Left Ventricular and Myocardial Function in Mice Expressing Constitutively Pseudophosphorylated Cardiac Troponin I


Rationale: Protein kinase (PK)C-induced phosphorylation of cardiac troponin (cTn)I has been shown to regulate cardiac contraction.

Objective: Characterize functional effects of increased PKC-induced cTnI phosphorylation and identify underlying mechanisms using a transgenic mouse model (cTnIPKC-P) expressing mutant cTnI (S43E, S45E, T144E).

Methods and Results: Two-dimensional gel analysis showed 7.2±0.5% replacement of endogenous cTnI with the mutant form. Experiments included: mechanical measurements (perfused isolated hearts, isolated papillary muscles, and skinned fiber preparations), biochemical and molecular biological measurements, and a mathematical model-based analysis for integrative interpretation. Compared to wild-type mice, cTnIPKC-P mice exhibited negative inotropy in isolated hearts (14% decrease in peak developed pressure), papillary muscles (53% decrease in maximum developed force), and skinned fibers (14% decrease in maximally activated force, Fmax). Additionally, cTnIPKC-P mice exhibited slowed relaxation in both isolated hearts and intact papillary muscles. The cTnIPKC-P mice showed no differences in calcium sensitivity, cooperativity, steady-state force-MgATPase relationship, calcium transient (amplitude and relaxation), or baseline phosphorylation of other myofilamental proteins. The model-based analysis revealed that experimental observations in cTnIPKC-P mice could be reproduced by 2 simultaneous perturbations: a decrease in the rate of cross-bridge formation and an increase in calcium-independent persistence of the myofilament active state.

Conclusions: A modest increase in PKC-induced cTnI phosphorylation (≈7%) can significantly alter cardiac muscle contraction: negative inotropy via decreased cross-bridge formation and negative lusitropy via persistence of myofilament active state. Based on our data and data from the literature we speculate that effects of PKC-mediated cTnI phosphorylation are site-specific (S43/S45 versus T144). (Circ Res. 2009;105:1232-1239.)

Key Words: transgenic mice protein kinase C cardiac troponin I calcium (cellular) model-based analysis

The trimeric protein cardiac troponin (cTn) is associated with the sarcomeric thin filament and is a key protein that regulates cardiac contraction. Calcium binds to cTn causing a conformational shift in tropomyosin and allowing actin and myosin to form a force generating cross-bridge. cTn is more than a simple on-off switch for contraction; it can exert more complex control through its phosphorylation. For example, the inhibitor subunit of cTn, cTnI, has shown significant modulatory capacity when phosphorylated by protein kinase (PK)A and PKC. Some of these aspects of control include filament sliding speed, calcium sensitivity, cross-bridge cycling, and MgATPase activity. These affect global force production and relaxation responses to afterload, frequency and length. There are at least five phosphorylatable sites on cTnI: serines at 23, 24, 43 and 45 (S23, S24, S43, S45) and a threonine at 144 (T144). The sites nearest the N-terminal (S23, S24) are primarily phosphorylated by PKA, whereas the other 3 sites (S43, S45, T144) are primarily phosphorylated by PKC. There may be other phosphorylation sites, including S1507 and S76/T778 (in human) as well as other kinases that act on cTnI, including p21-activated kinase2 and Mst1 (mammalian sterile 20-like kinase1). In the present study, we have focused on PKC phosphorylation for 2 reasons: (1) PKC is upregulated during heart failure10,11; and (2) its effects are largely dominant over those of PKA.4

We previously created a transgenic (TG) mouse with mutated PKC phosphorylation sites on cTnI: S43 and S45 were replaced with alanines to mimic nonphosphorylatable sites (cTnIS43/S45NP). Decreased phosphorylation at these sites causes a positive inotropic response12,13 no change in

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were quickly excised. Left ventricular (LV) pressure was measured.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Serine 43 and 45 and threonine 144 residues on cTnI were replaced with glutamic acid to simulate constitutive phosphorylation. Steps to create the TG mouse were similar to the creation of the cTnIPKC-P mice, wherein S43, S45, and T144 on cTnI were replaced with glutamic acids to simulate constitutive phosphorylation. Note this new model mutated the threonine at position 144, which the cTnI43/45/144-NP mouse did not. Authentic phosphorylation of these 3 sites by PKC produced responses that closely mimic the effect of pseudophosphorylation using glutamic acid in reconstituted fibers. Based on previous work with the cTnI43/45/144-NP mouse, we hypothesized that the cTnIPKC-P mouse will exhibit negative inotropy and unchanged relaxation. Our goal was to test this hypothesis and determine how activator calcium, myofilament activation, and myofilament contraction contribute to the observed functional effects.

