mutant and wild-type TnI proteins in adult myocytes. A striated pattern of immunostaining was observed in controls (Figure 2A) and myocytes expressing cTnIFLAG or cTnIR1446GFLAG (Figures 2C and 2E), using a mAb recognizing all isoforms of TnI. A striated immunolabeling profile was also observed with M2 anti-FLAG mAb in myocytes expressing cTnIFLAG and cTnIR1446GFLAG (Figures 2D and 2F), but not control myocytes (Figure 2B). The consistent striated labeling pattern observed across the length and depth of myocytes expressing cTnIFLAG and cTnIR1446GFLAG, and the absence of cytosolic accumulation of epitope-tagged protein further indicates there is specific incorporation of each mutant TnI protein into the myofilaments.

TnI Competition Assay

The differences in cTnIR1446GFLAG replacement of cTnI compared with ssTnI\(^{14}\) and cTnIFLAG (Figure 1B) raised the possibility that cTnIR1446G may not compete with equal effectiveness for sites within the myofilament. Cardiac myocytes exquisitely regulate total contractile protein.\(^{21}\) One potential therapeutic strategy for HCM mutations may be to competitively replace the human cTnIR145G mutation in the cardiac sarcomere via expression of wild-type cTnI. To test this idea in vitro, competition experiments were performed to determine whether vector-mediated cTnI expression could effectively out-compete cTnIR1446G expression/incorporation at the level of the sarcomere (Figure 3). Adult myocytes were cotransduced with a range of AdcTnIFLAG doses, along with cTnI or cTnIR1446G at a fixed titer. Western blot analysis indicated that cTnIFLAG expression is far lower in cTnI- compared with cTnIR1446G-expressing myocytes. Thus, normal cTnI appears to have an advantage over mutant TnI for expression at the level of the sarcomere, over a broad range of transcriptional activation.

Myofilament Ca\(^{2+}\)-Activated Tension in Myocytes Expressing cTnIR1446G and ssTnI

The functional relationship between Ca\(^{2+}\) and steady state isometric tension was directly assessed in single, permeabil-
ized myocytes after TnI gene transfer. The position of the tension-pCa curve (pH 7.0), as measured by the pCa50, was comparable in control and AdcTnI-treated myocytes (Figure 4A). In contrast, the cTnIR146G mutation significantly increased pCa50 relative to control values, in agreement with earlier work in transgenic mouse myocardium.6 Replacement of cTnI with ssTnI also increased myofilament Ca2+/H11001 sensitivity, as demonstrated previously,4 and this increase was similar in magnitude to the increase observed with cTnIR146G (Figure 4A). Thus, under normal physiological activating conditions, cTnIR146G and ssTnI each cause heightened myofilament Ca2+ sensitivity.

Myocardial ischemia-mediated acidosis has been observed in patients with HCM.22,23 Evidence of ischemia has been detected in asymptomatic patients10 and transgenic mice.6 However, the effect of intracellular acidification on the contractile responses of myocytes expressing cTnIR146G is unknown. Thus, the tension-pCa relationship under acidic conditions (pH 6.2) was determined in myocytes expressing ssTnI, cTnI, and cTnIR146G (Figure 4B). Myocytes expressing cTnI and cTnIR146G responded to acidic pH with a large decrease in the pCa50, whereas the pH response was markedly blunted in myocytes expressing ssTnI (Figure 4B). We propose this difference in myofilament pH sensitivity between myocytes expressing cTnIR146G and ssTnI (Figure 4C) has important implications for the cardiac response to ischemia in patients carrying the HCM mutation (see Discussion).

Replacement of Endogenous cTnI With ssTnI Containing the Mutation Analogous to cTnIR146G

Acidosis decreases force generation in myocytes expressing normal and mutant cTnI. Thus, a mutant ssTnI, ssTnIR115G, was generated to determine whether the mutation itself alters myofilament pH sensitivity. Before functional studies, mutant ssTnI expression and incorporation were examined. In contrast to epitope-tagged cTnIR146G, comparable expression of ssTnI and ssTnIR115G was observed in adult myocytes (Figure 5A). Total TnI was unchanged in myocytes expressing ssTnI or ssTnIR115G, relative to a silver-stained portion of the gel, TnT, or Tm detection on Western blots (see online Table). Wild-type ssTnI and ssTnIR115G also were expressed to comparable levels in intact and membrane-permeabilized myocytes (Figure 5A). In immunolabeling experiments, cTnI-specific mAb labeling disappeared with ssTnI or ssTnIR115G expression (Figure 5B, right panel), whereas positive labeling continued to be present using a nonisoform-specific anti-TnI mAb (Figure 5B, left panel). These results indicate that, in contrast to cTnIR146G, ssTnIR115G and wild-type ssTnI each replace endogenous myofilament cTnI with similar efficiency.

