Frequency- and Afterload-Dependent Cardiac Modulation In Vivo by Troponin I With Constitutively Active Protein Kinase A Phosphorylation Sites

Eiki Takimoto,* David G. Soergel,* Paul M.L. Janssen, Linda B. Stull, David A. Kass, Anne M. Murphy

Abstract—Acute β-adrenergic stimulation enhances cardiac contractility, accelerates muscle relaxation, and amplifies the inotropic and lusitropic response to increased stimulation frequency. These effects are modulated by phosphorylation of calcium handling and myofilament proteins such as troponin I (TnI) by protein kinase A (PKA). To more directly delineate the role of TnI PKA phosphorylation, transgenic mice were generated that overexpress cardiac TnI in which the serine residues normally targeted by PKA are mutated to aspartic acid to mimic constitutive phosphorylation (TnIDD22,23). Native cardiac TnI was near completely replaced in one transgenic line as assessed by in vitro phosphorylation, and this led to reduced calcium sensitivity of myofibrillar MgATPase, as expected. TnIDD22,23 mice had mildly enhanced basal systolic and diastolic function, and displayed marked augmentation of frequency-dependent inotropy and relaxation, with a peak frequency response 2-fold greater in mutants than controls (P<0.005). Increasing afterload prolonged relaxation more in nontransgenic than TnIDD22,23 (P<0.02), whereas contractile responses to afterload were similar between these strains. Isoproterenol treatment eliminated the differential force-frequency and afterload response between TnIDD22,23 and controls. In contrast to in vivo studies, isolated isometric trabeculae from nontransgenic and TnIDD22,23 mice had similar basal, isoproterenol-, and frequency-stimulated function, suggesting that muscle shortening may be important to TnI PKA effects. These results support a novel role for cardiac TnI PKA phosphorylation in the rate-dependent enhancement of systolic and diastolic function in vivo and afterload sensitivity of relaxation. These results have implications for cardiac failure in which force-frequency modulation is blunted and afterload relaxation sensitivity increased in association with diminished PKA TnI phosphorylation. (Circ Res. 2004;94: 777-786.)

Key Words: troponin I ■ protein kinase A ■ cardiac function ■ myofilament

Stimulation of cardiac β-adrenergic receptors augments myocardial contractility, accelerates the rate of force development and relaxation, and amplifies the positive inotropic and lusitropic effects of higher stimulation frequency.1 In addition, the load dependence of cardiac relaxation is influenced by cardiac contractility and level of β-stimulation.2,3 These effects are thought to be mediated by the phosphorylation of target proteins by protein kinase A (PKA), including the myofilament proteins cardiac troponin I (cTnI) and myosin binding protein-C (MyBP-C), sarcoplasmic reticulum (SR) regulatory proteins phospholamban (PLB) and ryanodine receptor channel, and the sarcolemmlal calcium channel. The net result is greater availability of intracellular calcium during systole, more rapid calcium removal from the cytosol into the SR, and enhanced calcium dissociation from myofilaments during diastole (reviewed by Bers4). Of these PKA target proteins, strong evidence exists for potent regulation by PLB and calcium channel phosphorylation; however, the role of TnI remains less clear.

TnI is the inhibitory element of the troponin complex that regulates contraction.5-7 Unlike skeletal TnI, the cardiac isoform contains a unique 31 amino acid domain at the amino terminus with two PKA-targeted phosphorylation sites (positions 22 and 23 in mouse, excluding the initiating methionine). PKA phosphorylation of cTnI desensitizes myofilament MgATPase activity to calcium,8 shifting the force-pCa relationship rightward and enhancing relaxation by increasing the dissociation rate of Ca2+ from TnC.9,10 The relative importance of this effect, however, remains somewhat controversial. Li et al11 studied PLB-null mice and found positive lusitropic effects with isoproterenol only at maximal stimulation and higher afterload, suggesting that PLB phosphory-
lation played the dominant role. In contrast, Kentish et al,12 studied transgenic mice with slow skeletal TnI replacing cTnI and used flash photolysis of diazo-2 to rapidly remove Ca\(^{2+}\) from skinned cardiac muscle fibers. Muscle expressing skeletal TnI (which lacks PKA sites) had no acceleration of relaxation in \(-\alpha\)-adrenergic stimulation, suggesting that cTnI phosphorylation played an important role in intrinsic relaxation. Recent studies further suggest that muscle afterload may be important to adrenergic-mediated effects on relaxation.13 This may underlie observations by some investigators that PKA or isoproterenol stimulation has minimal to no effect on unloaded shortening velocity in cardiac muscle and myocyte preparations.14–16