Results

Characterization of the Mouse Model

The cTnI_{PKC-P} mice showed no differences in body mass (WT: 30.7 ± 1.6 g; cTnI_{PKC-P}: 31.3 ± 1.0 g), LV mass (WT: 102.4 ± 6.7 mg; cTnI_{PKC-P}: 103.7 ± 4.2 mg), or their ratio (WT: 296 ± 9; cTnI_{PKC-P}: 303 ± 9). There were also no overt signs of heart failure, including lethargy or differences in feeding.

Two-Dimensional Difference in Gel Electrophoresis Analysis

Proteins from WT and cTnI_{PKC-P} myofibrils were separated using 2D-DIGE (Figure 1A). The 2D-DIGE gels were capable of separating cTnI species with posttranslational modifications and unmodified species (U: unmodified cTnI; Px: posttranslational modified cTnI, where x = number of possible modifications). In cTnI_{PKC-P} samples, there was an additional spot near P3, indicated by an asterisk in Figure 1A, which was not present in the WT samples. We postulated this spot represented mutant cTnI.

To confirm this spot was constitutively pseudophosphorylated mutant cTnI, we treated WT and cTnI_{PKC-P} myofibrils with PPIA and PPA2A to dephosphorylate cTnI (Figure 1B). The WT cTnI spot profile was reduced to the U (unmodified) spot, indicating: (1) that phosphorylation was the primary posttranslational modification, (2) complete dephosphorylation, and (3) that at least 1 phosphorylation site was associated with each Px spot. In the phosphatase-treated cTnI_{PKC-P} samples, there were 2 spots: U and mutant cTnI (indicated by an asterisk in Figure 1B). The ratio of the intensity of the mutant cTnI spot to the total intensity (i.e., sum of unmodified spot [U] and mutant spot) represents the percentage replacement, which was 7.2 ± 0.5% (n = 9) (Online Table I).

To verify the protein identity of the spots, membrane transfers of the 2D-DIGE gels were probed with a specific pan cTnI antibody. Figure 1C illustrates multiple spots in the untreated samples, whereas only one spot is visible in the phosphatase-treated sample, a pattern similar to that in the 2D-DIGE gels (Figure 1A and 1B, second images). All 6 spots are identified in the Online Figure IB. The mutant cTnI spot was also confirmed by Western analysis (Online Figure IA).

There were no differences in the relative spot intensities (U, P1–P5) of cTnI in the WT and cTnI_{PKC-P} samples, except the presence of the mutant cTnI spot (Figure 1A and Online Table I). This indicates that the basal pattern of actual phosphorylation of cTnI was unchanged in the TG mouse. Moreover, there were no differences in the phosphorylation levels of cTnT, tropomyosin, myosin regulatory light chain,
The slope of the cTnIPKC−P mice (WT: 2.9 ± 0.067 mm Hg · ms⁻¹ · mL⁻¹, n = 6; cTnI_PKC-P: 2.5 ± 0.1 mm Hg · μL⁻¹, n = 6, P = 0.02), with no change in the intercept, indicating a 14% reduction in LV contractile state over all lengths (Figure 3A). Additionally, there was a decrease in the slope of the dσ/dt_max−volume relationship (WT: 0.084 ± 0.004 mm Hg · ms⁻¹ · μL⁻¹, n = 6; cTnI_PKC-P: 0.074 ± 0.003 mm Hg · ms⁻¹ · μL⁻¹, n = 6, P = 0.03), with no change in the intercept, indicating a 12% decrease in the kinetic aspects of contraction (Figure 3B).

The cTnI_PKC-P mice exhibited slowed relaxation as evidenced by a parallel, upward shift of the T_relax−volume relationship (intercept values: WT: 16 ± 1 ms, n = 6; cTnI_PKC-P: 21 ± 3 ms, n = 6, P = 0.005), with no change in the slope, indicating a 20% increase (maximum) in the time for the LV to relax (Figure 3C). Consistent with this observation, there was a decrease in the magnitude of the slope of the dt_max−volume relationship (WT: −0.067 ± 0.003 mm Hg · ms⁻¹ · μL⁻¹, n = 6; cTnI_PKC-P: −0.058 ± 0.003 mm Hg · s⁻¹ · mL⁻¹, n = 6, P = 0.03), with no change in the intercept, indicating a 16% reduction in the kinetic aspects of relaxation (Figure 3D).

The cTnI_PKC-P mice showed no differences in passive properties compared to WT mice. There were no statistical differences in P_stiff (Figure 2D, triangles) or σ_stiff (Online Figure I) over the entire range of volumes studied.

