Cardiac Transgenic and Gene Transfer Strategies Converge to Support an Important Role for Troponin I in Regulating Relaxation in Cardiac Myocytes

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Abstract—Elucidating the relative roles of cardiac troponin I (cTnI) and phospholamban (PLN) in β-adrenergic–mediated hastening of cardiac relaxation has been challenging and controversial. To test the hypothesis that β-adrenergic phosphorylation of cTnI has a prominent role in accelerating cardiac myocyte relaxation performance we used transgenic (Tg) mice bearing near complete replacement of native cTnI with a β-adrenergic phospho-mimetic of cTnI whereby tandem serine codons 23/24 were converted to aspartic acids (cTnI S23/24D). Adult cardiac myocytes were isolated and contractility determined at physiological temperature under unloaded and loaded conditions using micro-carbon fibers. At baseline, cTnI S23/24D myocytes had significantly faster relaxation times relative to controls, and isoproterenol stimulation (Iso) had only a small effect to further speed relaxation in cTnI S23/24D myocytes (delta Iso: 7.2 ms) relative to the maximum Iso effect (31.2 ms) in control. The Ca²⁺ transient decay rate was similarly accelerated by Iso in Tg and nontransgenic (Ntg) myocytes. Gene transfer of cTnI S23/24D to myocytes in primary culture showed comparable findings. Gene transfer of cTnI with both serines 23/24 converted to alanines (cTnI S23/24A), or gene transfer of slow skeletal TnI, both of which lack PKA phosphorylation sites, significantly blunted Iso-mediated enhanced relaxation compared with controls. Gene transfer of wild-type cTnI had no effect on relaxation. These findings support a key role of cTnI in myocyte relaxation and highlight a direct contribution of the myofilaments in modulating the dynamics of myocardial performance. (Circ Res. 2007;101:377-386.)

Key Words: contraction calcium heart

β-adrenergic stimulation of the heart causes a dramatic increase in heart performance.¹ This represents a vital mechanism of the mammalian “fight or flight” response wherein modulation of heart performance responds on a beat-to-beat basis to variations in physiological demands. During β-adrenergic stimulation heart rate (chronotropy), pressure development (inotropy) and muscle relaxation rate (lusitropy) are all increased. To accomplish a marked increase in cardiac output in the face of reduced time for ventricular filling, the speed of myocardial relaxation must increase.

The molecular determinants of enhanced cardiac muscle relaxation during β-adrenergic stimulation are important to define and are still being debated. Downstream of β-adrenergic receptor stimulation and subsequent activation of protein kinase A (PKA) are several cardiac phosphoproteins that have been implicated in hastening relaxation.²,³ Phospholamban (PLN) is known to regulate the activity of the SERCA2a calcium ATPase pump on the sarcoplasmic reticulum (SR), such that pump activity is increased on phosphorylation of PLN leading to faster sequestration of calcium into the SR.²,⁴ It is also well known that PKA phosphorylates cTnI in the sarcomere.²,⁵ Troponin I is a sarcomeric protein that is essential for regulating myofilament function.⁶,⁷ PKA-mediated cTnI phosphorylation causes a desensitization of the contractile apparatus to activation by calcium⁷ which, in turn, is hypothesized to facilitate faster muscle relaxation rates because the thin filament is inactivating more readily.⁸

Broadly stated, there are 2 prevailing viewpoints regarding the molecular determinants of cardiac lusitropy during β-adrenergic stimulation. Studies centered on the PLN knockout (PLNKO) mouse conclude that PLN phosphorylation plays virtually the entire role, whereas cTnI phosphorylation has no role or at best a minor role in β-adrenergic–mediated increased relaxation rates.⁹–¹² A differing view is that cTnI and PLN are both important in determining relaxation function.¹³,¹⁴ Resolution of this controversy is important because it will facilitate the development and rational design of potential therapeutic strategies designed to improve relaxation function in disease, including diastolic heart failure.

While acknowledging the wealth of vital insights gained from the PLNKO mouse, it can be asked whether results obtained in the context of the PLN null background can
directly translate to the normal PLN expressing heart. The starting conditions for both TnI and PLN are likely critical in evaluating the modulation of myocardial relaxation performance. For example, TnI phosphate content is already significant at baseline in the PLNKO mouse,11 which has implications for assessing the physiological effects of the full dynamic range of TnI phosphate content.