The present study was designed to better elucidate the functional role of PKA-mediated phosphorylation of cTnI both in vivo and in vitro. Based on evidence that aspartic acid (D) substitutions for the serines targeted by PKA are good mimics for changes induced by native phosphorylation17 and that cTnI specifically altered by such mutations (TnIDD\(_{22,23}\)) exhibits decreased force-calcium sensitivity in vitro,18 transgenic mice were generated with cardiac overexpression of TnIDD\(_{22,23}\) using an \(\alpha\)-myosin heavy chain promoter. Functional analysis was performed in vitro and in vivo to determine the physiological and biochemical influence of this altered protein, permitting a detailed examination of the role of TnI phosphorylation in the intact heart.

Materials and Methods

Transgenic Model

The expression vector contained the 5.5-kb murine \(\alpha\)-myosin heavy chain promoter was provided by Dr Jeffrey Robbins. A PCR-amplified cDNA encoding rat cardiac TnI with Ser22 and Ser23 changed to aspartic acid residues was cloned into the \(\text{SalI}\) site (Figure 1A), confirmed by sequencing, and the \(\text{SalI}\) digested insert was injected into mouse pronuclear embryos (C57BL/6\(\times\)SJL) as described.19 Two founders were bred with nontransgenic C57BL/6 mice. Offspring were genotyped as described.19 Both lines of transgenic mice appeared healthy with no symptoms of heart failure and had normal life spans and breeding potential. For experimental studies, the transgenic mice were compared with sib or age-matched C57BL/6 mice. Studies of the mice were in accordance with institutional guidelines.

Myofibrillar MgATPase Activity Measurement

Myofibrils were prepared from cardiac ventricle as described20 with careful use of protease inhibitors. Assays were performed as described in the expanded Materials and Methods in the online data supplement available at http://circres.ahajournals.org.

Phosphorylation Studies

Myofibrils were isolated as previously described, then resuspended in 50 mmol/L Tris-HCl (pH 7.5) buffer containing protease inhibitors, 20 \(\mu\)mol/L cAMP, and 32P-\(\gamma\)-ATP for 30 minutes at 30°C. Ten micrograms of protein was separated by gel electrophoresis and exposed to film. Arrows indicate location of myosin binding protein-C (MyBP-C) and TnI on the gels. Only the NTG myofibrils incorporated any significant isotope. Densitometry scans indicated the transgenic line had at least 95% replacement of native TnI with TnIDD\(_{22,23}\) when normalized to MyBP-C incorporation.

### Figure 1

A, Peptide sequence of the cardiac-specific region of TnI demonstrating the location of the two adjacent serine residues that have been mutated in the transgenic construct to encode aspartic acid residues (D). B, PKA-dependent phosphorylation of myofibrillar proteins. Myofibrils were isolated from hearts of nontransgenic (NTG) control littermates and high-expressing TnIDD\(_{22,23}\), transgenic mice in the presence of protease inhibitors and were preincubated with either alkaline phosphatase (AP) to remove residual endogenous phosphates or sodium fluoride (NaF) to inhibit dephosphorylation. Myofibrils were then washed in buffer, and 100-\(\mu\)g aliquots were exposed to 50 U purified protein kinase A catalytic subunit, 20 \(\mu\)mol/L cAMP, and 32P-\(\gamma\)-ATP for 30 minutes at 30°C. Ten micrograms of protein was separated by gel electrophoresis and exposed to film. Arrows indicate location of myosin binding protein-C (MyBP-C) and TnI on the gels. Only the NTG myofibrils incorporated any significant isotope. Densitometry scans indicated the transgenic line had at least 95% replacement of native TnI with TnIDD\(_{22,23}\) when normalized to MyBP-C incorporation.
stopped by adding SDS-PAGE loading buffer and heating at 65°C for 10 minutes. Proteins were separated by gel electrophoresis and the gel exposed to film. Myofilibr preparations were also performed using phosphatase inhibitors 2 mmol/L calyculin A and 0.1 μmol/L orthovanadate in order to maintain endogenous phosphorylation during the myofilibr isolation process.