Treatment with 1 μmol/L isoproterenol increased developed pressures to the same degree in both WT and cTnI_PKC-P (Table 1). Isoproterenol also shortened rise and relaxation times to the same degree in WT and cTnI_PKC-P animals (Table 1).

**Isolated Papillary Muscle Experiments**

Figure 4A shows representative force and calcium data from one WT and one cTnI_PKC-P experiment. The cTnI_PKC-P mice exhibited decreased force production, indicated by a downward shift of the developed force (F_max−length relationship (intercept values: WT: 1.88 ± 1.22 mN · mm⁻², n = 5; cTnI_PKC-P: −0.51 ± 0.35 mN · mm⁻², n = 6, P = 0.04, Figure 4B). There was also slowed relaxation in the cTnI_PKC-P mice, as evidenced by an
Discussion

We created a new TG mouse model (cTnIPKC-P) wherein the 3 PKC phosphorylation sites on cTnI were mutated to glutamic acid to simulate constitutive pseudophosphorylation. Despite low integration of mutant protein (~7%), TG mice show significant functional changes, indicating high sensitivity of cardiac contraction to PKC cTnI phosphorylation. There are 3 main experimental findings of the present study. Compared to WT mice, cTnIPKC-P mice exhibited: (1) decreased active contraction and slowed relaxation, (2) a decreased sensitivity of cardiac contraction to PKC cTnI phosphorylation, (3) decreased maximal activated force without changes in calcium sensitivity or tension cost. We discuss each of these observations individually below, followed by an integrative interpretation that reconciles these experimental findings.

Table 1. Summary of Isoproterenol Treatment Data

<table>
<thead>
<tr>
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<th>WT (n=7)</th>
<th>TG (n=7)</th>
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<tbody>
<tr>
<td>ΔPdev (mm Hg)</td>
<td>26±2</td>
<td>29±3</td>
</tr>
<tr>
<td>ΔdP/dtmax (mm Hg · ms⁻¹)</td>
<td>3.37±0.35</td>
<td>3.20±0.21</td>
</tr>
<tr>
<td>ΔTrise (ms)</td>
<td>14±1</td>
<td>14±1</td>
</tr>
<tr>
<td>ΔTrelax (ms)</td>
<td>68±13</td>
<td>56±12</td>
</tr>
</tbody>
</table>

Values are means±SEM. Pdev indicates developed pressure; Trise, rise time; Trelax, relaxation time. There were no differences in the response to isoproterenol treatment between the WT and TG mice at Vmax (volume that produced maximum Pdev).
TG Mouse Model

The cTnIPKC-P mouse showed ~7% replacement of endogenous cTnI with mutant cTnI. There were no differences in the basal actual phosphorylation pattern of cTnI. Therefore, there is an increase in total phosphorylation of cTnI at the PKC sites: the summation of basal actual phosphorylation (unchanged in cTnIPKC-P mice) and pseudophosphorylation (increased by ~7% in cTnIPKC-P mice). In spite of the relatively low level of replacement, there were significant functional effects. This is an unexpected and remarkable finding, suggesting a high sensitivity of cardiac contraction to PKC-mediated cTnI phosphorylation and potentially important physiological and pathophysiologic roles for this posttranslational regulatory process.

Recent evidence indicates that basal in vivo cTnI phosphorylation at the PKC sites is very low. Thus, the percentage replacement in our TG mice, although small, may represent a physiologically relevant level of increased PKC cTnI phosphorylation. Our previous mouse model, cTnIS43/S45-NP (serine 43 and 45 mutated to alanine), showed ~50% replacement of endogenous cTnI by the mutant cTnI. However, given the low basal phosphorylation state, a higher replacement of nonphosphorylatable sites would be required to observe functional effects.

There were also no alterations in phosphorylation of tropomyosin, TnT, essential light chain, or myosin binding protein-C, indicating that the observed effects were from cTnI PKC pseudophosphorylation alone.

Contraction

There was evidence of reduced contraction in the cTnIPKC-P mice compared to WT mice at all 3 levels studied: (1) isolated papillary muscles.
heart experiments showed depressed developed pressures, (2) intact papillary muscles exhibited lower developed force, and (3) skinned fibers showed significantly lower $F_{\text{max}}$. In reconstituted fibers where the cTn PKC phosphorylation sites were rendered constitutively pseudophosphorylated, a decrease in $F_{\text{max}}$ was also seen. The cTnIS$_{43}$/S$_{45}$-NP mouse model showed an increase in developed pressures at high extracellular calcium levels (3.5 mmol/L $[\text{Ca}]$). Together with previous data, the new TG model supports the hypothesis that phosphorylation of cTn by PKC lowers the ability of the myocardium to generate active force, both under dynamic and steady-state activations.