We hypothesize that the best experimental context to evaluate the relative roles of cTnI and PLN in defining β-adrenergic–mediated increased relaxation rates is the intact myocyte with both PLN and troponin present. Accordingly, in the present work, we used 2 complementary genetic approaches, transgenics in mice and gene transfer in rat adult cardiac myocytes, to engineer in the sarcomere either a PKA nonphosphorylatable TnI or a PKA phospho-mimetic of cTnI. Single cardiac myocyte contractility was determined at physiological temperature and in the absence and presence of physiological loading using micro-carbon fiber technology. Results from both experimental approaches converge to indicate an important role for cTnI in regulating cardiac myocyte relaxation performance.

Materials and Methods

Animals

The process of generating Tg mice as well as the molecular analysis and comparison between Tg and nontransgenic (Ntg) mice was previously reported.15 For gene transfer studies we used adult Sprague-Dawley rats (Harlan, Indianapolis, Ind).

Recombinant Adenovirus Construction and Adult Cardiac Myocyte Isolation/Primary Culture

The procedures used in this study were approved by the University of Michigan Committee on the Use and Care of Animals. Veterinary care was provided by the University of Michigan Unit for Laboratory Animal Medicine, and the animal care use program conforms to the standards of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 86-23). The methods for adenoviral construction16,17 and adult cardiac myocyte isolation were as described elsewhere16,18 and in the supplemental materials (available online at http://circres.ahajournals.org). Mouse cardiac myocytes from Ntg and Tg were plated on cover slips and used in experiments to measure twitch kinetics and calcium transients. The myocytes used for Western blot analysis were cultured for 4 days, as previously described.17 Western blot experiments to detect exogenous cTnI expression were performed as described previously17,19,20 and detailed in the online supplement.

Measurement of Single Cardiac Myocyte Twitch Force

The experimental system for measurement of length and tension (at 37°C) was developed with modifications from a previously reported method using micro-carbon fibers.16 Both ends of a rod-shaped myocyte were firmly held with a pair of carbon fibers which in turn were connected to glass rods. One rod was connected to a sensitive force transducer system (50 μN/volt, Aurora Scientific Inc.), and the other was connected to a piezoelectric transducer (P-173, PI Polytect) to control myocyte length. The carbon fibers used in this study were rigid (diameter, 40 μm; compliance, 0.02 m/N). Force signal from the force transducer was converted to digital data and recorded at 1000 Hz sampling rate with an analog-to-digital recording system (Accura 100, Nicolet). The video image of a cardiac myocyte with carbon fibers attached at both ends was used to measure sarcomere length. Sarcomere length was adjusted to 1.80 to 1.85 μm by manipulating the piezoelectric transducer.18 Calcium kinetics as described in online supplement.

Membrane Permeabilized Cardiac Myocyte Tension-pCa

Protocols were as previously detailed for contractile studies on permeabilized myocytes with and without PKA.21

Statistical Analysis

All data are presented as mean±SEM, and analyzed using Student t test to determine the difference between mean values of Ntg and Tg. To assess the difference among gene transfer groups (cTnI, ssTnI, cTnI S23/24A, and cTnI S23/24D), One-way ANOVA tests were performed with a Newman-Keuls multiple comparisons post-test.

Results

Transgenic Studies

Calcium Sensitivity of Tension in Cardiac Myocytes From cTnI S23/24D Mice

We used cTnI S23/24D transgenic (Tg) mice that have ≥95% stoichiometric replacement of native cTnI.15 In Tg animals, the relative phosphorylation states of the ryosine receptor, phospholamban, the myosin light chains, and voltage-dependent Ca2+ channel were not altered from control.15 Independently, previously it was shown that MyBP-C phosphate status was not altered in cTnIS23/24D Tg mice.22 In agreement, Pro-Q Diamond staining, when adjusted for loading, showed comparable phosphoprotein detection in Tg and Ntg mice (Figure 1). Figure 1 shows representative tension-pCa relationships from cTnI S23/24D and control cardiac myocytes before and after exposure to PKA. Before PKA treatment, the tension-pCa plot of Tg myocytes was shifted rightward relative to nontransgenic (Ntg) myocytes, with the Tg group showing significantly lower pCa50 than the Ntg group (Figure 1B). In the presence of PKA catalytic subunit, the pCa50 of the Ntg was significantly reduced in agreement with previous work (Delta pCa50=0.21±0.03, P<0.01)3,7,23; however, there was no significant effect of PKA on Tg myocytes (Delta pCa50=0.06±0.035, ns) such that the PKA-treated Ntg and Tg tension-pCa data converged and their pCa50s similar (P>0.05).