Ca2+-Activated Tension in Myocytes Expressing ssTnIR115G

Functional studies on permeabilized myocytes were performed to determine whether ssTnIR115G would influence
pCa₀ results were compared at each pH using a 1-way ANOVA followed by a post hoc Newman-Keuls test, with P<0.05 considered significantly different from control (−) or cTnl (+). Maximum tension decreased remarkably at pH 6.2 for each group of myocytes (results not shown), in agreement with earlier findings from cTnl- and ssTnl-expressing myocytes.14 C, Comparison of pCa₀ results in ssTnl vs cTnlR146G-expressing myocytes at pH 7.0 and pH 6.2. Results were compared by subtracting the average pCa₀ for myocytes expressing ssTnl from pCa₀ values obtained for myocytes expressing cTnlR146G. Statistical analysis was performed using a 1-way ANOVA and a post hoc Newman-Keuls test; *P<0.05 considered significantly different from the zero baseline. In B, P<0.05 considered significantly different from all other groups.

Ca²⁺-activated tension relative to ssTnl or cTnlR146G. Expression of cTnlR146G, ssTnl, or ssTnlR115G increased the pCa₀ to a similar extent (pCa₀: ssTnl 6.13±0.08, n=5; ssTnlR115G 6.06±0.04, n=8; cTnlR146G 6.15±0.06, n=10). This result indicates ssTnlR115G does not additionally increase the myofilament Ca²⁺ sensitivity produced with ssTnl alone, and likely works through a common mechanism. Unexpectedly, the normal “protective” effect of ssTnl on myofilament Ca²⁺ sensitivity at acidic pH was attenuated in myocytes expressing ssTnlR115G (Figure 5C; pCa₀ at pH 6.20=5.15±0.09, n=6) and was more similar to cTnlR146G (Figure 4B). Thus, regardless of Tnl isoform, the HCM-linked mutation greatly diminishes submaximal tension in response to acidic pH. The slope of the tension-pCa relationship measured from the Hill coefficient (nₕ) also was comparable in control, cTnlR146G-, and ssTnlR115G-expressing myocytes (nₕ: Control 2.53±0.32, n=8; cTnl 2.41±0.27, n=7; cTnlR146G 2.24±0.21, n=10; ssTnlR115G, 2.00±0.20, n=8), and these values were significantly greater than the slope observed in myocytes expressing ssTnl (nₕ 1.27±0.11, n=5; P<0.05). The comparable nₕ values in myocytes expressing ssTnlR115G and cTnlR146G provides evidence that the mutation influences myofilament cooperativity in an isoform-independent manner. Finally, maximum tension values in myocytes expressing cTnlR146G, ssTnl, or ssTnlR115G were unchanged relative to control values (in kN/m²: control 15.4±1.4, n=8; cTnl 17.5±3.4, n=7; cTnlR146G 17.9±3.4, n=8; ssTnl 17.1±2.6, n=4; ssTnlR115G 13.1±2.1, n=8).

Discussion

The heightened myofilament Ca²⁺ sensitivity caused by HCM-associated mutant sarcomeric proteins has been proposed to be a key element in the development of organ-level disease.3 The present study shows that under physiological activating conditions, ssTnl and cTnlR146G similarly enhance myofilament Ca²⁺ sensitivity of tension in adult cardiac myocytes. This presents a paradox whereby both ssTnl and cTnlR146G heighten Ca²⁺ sensitivity, although only cTnlR146G leads to organ-level pathology and premature death in mice.6,9 We further show that myofilament pH sensitivity is an additional factor that, together with enhanced myofilament Ca²⁺ sensitivity, may be important for the development of cardiac dysfunction caused by the HCM-associated cTnlR146G mutation. Specifically, under acidic pH conditions, enhancement of calcium sensitivity of contraction is lost in cTnlR146G compared with ssTnl (Figure 4C).

Working Model: Progression From Enhanced Myofilament Ca²⁺ Sensitivity to Cardiac Dysfunction