**In Vivo Ventricular Function Studies**

Left ventricular pressure-volume (PV) studies were performed as previously described. Assessment of force-frequency and afterload responses, treatment with isoproterenol and statistical methods are described in the expanded Materials and Methods.

**Isolated Muscle Studies**

Isolated muscle studies were performed as described with details in the expanded Materials and Methods.

**Phospholamban (PLB) and PLB Phosphorylation**

Myocardial protein homogenates were prepared in the presence of protease inhibitors as described above as well as phosphatase inhibitors (Cell Signaling Technologies, Inc). Samples consisting of 5 to 15 μg of total protein were by gel electrophoresis, transferred to nitrocellulose membrane, and stained with mouse anti-phospholamban (Affinity Bioreagents) to determine total PLB followed by rabbit anti-phospho-Ser16-phospholamban (Upstate Cell Signaling Solutions) to determine the degree of phosphorylation at this site. After incubation with secondary antibodies, ECL was performed per manufacturer protocol (Amersham Biosciences).

**In Vitro Phosphorylation**

Myofilaments isolated from TnIDD22,23 (high-expressing line) demonstrated markedly lower 32P incorporation than in nontransgenic mice (NTG) at the molecular weight corresponding to cTnI, whereas the signal corresponding to MyBP-C was similar (Figure 1B). Quantitative analysis indicated TG mice had approximately 95% replacement of native cTnI with mutant TnIDD22,23. The total amount of TnI protein is similar in TnIDD22,23 and NTG as determined by Western blot (data not shown). Pretreatment of purified myofilibr with alkaline phosphatase did not significantly increase 32P incorporation by PKA, indicating the standard myofilibr preparation removed most endogenous phosphorylation. However, isolation of myofilaments in the presence of the phosphatase inhibitor NaF (10 mmol/L) did not alter 32P incorporation in TnIDD22,23, but reduced 32P incorporation on average by 17% to 44% (mean=26%, n=3) in NTG hearts when compared with maximal phosphorylation obtained after alkaline phosphatase treatment. This “back phosphorylation” experiment suggested the combined TnI PKA phosphorylation sites were approximately 74% unoccupied at baseline. Similar results were obtained when myofilibr were isolated using the phosphatase inhibitors 2 mmol/L calyculin A and 0.1 μmol/L orthovanadate. Myofilibr isolated from diaphragm (containing ssTnI) and skeletal muscle (containing sTnI) tested the specificity of the cardiac isoform reaction, and neither demonstrated 32P incorporation in response to PKA. Taken together these results indicate that the myofilaments from the high-expressing TnIDD22,23 line had virtually complete replacement of native TnI with the transgenic construct mimicking constitutive phosphorylation.

A second line of mice had approximately 50% replacement of TnI with TnIDD22,23 as determined by phosphorylation experiments, and this line was utilized only for MgATPase experiments.