Unloaded Shortening of Single Cardiac Myocytes From cTnI S23/24D Mice

We recorded sarcomere length change during single twitch contraction of membrane intact cardiac myocytes under unloaded conditions at physiological temperature with and without β-adrenergic stimulation (Figure 2A and 2B). Peak fractional sarcomere shortening, calculated as a percentage of baseline sarcomere length, was similar between Tg and Ntg myocytes without isoproterenol (Iso) (%FS, 9.9±0.5 versus 9.7±0.6, Tg versus Ntg statistically no difference, n=17 cells each; Figure 2C). Importantly, at baseline conditions, the Tg myocytes showed markedly faster relaxation kinetics than Ntg myocytes at 37°C (time to 75% relaxation [T0.75R], 44.0±3.3 versus 68.5±5.6 ms, P<0.01, n=17 each; Figure 2D). Treatment with 50 nmol/L Iso similarly increased myocyte contraction (SL amplitude) in Tg and Ntg myocytes (%FS, 15.4±0.5 versus 16.1±0.9, not statistically different, n=17; Figure 2C). In the presence of Iso, there was a greater magnitude increase in relaxation rate in Ntg versus Tg myocytes, such that baseline differences between groups were eliminated (T0.75R, 36.8±1.8 versus 39.0±1.5 ms,
statistically no difference, n=17; Figure 2D; additional relaxation data in supplemental Figure I). As shown in Figure 3, there was no significant difference in the Ca\textsuperscript{2+} transient peak or in kinetics as indicated by Fura-2 ratios between Tg and Ntg cardiac myocytes in the absence (time to 50% decay [T0.5d], 97±7.1 versus 106.9±7.6 ms; tau, 129±14 versus 143±11.8 ms) or presence of Iso (T0.5d, 73.7±4.9 versus 64.5±4.6 ms; tau, 72.5±7.5 versus 79.1±6.6 ms).

**Isometric Twitch Tension of Single Cardiac Myocytes**

Micro-carbon fiber system\textsuperscript{18} was used to attach and record isometric twitch tension in membrane intact adult single cardiac myocytes. In these experiments sarcomere length set at 1.80 to 1.85 μm. Peak amplitude of isometric twitch tension development was similar between the Tg and Ntg groups (4.6±0.4 versus 4.4±0.3 mN/mm\textsuperscript{2}, n=8 each; Figure 4). As with unloaded myocytes, under baseline conditions the Tg myocytes showed faster decay in isometric twitch tension relative to controls (T0.75R, 41.1±0.27 versus 45.9±1.2 ms, P<0.01, n=8). Treatment with 50 nmol/L Iso increased peak tension development of Tg and Ntg myocytes to a similar extent (7.1±0.3 versus 6.9±0.3 mN/mm\textsuperscript{2}, n=8 each), and eliminated the difference in tension decay between the 2 groups (T0.75R, 39.5±0.3 versus 39.6±0.4 ms, statistically no difference, n=8; Figure 4; additional relaxation data in supplemental Figure II).

**Gene Transfer Studies**

**TnI Phospho-Mimetics and ssTnI**

To estimate the extent of cTnI replacement in TnI gene transfer experiments we engineered a FLAG epitope into wild-type\textsuperscript{24} and serine mutant cTnIs. Western blot analysis showed evidence of stoichiometric replacement of endogenous cTnI by vector-derived cTnI FLAG, cTnI S23/24A FLAG, cTnI S23/24D FLAG, and ssTnI at day 4 after gene transfer (Figure 5). Replacement ratios at this time point after gene transfer were approximately 50% for cTnI FLAG, cTnI S23/24A FLAG, and cTnI S23/24D FLAG, and 70% (ssTnI). These data are consistent with previous studies showing stoichiometric replacement of TnI with TnI isoforms, mut- ants, and chimeras.\textsuperscript{17,19,20,24}