The development of ventricular arrhythmia resulting from myocyte disarray is often assumed to be the primary entity involved in the premature deaths in HCM patients.24 However, multiple clinical studies have pointed to the possibility that localized or regional myocardial ischemia could also be a factor in the development of cardiac dysfunction, arrhythmia, and/or death.10,23,25 In support of this idea is the recent finding of myocytes with evidence of hypoxia in cTnlR146G mouse lines where hearts did not yet exhibit overt pathology.6 A model incorporating divergent myofilament pH sensitivity in conjunction with the enhanced myofilament Ca²⁺ sensitivity in cTnlR146G- versus ssTnl-expressing myocytes is proposed below. Our working hypothesis is that enhanced myofilament Ca²⁺ sensitivity could work in parallel with greater pH sensitivity to cause progressive changes in myocardium-expressing cTnlR146G, but not ssTnl. The shared heightened myofilament Ca²⁺ sensitivity of tension in myocytes expressing ssTnl or cTnlR146G will cause a slight slowing of myocardial relaxation, as demonstrated in transgenic mice expressing either Tnl protein.6,9 This relaxation delay may only lead to subtle changes in working myocardium to compensate for delayed relaxation, including remodeling of the sarcomere or Ca²⁺ handling protein content or function1 and/or alterations in the adrenergic signaling response.26,27 Short intervals of mild local ischemia resulting from myocardial bridging (eg, a band of overlying muscle that can result in systolic compression of a coronary artery23), exercise, and/or stress22,23 within myocytes expressing cTnlR146G or ssTnl would initiate the divergent pathway leading to dysfunction in cTnlR146G-expressing myocardium. Specifically, the acidosis accompanying ischemia is predicted to decrease cardiac function to a greater extent in
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both sets of myocytes labeled with the MAB 1691 Ab, which indicates

that nascent TnI protein is replacing endogenous cTnI. Bar

detecting with TI-1 mAb is present in myocytes expressing cTnI but is

detected with secondary Ab conjugated to FITC; Right panels,

myocytes expressing cTnI (top) and ssTnIR115G (bottom). Left panels,

within the cytosol. B, Representative confocal projection images of

that exogenous contractile protein does not detectably accumulate

during cardiac ischemia.11,13 The working model does not

attempt to incorporate the important connection between

hypertrophy and mortality, due to the complexity of this

relationship. Most importantly, the possible role of other

factors in addition to acidic pH, such as altered crossbridge

kinetics and/or cellular signaling,26,28 may require consider-

ation as this model is refined in the future.

Implications of TnI Competition Assay
Our results indicate there is diminished ability of cTnIR146G to
replace endogenous cTnl within the sarcomere. In trans-
genic mice, cTnIR146G mRNA increased 2- to 10-fold,6 and thus, the reduced presence of cTnIR146G within the sarco-

mere is presumably due to a reduced ability of mutant cTnI to
incorporate in myofilaments relative to wild-type cTnI. In the

TnI competitive assay (Figure 3), titration of recombinant

vectors containing wild-type and mutant TnIs supports this

interpretation. This information may be useful in developing

gene or protein-based therapeutic strategies for HCM. A

possible explanation for the reduced myofilament incorpora-
tion of cTnIR146G relative to the other TnI proteins (Figures

1 and 5) is the presence of subtle conformational differences

of the mutant TnI that influence the affinity of this protein for

the myofilament binding sites. Previously, cTnI and

cTnIR146G binding to immobilized TnC were reportedly not
different,29 although the use of Tn concentrations associated

with maximal TnC binding may have obscured differences in

affinity. Regardless of the mechanism involved in reducing

myofilament incorporation of this mutant TnI, our results

indicate there is a potential treatment strategy available to

minimize incorporation of mutant TnI in the sarcomere. Experimen-
tal approaches could involve gene or protein-based

delivery/expression of normal TnI and/or partial suppression

of the mutant allele. In addition, abolishing the pH sensitivity

of cTnI through gene transfer may prevent maladaptive

hypertrophy associated with mutations in different contractile

proteins.

Molecular Switch Functions of TnI
The R145G mutation in human cTnI lies within the inhibitory
peptide (IP; Figure 1A), a region postulated to act as an

important molecular switch within TnI that toggles from actin
to troponin C in the presence of Ca\textsuperscript{2+}.30 The IP region

is highly conserved among TnI isoforms, with a single substi-
tution of Pro in ssTnI for Thr in cTnI at codon 144 in the rat

sequence (Figure 1A). Previous studies on the IP region

indicated this proline substitution does not change Ca\textsuperscript{2+}-acti-

vated force properties.31 In contrast, functional results ob-
tained in the present study indicate a single amino acid

substitution (R → G), with a net decrease in positive charge, is

sufficient to change myofilament Ca\textsuperscript{2+} sensitivity of tension

(Figure 3). The heightened acidic pH response observed with
cTnIR146G and ssTnIR115G, relative to ssTnI (Figures 3 and
4), indicates loss of positive charge at this residue also may

influence myofilament pH sensitivity in an isoform-
independent manner. Other amino acid differences between cTnI and ssTnI may be involved in producing a similar change in myofilament Ca\(^{2+}\) and pH sensitivities of tension. The similar Ca\(^{2+}\) sensitivity and enhanced pH sensitivity observed in myocytes expressing ssTnIR115G or cTnIR146G relative to ssTnI-expressing myocytes support this view.

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