**Myofibrillar MgATPase Activity**

Maximal MgATPase activity was not significantly different in TnIDD22,23 myofilibr from high-expressing lines (147±11 nmol inorganic phosphate/minute per milligram protein) compared with NTG (135±9 nmol inorganic phosphate/minute per milligram protein). The minimal activities also did not differ between groups (23±2 for the TnIDD22,23 myofilibr versus 23±3 for the NTG). For the TnIDD22,23 myofilibr, the mean MgATPase activity had an EC50 for calcium of 5.75±0.32 μmol/L for TG, whereas NTG had an EC50 of 4.75±0.31 μmol/L (P<0.05), demonstrating desensitization of the TG myofilaments to calcium (Figure 2). This EC50 shift is similar to that produced by native PKA phosphorylation in murine skinned fibers and by introduction of recombinant TnI PKA site DD mutant in skinned fibers. The Hill coefficient was not significantly different between TnIDD22,23 lines and NTG. Myofibrils from the lower expressing TnIDD22,23 line had no significant shift in EC50 compared with NTG myofilibr (data not shown), and physiological experiments were performed on the high-expressing TnIDD22,23 line.

**In Vivo Physiology**

At baseline, TnIDD22,23 hearts generated mildly, but significant, enhancement in systolic function (dP/dtmax and dP/dtmin/IP), higher LV end-systolic pressures, and more negative dP/dtmin (Table). The latter suggested enhanced relaxation, which was supported by a significantly reduced relaxation time constant (τ) based on a logistic model. The LV end-systolic pressure was mildly elevated in the TnIDD22,23.

In comparison to subtle changes in basal function, marked differences of both systolic and diastolic function were observed at faster pacing rates (Figure 3). Representative steady-state PV loops revealed little difference between TnIDD22,23 and nontransgenic at lower heart rates, but more enhanced function (leftward shift of the PV loop and end-systolic pressure-volume point) at faster rates in TnIDD22,23 versus nontransgenic hearts (Figure 3A). Summary data for systolic and diastolic function-heart rate responses are shown in Figure 3B. Both rate-dependent enhancement of systolic load-independent parameters of maximal dP/dt normalized for IP, and maximal power normalized for end diastolic volume, as well as the acceleration of relaxation were enhanced at faster rates in the TnIDD22,23 animals.

We next tested whether nontransgenic and TnIDD22,23 hearts displayed different systolic and diastolic responses to β-adrenergic stimulation with isoproterenol. As shown in Figure 4, both types of heart displayed similar systolic augmentation with increasing isoproterenol dose (≈50% over baseline at 80 ng/kg per minute). Relaxation rates were less affected in both groups, perhaps in part as basal rates were already quite rapid. At maximal isoproterenol stimulation, we varied heart rate over a broad range (using an I1 blocker to inhibit spontaneous sinus rate). Isoproterenol stimulation resulted in a similar flattened force-frequency relationship (FFR) for TnIDD22,23 and nontransgenic mice, supporting a role for TnI phosphorylation to the normal rate response.
Afterload Relaxation Dependence

Results of altering ventricular afterload induced by graded aortic constriction on contraction and relaxation are displayed in Figure 5. Raising afterload reduced stroke volume at higher pressures (Figure 5A) as expected and was quantified from the loop data using effective arterial elastance (Ea). At constant heart (used in this protocol), Ea incorporates mean resistive as well as pulsatile loading properties of the vasculature. In nontransgenic mice, raising afterload delayed LV relaxation demonstrated by the slower decline in pressure (Figure 5B, data are displayed normalized to peak pressure and cycle length); however, this was far less so in TnIDD22,23 hearts. Figure 5C shows summary data relating the percent change in afterload to relaxation time constant-normalized to baseline. TnIDD22,23 mice had significantly shallower relations (covariance analysis) using both the monoexponential ($\tau_1$) and logistic ($\tau_2$) relaxation models. In contrast, systolic function responded minimally and similarly to afterload in both mouse strains (data not shown).

The lusitropic response to afterload during isoproterenol stimulation is displayed in Figures 5D and 5E. Isoproterenol essentially eliminated relaxation delay induced by afterload in both nontransgenic and TnIDD22,23 (Figure 5D). This is confirmed by the group covariance analysis (Figure 5E).