**Ca\textsuperscript{2+} Sensitivity of Tension in Permeabilized Myocytes**

Representative tension-pCa curves for cTnI S23/24A, cTnI 23/24D, and cTnI wild-type gene transfer myocytes are shown in Figure 6A. Membrane permeabilized single cardiac myocytes expressing cTnI S23/24D shifted rightward compared with cTnI controls. Tension-pCa plots were similar in cTnI and cTnI S23/24A myocytes. Cardiac myocytes expressing cTnI S23/24D showed significantly lower pCa\textsubscript{50} and maximum isometric tension than either cTnI or S23/24A groups (Figure 6B and 6C). The magnitude shift in pCa\textsubscript{50} by S23/24D is comparable to that seen in control myocytes after PKA treatment in control myocytes (Figure 1). Neither epitope Flag-tagged cTnI nor cTnI wild-type had significant effects on contraction in agreement with previously collected data.\textsuperscript{20,24}
Relaxation Performance in cTnI Mutant or ssTnI Expressing Myocytes

We recorded unloaded sarcomere shortening dynamics (Figure 7A and 7B), and compared time to 75% relaxation from peak shortening of unloaded single cardiac myocytes expressing cTnI, cTnI S23/24A, cTnI S23/24D, and ssTnI, 4 days after gene transfer (Figure 7C). In the absence of Iso, the ssTnI group showed significantly slower relaxation than the cTnI control group (128.9 ± 5.4 versus 75.4 ± 4.0 ms, ssTnI versus cTnI, P < 0.05), whereas the cTnI S23/24D group showed significantly faster relaxation (61.3 ± 4.1 ms) than the cTnI group (P < 0.05). The cTnI S23/24A group trended toward slower relaxation (84.4 ± 6.2 ms) than the cTnI group (P > 0.05). With 50 nmol/L Iso, the ssTnI (107.5 ± 9.3 ms) and cTnI S23/24D (71.5 ± 4.5 ms) groups were accelerated in relaxation but the magnitude change (delta) was significantly less than in cTnI controls (50.0 ± 1.8 ms). In companion studies, Iso similarly increased the intracellular Ca²⁺ decay rate in control and S23/24A myocytes (Figure 7), analogous to results in S23/23D Tg myocytes (Figure 3).

Estimating Relative Contributions of TnI and PLN to Enhance Relaxation Performance

Plots of the time to 75% relaxation in the absence and presence of Iso in control and genetically engineered cardiac myocytes are shown in Figure 8A and 8B. We followed the analytical formalism used previously by Bers and colleagues from their studies estimating the relative contributions of TnI and PLN to speed relaxation in PLN-deficient mice. Accordingly, we used results ± Iso to establish upper and lower limits on myocyte relaxation rates to calculate the maximum change for all groups tested (delta max). The change in relaxation rate with Iso in each group (delta Iso) was then divided by delta max for that set of experiments (eg, Tg loaded or Tg unloaded) to give the percentage of relaxation attributable to PLN. This percent taken from 100% gives the percent attributable to TnI. For example, in the unloaded Tg myocyte study results for the upper (68 ms; Ntg value) and lower (36.8 ms; Tg value) limits gave a delta maximum of 31.2 ms (delta max). The delta Iso for cTnI S23/24D myocytes was 7.2 ms (44 to 36.8 ms) divided by the delta max of 31.2 ms (delta max) gives 23.1% of relaxation due to PLN with 76.9% due to TnI (Figure 8C).

Discussion

This study used 2 complementary genetic strategies, transgenesis and acute gene transfer, to provide new insights into the mechanistic basis of myocardial relaxation during β-adrenergic stimulation. Using membrane intact adult cardiac myocytes studied at physiological temperature with or without physiological loading, TnI transgenesis and acute genetic engineering studies converged to support a key role of...
cTnI in conferring Iso-mediated enhanced cardiac relaxation performance (Figure 8C). The overarching premise of these studies is that if one assumes a dynamic and tightly coupled interplay between SR-based Ca\(^{2+}\) uptake and the myofilament-based effectors of contractility,\(^{25}\) then it should be possible to modify each component independently to either hasten or slow relaxation performance. We report a slowing of relaxation by gene transfer of non-PKA phosphorylatable TnIs (ssTnI or cTnI S23/24A), and a marked acceleration of relaxation by PKA phospho-mimetic cTnI S23/24/D. The greater acceleration of relaxation by cTnI 23/24D in Tg myocytes compared with gene transfer of cTnI 23/24D may be explained by the greater stoichiometric replacement of cTnI in the Tg mouse model (\(95\%\)) relative to adenoviral gene transfer (\(50\%\)). This TnI dose-response effect gains support from the present ssTnI gene transfer results where greater replacement of endogenous cTnI by ssTnI is achieved and a greater relative contribution of TnI is obtained. In transgenic cTnI S23/24D single living myocytes, relaxation rate increased markedly, and was 76.9% of the total possible increase in relaxation relative to the Iso stimulated value in controls.