### Hemodynamic Measures on TnIDD22,23 and Nontransgenic (NTG) Mice

<table>
<thead>
<tr>
<th></th>
<th>NTG (n=8)</th>
<th>TnIDD22,23 (n=9)</th>
<th>$P$</th>
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</thead>
<tbody>
<tr>
<td>HR, bpm</td>
<td>$492 \pm 17$</td>
<td>$516 \pm 12$</td>
<td>0.23</td>
</tr>
<tr>
<td>LVPes, mm Hg</td>
<td>$97 \pm 2$</td>
<td>$106 \pm 2$</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ejection fraction</td>
<td>$0.63 \pm 0.07$</td>
<td>$0.63 \pm 0.04$</td>
<td>0.97</td>
</tr>
<tr>
<td>$dP/dt_{max}$, mm Hg/s</td>
<td>$9985 \pm 629$</td>
<td>$12496 \pm 572$</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>$dP/dt_{min}$, mm Hg/s</td>
<td>$-9400 \pm 545$</td>
<td>$-11321 \pm 361$</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ea</td>
<td>$6.9 \pm 0.9$</td>
<td>$6.9 \pm 1.0$</td>
<td>0.97</td>
</tr>
<tr>
<td>$PWR_{max}/EDV$, W</td>
<td>$30.9 \pm 5.2$</td>
<td>$30.2 \pm 3.4$</td>
<td>0.91</td>
</tr>
<tr>
<td>$\tau_2$, logistic regression</td>
<td>$5.04 \pm 0.27$</td>
<td>$4.38 \pm 0.14$</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>$dP/dt/IP$</td>
<td>$162 \pm 9$</td>
<td>$195 \pm 10$</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>
showing that both TnIDD22,23 and nontransgenic mice had no significant change in $\tau_1$ or $\tau_2$ despite increasing afterload. These data support an important role for TnI phosphorylation in the interaction between afterload and chamber relaxation.

Figure 3. In vivo force-frequency response is enhanced in TnIDD22,23 (TG) mice. Hearts were studied using a miniaturized pressure-volume catheter. A, Representative pressure-volume loops in a TnIDD22,23 transgenic (TG) and nontransgenic (NTG) control at atrial pacing rate of 400 and 800 bpm. There is a greater leftward shift (inotropy) with faster rate in the TG animal. B, Mean results for rate response of systolic and diastolic function. $dP/dt_{\max}/IP$ indicates maximal rate of pressure rise normalized to instantaneous developed pressure; $PW_{max}/EDV$, maximal power normalized to end-diastolic volume; $dP/dt_{\min}$ (peak negative $dP/dt$), and $\tau_1$, relaxation time constant (logistic model). $P$ values are for group interaction term (analysis of covariance). TnIDD22,23 mice showed greater rate-dependent augmentation of systolic and diastolic function versus controls.

Figure 4. Response to isoproterenol in vivo. A, Hearts were studied at a fixed heart rate of 550 bpm and exposed to increasing infusion rates of isoproterenol. Infusion of isoproterenol results in augmentation of $dP/dt_{\max}/IP$ at this fixed heart rate, without significant difference between TG and NTG. Tau response is relatively flat and not different between TG and NTG. B, Rate response in the presence of isoproterenol (at infusion rate of 80 ng/kg per minute). In the presence of isoproterenol, the response to increasing heart rate is relatively flat and there is no significant difference in $dP/dt_{\max}/IP$ nor Tau response to heart rate between TG and NTG.
Figure 5. Relaxation dependence on afterload is reduced in TnIDD22,23 (TG) mice in vivo. A, Representative pressure-volume loops demonstrating afterload increase in the murine heart and quantitation of the loading change by effective arterial elastance (Ea). B, Example LV pressure decay time plots for TG versus NTG hearts, with each curve normalized so that peak pressure and cycle length are 1.0. Relaxation became significantly more delayed with increased afterload in NTG compared with TG. C, Regression analyses of relaxation-afterload dependence. Data are percent change in relaxation time constant versus afterload (Ea); Tau(1) and Tau(2) are relaxation time constants based on a monoeponential or logistic decay model, respectively. Relaxation prolonged with higher afterload significantly more in NTG than TG hearts. P values: Load reflects overall relation between Tau and afterload, and Genotype the interaction term reflecting significant differences between NTG and TG. D, Example LV pressure decay time plots for TnIDD22,23 transgenic mice (TG) versus controls (NTG) in the presence of isoproterenol. Isoproterenol abolishes the delay in relaxation with increase afterload in NTG, thus eliminating the differential response between TG and NTG. E, Regression analyses in the presence of isoproterenol. Isoproterenol abolishes the prolonged relaxation in response to afterload (P>0.7 to 0.8), and eliminates the differential response between the TG and NTG (P>0.7 to 0.8).
Isolated Muscle Studies
In contrast to in vivo studies in which the muscle shortens during systole, studies performed in isometric (high afterload) isolated muscle did not reveal any differences between TnIDD22,23 and nontransgenic trabeculae. The FFR was positive in both TnIDD22,23 and nontransgenic trabeculae. Maximum force was 32.5 ± 4.7 mN/mm² at 8 Hz in TnIDD22,23 versus 34.9 ± 6.7 mN/mm² in nontransgenic. The time to 50% relaxation (RT 50) declined similarly in both strains at each of the varied stimulation frequencies (Figures 6A and 6B). Stimulation with isoproterenol (from 1 nmol/L to 1 μmol/L) produced a dose-dependent increase in tension from 20 to 50 mN/mm² that was also similar in TnIDD22,23 and nontransgenic (Figures 6C and 6D), with an EC₅₀ near 10 nmol/L in both.

Assessment of PLB and PLB-Ser 16 Phosphorylation
One potential factor that could alter myocardial behavior in TnIDD22,23 was secondary changes in the level of PLB content and/or phosphorylation at Ser16, the residue phosphorylated by PKA. Figure 7 displays results of immunoblot studies for PLB and for phosphorylation at PLB-Ser16 (n = 3), and reveals no differences in either total or phosphorylated protein.

Discussion
This study provides novel evidence supporting a prominent role for cardiac TnI PKA phosphorylation in the enhancement in vivo systolic and diastolic function. Whereas basal differences were modest, the TnIDD22,23 mice had an enhanced response to beat frequency and a modulation of myocardial relaxation in response to afterload. Notably, treatment with isoproterenol in vivo eliminated the differential between TnIDD22,23 and nontransgenic mice in the response to increased heart rate and imposition of afterload, supporting the role of PKA phosphorylation of TnI in these responses. These findings are of particular significance because of the association of heart failure with decreased phosphorylation of TnI at PKA sites.31–33

Studies regarding the role of PKA-site TnI phosphorylation have principally focused on cardiac muscle relaxation effects, and the results have been the subject of some debate. Desensitization of myofibrils to Ca²⁺ appears to oppose the inotropic effect produced by PLB and sarclemmal L-type calcium channel phosphorylation. However, TnI phosphorylation could contribute to augmented function from β-stimulation by enhancing relaxation and crossbridge cycling, and accelerating unloaded shortening velocity. Studies in genetically modified animals have suggested that TnI phosphorylation by PKA does have a role in augmenting relaxation and the frequency of minimum dynamic stiffness (a surrogate for augmenting crossbridge kinetics). Other studies performed in isolated trabeculae have found that PKA phosphorylation does not alter unloaded shortening, and the precise experimental conditions may be important in this regard.

Although most published work has focused on effects of TnI phosphorylation on relaxation, there are data suggesting myofilament protein phosphorylation by PKA can increase skinned myocyte power-output by a mechanism involving the kinetics of loaded shortening. Layland and Kentish reported positive inotropic effects of β-adrenergic stimulation in rat trabeculae with pharmacologically inhibited SR. Although, in this particular study, increased sarclemmal Ca influx from L-type Ca channel phosphorylation may have contributed to the inotropic effect. In contrast, the present model selectively targeted TnI and supports a role for TnI phosphorylation in modulating both systolic and diastolic function in vivo, particularly as a function of heart rate and afterload.