Mechanistically, modifications in TnI to alter thin filament regulation likely account in part for these findings. Thus, ssTnI myocytes have a primary effect to increase force output at submaximal [Ca\(^{2+}\)]s and delay inactivation of the thin filament to slow relaxation. In comparison, cTnI S23/24D decreases force at submaximal [Ca\(^{2+}\)]s and accelerates thin filament inactivation and or cross-bridge detachment\(^{23,26}\) to directly speed relaxation. One caveat to this assessment is that cTnI S23/24A myocytes had no change in Ca\(^{2+}\) sensitivity of tension or in relaxation performance at baseline conditions thus serving as a non-PKA phosphorylatable variant of wild-type TnI. Interestingly, the cTnI S23/24A myocytes had a significantly blunted speeding of relaxation by Iso even though the Ca\(^{2+}\) decay rate was accelerated similar to control (Figure 7). This finding further supports a key role of TnI in Iso-mediated enhanced relaxation performance in cardiac myocytes.

**Comparison to Earlier Work**

Since the seminal discovery by Kranias and Solaro\(^{2}\) that cTnI and PLN are phosphorylated and temporally coupled to enhanced myocardial performance during adrenergic stimulation there has been great and continued interest in dissecting the relative roles of these proteins in conferring relaxation performance in cardiac muscle. In 1994, elegant genetic
Ablation strategies gave rise to mice completely lacking PLN. These mice had markedly hastened myocardial relaxation which was initially reported to not be further accelerated during β-adrenergic stimulation. Subsequent in vivo studies on these animals did report some further but small enhanced relaxation performance, presumably attributable to cTnI phosphorylation. Unloaded isolated muscles and myocytes from PLNKO mice showed little to no effect of isoproterenol to affect the already rapid relaxation. Under isometric loading conditions, PLNKO papillary muscle had only a ~17% increase in relaxation. Collectively, results from the PLNKO mice have been interpreted to indicate that PLN has a dominant effect and cTnI no effect, or at best only a minor effect, on relaxation performance. One issue to consider in interpreting these experiments is that, in PLNKO mice, TnI was already significantly phosphorylated so that the full dynamic range of TnI’s potential effects on relaxation would be masked. Thus, the fractional contribution of PLN and TnI to relaxation rate will surely depend on the initial conditions.

The question can also be posed whether results from PLNKO mice are informative in helping understand the basis of relaxation in normal hearts where both PLN and TnI are present. For example, in a converse experiment where tropinin is disabled, tension develops in the absence of activating [Ca²⁺]. Gene ablation studies confirm that TnI, at least in some form, is absolutely required to sustain life of the animal. Although these studies indicate an indispensable role of TnI in striated muscle, they cannot be taken as evidence of the relative importance of TnI and PLN in setting relaxation in the context of normal heart performance. Other experiments have used transgenesis of ssTnI to replace endogenous cTnI. These animals survive, although they have diastolic dysfunction, owing to significant slowing of relaxation by this Ca²⁺-sensitizing ssTnI molecule. Studies on ssTnI mice ranging from isolated cardiac myocytes in vitro to whole heart and in vivo report a significant contribution of TnI in underlying β-adrenergic-mediated relaxation. In these studies, evidence is provided that PKA-mediated phosphorylation of PLN is intact, thus further supporting a direct role for TnI in governing relaxation performance. More recently, Tg mice were generated bearing a modified cTnI where PKA target sequence serines 23 and 24 were mutated to aspartic acid residues as a chemical mimetic of phosphate incorporation. Organ-level and whole animal studies on these mice, independently generated in 2 separate laboratories, provide evidence that phospho-mimetic cTnI directly increases relaxation performance. These were the first studies performed on intact cardiac muscle tissue and at the whole organ level to demonstrate modified cTnI to accelerate relaxation and thus support a role for TnI in conferring, at least in part, relaxation performance of the heart.

Figure 4. Micro-carbon fiber-based recording of isometric twitch tension in single cardiac myocytes from S23/24D mice. A and B, Representative traces of isometric twitch tension in membrane intact single cardiac myocytes from Ntg and Tg mice in the presence and absence of 50 nmol/L isoproterenol. In panel B, these representative tension traces have been normalized to their peak value (set at 1.0), and drawn to show only the relaxation portion of the trace (time set at 0 at peak tension) to highlight relaxation performance in Ntg and Tg myocytes. Summaries of peak isometric twitch tension (C) and time to 75% tension decay from peak tension (D) for Ntg and Tg cardiac myocytes. Values are mean±SEM, n=8 per group. *Significantly different between Ntg and Tg, P<0.05.

Figure 5. Western blot analysis of TnI expression in adult cardiac myocytes after cTnI S23/24D, cTnI S23/24A, cTnI FLAG, and ssTnI gene transfer. The proteins were analyzed from the cultured myocytes 4 days after gene transfer.
A vital role of TnI and the myofilaments in defining relaxation performance is also supported by physiological and mathematical modeling studies. Jannsen et al. used phase-plane analysis of tension and Ca\(^{2+}\) in thin trabeculae studied at physiological temperature. Physiological pacing revealed Ca\(^{2+}\) decay was more rapid than that of force, translating to a leftward shift in the phase plane with respect to the steady-state tension-Ca\(^{2+}\) curve. This is consistent with the hypothesis that the myofilaments represent the slowest step in excitation-contraction coupling governing relaxation in normal heart muscle. Detailed mathematical models have been developed and similarly point to the important role the myofilaments play in relaxation performance. Factorial analysis pointed to Ca\(^{2+}\) unbinding from troponin C as a significant determinant of cardiac relaxation. This derives significance from biochemical studies showing the PKA significantly accelerates the unbinding of Ca\(^{2+}\) from troponin C. Collectively, these differing approaches point to an important role of the sarcomere and phosphorylation of TnI in determining cardiac relaxation performance.

Our present results do not exclude the possible roles of other sarcomeric proteins participating in \(\beta\)-adrenergic stimulation-mediated faster relaxation. The sarcomeric thick filament protein myosin binding protein C (MyBP-C) is phosphorylated by PKA and therefore could play a role in modulating contraction. The well-documented PKA-mediated decrease in myofilament Ca\(^{2+}\) sensitivity is fully negated in cardiac myocytes after gene transfer of the ssTnI, a nonphosphorylated substrate for PKA. This is evidence that MyBP-C phosphorylation does not impact this aspect of thin filament regulation thought to be vital for modifying relaxation performance. In ssTnI Tg mice that have normal MyBP-C phosphorylation in response to PKA, relaxation rates of permeabilized and membrane intact cardiac preparations were not affected by PKA or isoproterenol, respectively. This is evidence that TnI phosphorylation, not MyBP-C phosphorylation, is responsible for enhancing the intrinsic relaxation rate of cardiac myofilaments.

**Physiological Relevance**

Diastolic heart failure is an important clinical syndrome defined by poor relaxation performance of the heart. It has been estimated that \(\approx 40\%\) of heart failure patients have isolated diastolic failure with normal systolic performance. The absence of therapeutic strategies that directly target heart relaxation performance underscores the significance of defining components of excitation-contraction coupling that control myocardial relaxation. In human heart disease, PLN content and the SERCA2a/PLN ratio can be markedly altered. In attempting to define the relative contributions of PLN and cTnI to relaxation it is evident that the starting conditions for both PLN and TnI, both in terms of content/phosphorylation status, are important. Based on PLNKO animals and the present findings it appears that TnI plays a vital role in the normal myocyte expressing both SERCA2a/PLN, whereas PLN plays a dominant role in the context of the genetically engineered PLN-deficient (and TnI replete) heart that has unfettered SERCA2a function. Thus, experiments performed in the absence of PLN, and with normal TnI, shift...
the relative importance of cTnI–PLN toward PLN as Ca\(^{2+}\) is sequestered more rapidly and the thin filament regulatory system responds appropriately by inactivating actin-myosin interactions. This is not merely an academic discussion, as human patients have recently been identified as functional PLN nulls.\(^{32}\) Humans that are functional nulls for PLN perform poorly and transition into heart failure and premature death. In other forms of heart failure the stoichiometry of SERCA2a/PLN is altered.\(^{31}\) An important unanswered question becomes: what is the relative importance of cTnI and PLN when SERCA2a/PLN ratios are altered in human disease? Based on the present results, and those from PLNKO studies, it would seem that as PLN content is reduced, thus relieving SERCA2a inhibition, the relative contribution of PLN-cTnI shifts to PLN. This discussion assumes, however, that TnI phosphate content is low and not changed, an
assumption that would need to be confirmed experimentally. In otherwise normal myocardium, we hypothesize there is a tight integration between Ca$^{2+}$ handling and the myofilaments and therefore conclude that cTnI and PLN are both vital regulators of relaxation and together they “tune” overall relaxation performance in the heart.