Role of TnI in the Force-Frequency Relationship
Augmentation of systolic and diastolic function in response to higher stimulation frequency is a well-described property of cardiac muscle. In humans, there is nearly a 100% increase in intact heart contractility over the physiological frequency range, and similar responses have been reported in other large mammalian models. In mice, the response is diminished, particularly when it is assessed by parameters that minimize the preload-sensitivity with higher frequencies. However, β-adrenergic stimulation has been shown to enhance the FFR, independent of the chronotropic effects of β-adrenergic stimulation, whereas the relation is profoundly blunted by cardiac failure in association with altered calcium cycling. Although it has been widely held that phosphorylation of PLB modulating SR-ATPase activity and sarclemmal calcium channels to enhance Ca²⁺ entry per excitation is essential to this synergy, the current data supports a key involvement of TnI phosphorylation as well.

It is intriguing that whereas TnI phosphorylation had a prominent effect on the in vivo FFR, there was no impact on this relation in isolated muscle under isometric conditions. One major difference is the lack of muscle shortening in the latter preparation, yet shortening and load-interactions may be central to the mechanisms of TnI modulation. In the study of Layland and Kentish, comparisons were made between isometric versus work-loop contracting trabeculae, a methodology that mimics in vivo muscle shortening. Elevation of stimulation frequency had a more notable impact on relaxation in shortening muscles versus those stimulated at fixed length. The increase in crossbridge cycling by TnI phosphorylation was felt to reduce the work required to restretch during diastole at high frequency. The data of Layland and Kentish provide a potential explanation for the apparent discrepancy between the enhanced FFR in vivo in our studies with a lack of an enhanced FFR in vitro in nonshortening muscle preparations. However, it is important to note that the muscle studies performed in the present study are limited in that analyses in muscle preparations that permitted shortening and used varying afterload were not performed. Although accurate replication of in vivo loading in isolated muscle is nontrivial, such studies would be needed to directly compare in vitro to in vivo findings. Thus, further experimental testing is needed to understand this apparent discrepancy.

TnI Phosphorylation and the Load Dependence of Relaxation
Elevation of cardiac afterload prolongs cardiac relaxation and this effect is attenuated by β-adrenergic
Figure 6. Effect of increased stimulation frequency and isoproterenol on developed tension and relaxation kinetics in vitro. A and B, Isolated intact trabeculae were stimulated at increasing frequencies. A, Developed force at varying frequency of stimulation. B, RT_{50} at varying frequency of stimulation. Experiments were performed at 37.5°C and 1.5 mmol/L Ca^{2+} for 7 trabeculae in each group. ANOVA analysis demonstrated no significant difference between NTG and TnIDD_{22,23} muscles. C and D, Isoproterenol effect on tension development and relaxation kinetics. Isolated trabeculae were exposed to increasing concentrations of isoproterenol. Developed force (C) and RT_{50} (D). Experiments were performed at 37.5°C and 1.5 mmol/L Ca^{2+} for 8 trabeculae in each group. ANOVA analysis demonstrated no significant difference between NTG and TnIDD_{22,23} muscles.
nificant difference between NTG and TG in amount of total PLB or phospho-

Figure 7. Immunoblot of phospholamban (PLB) content in homogenates from TnIDD22,23 transgenic (TG) and NTG mice. Myofibril preparations from NTG and TG hearts were performed as described. Western blots of samples containing 5, 10, and 15 μg of total protein were performed using primary rabbit anti-phospho-Ser16 phospholamban antibody. ECL was performed after incubation with secondary antibody per manufacturer protocol (Amersham). The membranes were then stripped and reprobed using mouse primary anti-phospho-Ser16 phospholamban, and ECL was repeated after secondary antibody incubation. Bands were scanned and quantified; n=3/group. Representative blots are pictured. There is no significant difference between NTG and TG in amount of total PLB or phospho-

In summary, mice with TnI that mimics constitutive phosphorylation at the PKA sites have modest augmentation in baseline ventricular systolic and diastolic function. Most notably these mice have augmented force-frequency response and attenuated afterload-induced prolongation of relaxation, suggesting a role for TnI phosphorylation by PKA in these phenomena in vivo. The results have implications for heart failure in which force-frequency modulation is blunted and afterload relaxation sensitivity increased in association with diminished PKA TnI phosphorylation.