**Figure 8.** Estimate of the relative contributions of TnI and PLN to β-adrenergic-mediated faster relaxation. In all plots, green circles represent baseline 75% relaxation time with solid line representing magnitude of Iso-mediated improved 75% relaxation times (red circle). A, Transgenic studies using unloaded myocytes and loaded myocyte contraction. Dotted line in S23/24D group indicates direct TnI-mediated faster relaxation (ie, TnI contribution), and solid line connecting green and red circles indicates PLN contribution. B, Gene transfer studies using cTnI, cTnI S23/24D, cTnI S23/24A, and ssTnI. All gene transfer results are on unloaded myocytes. C, Summary of estimated cTnI and PLN contribution to relaxation. Left hatched bar indicates cTnI control, comprised of TnI + PLN effect = 100%. Filled in portion of vertical bars indicates estimated TnI contribution, open portion of bar represents PLN contribution. Error bars are ± SEM.

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**Disclosures**

None.

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Methods

Gene Transfer and Primary Culture Maintained in Electrical Stimulation Chambers. Two hours after rat myocytes were plated on cover slips in DMEM with 5% fetal bovine serum, the medium was replaced with serum-free DMEM plus P/S containing recombinant adenovirus with TnI cDNAs (cTnI, cTnI S23/24A, cTnI S23/24D, or ssTnI), and then incubated for an additional hour before adding 2 ml of DMEM with P/S.

Electrical stimulation. Cover slips containing the cardiac myocytes used for sarcomere shortening experiments were transferred to plexiglas stimulation chambers the day after isolation, and electrically stimulated at 0.5 Hz for 4 days in Media 199 (M199) supplemented with P/S, 10mM glutathione, 26.2mM sodium bicarbonate and 0.02% BSA (M199+), that was replaced every 12 hours.

Western blot. For each rat myocyte sample, the myocytes from two cover slips were scraped and stored in SDS-PAGE buffer at -20°C until use. For analysis of phosphoprotein content Pro-Q Diamond phosphoprotein stain was used. Samples were run on 12% SDS-PAGE gel was at 200-V constant voltage for 80 min in running buffer. The gel was fixed, washed in 100 ml of water for 10 min three times, and stained with 60 ml of Pro-Q Diamond phosphoprotein stain (Molecular Probes) for 75 min, and destained in 100 ml of Pro-Q Diamond destain solution for 30 min three times. After washout in water for 10 min the gel was imaged with Typhoon Trio imager (Amersham, 532nm laser excitation, 580nm emission filter). The gel was subsequently stained with 60 ml of SYPRO Ruby protein stain (Molecular Probes) for 8 h and washed in 100 ml water for 40 min. The gel was imaged with a UV image system.

Calcium kinetics. Cover slips with single cardiac myocytes from Tg and Ntg mice and cultured rat cardiac myocytes maintained in stimulation chambers for 4 days were transferred to a custom chamber with M199+ media kept at 37°C and mounted on the stage of an inverted microscope (Nikon). Some cover slips were used to measure the Ca2+ transient, and were incubated in M199+ medium containing 5 μM Fura-2 AM (Molecular Probe) for 4 minutes before they were transferred to the chamber. The Ionoptix fluorescence photometry and digital cell geometry detection system was used to measure sarcomere length and the Ca2+ transient (37°C). Ca2+ fluorescence ratio (360 nm/380 nm) signals were normalized to a maximal value (1.0) after subtracting background.
Supplemental Figure Legends

Supplement Fig. 1. Summary of 25% and 50% relaxation times from peak of unloaded shortening between Ntg and S23/34D Tg cardiac myocytes in the presence and absence of 50 nmol/L Iso. Values are mean +/- SEM, n = 17 in each group.

Supplement Fig. 2. Summary 25% and 50% relaxation time from peak twitch tension using micro-carbon fibers. Values are mean +/- SEM, N = 8 per group
Supplemental Figure 2

![Graphs showing T0.25R and T0.5R measurements with ISO and NTG/TG conditions.](image-url)