Acknowledgments

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Myofibrillar ATPase activity measurement

Assays were performed using incubation conditions established by varying the total concentration of metals, salts and ligands and maintaining ionic strength using stability constants compiled by Fabiato 1; and were performed at pH 7.0 with 50 mM imidazole, 50 mM KCl and 2 mM MgATP with 0.2 mg per ml of myofibrillar protein. Inorganic phosphate liberation was measured as described by Rarick et al. 2. To characterize the relationship of Mg ATPase activity to [Ca^{2+}], the raw data from each individual preparation was fitted to the Hill equation and maximal and minimal activation, EC_{50} and Hill coefficients were determined for each experiment using the Kaleidagraph statistical program. The mean values for the EC_{50} and Hill parameters, as well a maximal and minimal activities were compared between groups using Student’s t-tests as previously justified 3.

In vivo ventricular function studies

Force-frequency relations were derived as described 4, following injection of an I_{f} blocker (ULFS-49, 15-20 mg/kg i.p.) to generate bradycardia without altering ventricular function, and institution of atrial pacing via an esophageal lead. Steady-state data were collected at hearts rates ranging between 400-700 bpm.

To determine the influence of afterload on cardiac systolic and diastolic function, hearts were atrially paced at a fixed rate of 550 bpm, and graded aortic occlusions performed by pressing on the descending aorta with a custom manipulator. Steady-state data were collected 2 minutes after achieving 10, 30, 50 mmHg increases in systolic LVP.
Measures of chamber systolic and diastolic function were obtained as previously reported. Parameters included heart rate, LV end-systolic and end-diastolic pressures and volumes, dP/dt_{max} and dP/dt_{min}, and dP/dt max normalized for IP (instantaneous pressure), time constant of relaxation by the logistic regression (Tau 2) and monoexponential (Tau 1) methods, maximal ventricular power normalized to chamber preload (PWR_{max}/EDV), ejection fraction, and effective arterial elastance (Ea). Basal parameter comparisons were performed by unpaired t-test. Heart-rate and afterload dependencies were assessed by analysis of covariance, with genotype serving as the grouping factor.

Mice were also treated with increasing doses of isoproterenol (10, 20, 40, 80 ng/kg/min) infused via jugular vein at a fixed heart rate of 550 bpm. Measurements were performed for each dose after 3-5 minutes. Following the dose response-study, force-frequency relations and influence of increased afterload were examined as previously described in the presence of isoproterenol at 80ng/kg/min.

*Isolated muscle studies*

Mice were anesthetized and dissection of the thin, non-branching trabeculae was as described. The average dimensions of the muscles were 0.178 ± 0.02 mm wide, 0.13 ± 0.02 mm thick and 2.12 ± 0.02 mm long. Cross-sectional area was calculated assuming an ellipsoid shape.

Muscles were mounted with details of perfusion and stimulation protocols as noted. The muscles were superfused and studied at 37.5°C. Muscles were stretched to a length where a small increase in length resulted in about equal increases in resting
tension and active developed tension. This “optimal length” is slightly below the length
where active force development is maximal, and was selected to be comparable to the
maximally attained length in vivo (approximately 2.2 µm sarcomere length)\(^9\).

We assessed the effects of increasing a range of stimulation frequencies (2-12 Hz)
at 37.5°C. The effect of β-adrenergic stimulation was assessed at steady-state (1.5 mM
\([\text{Ca}^{2+}]_0\), 4 Hz) with muscles exposed to increasing isoproterenol (1 nM to 1 µM).
Developed force (\(F_{\text{dev}}\)) was determined and normalized to cross-sectional area.
Additionally, as a force-independent parameter of force decay kinetics, time from peak
force to 50% relaxation (\(R_{50}\)) was determined. Multiple analysis of variance
(MANOVA) was used to assess the significance of differences between interventions,
with post-hoc t-test when appropriate. A two-tailed P-value of <0.05 was considered
significant.

References:

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