### Relaxation

In isolated heart and intact papillary muscle experiments, cTnIPKC-P mice also showed negative lusitropy when compared to control mice. This is consistent with the results of Pi et al, who treated WT mice with the PKC activator endothelin-1 and observed an increase in relaxation time (negative lusitropy). Furthermore, in their TG animal, in which all 5 cTn phosphorylation sites were replaced with alanines, the effects of endothelin-1 were severely blunted. These results, combined with the results presented here, suggest that phosphorylation of the PKC sites on cTn has a negative lusitropic effect. By that same logic, the cTnIS$_{43}$/S$_{45}$-NP mouse would be expected to exhibit positive lusitropy. By that same logic, the cTnIPKC-P mice also showed negative lusitropy when compared to WT mice. Burkart et al conducted experiments on detergent-extracted cardiac fibers reconstituted with 3 forms of mutant cTn that were pseudo phosphorylated at the PKC sites: S$_{43}$/S$_{45}$, S$_{43}$/S$_{45}$/T$_{144}$, and T$_{144}$E. They showed that S$_{43}$/S$_{45}$ and S$_{43}$/S$_{45}$/T$_{144}$ fibers exhibited similar decreases in calcium sensitivity. However, there was no change in calcium sensitivity in T$_{144}$E fibers, suggesting that phosphorylation at this site plays no role. This is inconsistent with data from Wang et al, who treated WT mice with the PKC activator tertenol (1 mol/L) infusion: increasing developed pressure (R$_{Syst}$ or $R_{sys}$/R$_{act}$), instead of calibrated data, were used in analyzing calcium transients. The reasons for using this approach and its validity are discussed in Online Appendix 3.

### Calcium Sensitivity

cTnIPKC-P mice did not exhibit differences in calcium sensitivity compared to WT mice. Burkart et al conducted experiments on detergent-extracted cardiac fibers reconstituted with 3 forms of mutant cTn that were pseudo phosphorylated at the PKC sites: S$_{43}$/S$_{45}$/E, S$_{43}$/S$_{45}$/E/T$_{144}$E, and T$_{144}$E. They showed that S$_{43}$/S$_{45}$/E and S$_{43}$/S$_{45}$/E/T$_{144}$E fibers exhibited similar decreases in calcium sensitivity. However, there was no change in calcium sensitivity in T$_{144}$E fibers, suggesting that phosphorylation at this site plays no role. This is inconsistent with data from Wang et al, who treated WT mice with the PKC activator tertenol (1 mol/L) infusion: increasing developed pressure (R$_{Syst}$ or $R_{sys}$/R$_{act}$), instead of calibrated data, were used in analyzing calcium transients. The reasons for using this approach and its validity are discussed in Online Appendix 3.

### Differential Interpretation

Our goal was to identify the underlying changes in myofilamental processes that can simultaneously explain all of our results. We can group the contraction/relaxation processes into 3 main categories: (1) activator calcium, (2) myofilament calcium–handling proteins (eg, sarcoplasmic reticulum ATPase pump, L-type calcium channels). These results show that the mutation we introduced in cTn did not affect these PKA-dependent pathways.

### Intracellular Calcium Transients

There were no differences in intracellular calcium amplitude or relaxation between WT and cTnIPKC-P mice. We have previously reported that cTnI$_{S_{43}$/S$_{45}$-NP} mice do not exhibit any differences in intracellular calcium at normal extracellular calcium levels. Others have also shown that there are no changes in intracellular calcium with PKC phosphorylation of cTnI. Normalized fluorescence values ($R_{Syst}$ or $R_{sys}$/R$_{act}$), instead of calibrated data, were used in analyzing calcium transients. The reasons for using this approach and its validity are discussed in Online Appendix 3.

### Summary of Skinned Fiber Data

<table>
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<tr>
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<th>WT (n=10)</th>
<th>TG (n=10)</th>
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<tbody>
<tr>
<td><strong>Force pCa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max (mN mm$^{-2}$)</td>
<td>65.7±1.6</td>
<td>56.6±2.5*</td>
</tr>
<tr>
<td>$pCa_{50}$</td>
<td>5.74±0.01</td>
<td>5.76±0.01</td>
</tr>
<tr>
<td>$\eta_{1}$</td>
<td>2.98±0.13</td>
<td>2.84±0.11</td>
</tr>
<tr>
<td><strong>MgATPase pCa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max (pmol mm$^{-3}$ sec$^{-1}$)</td>
<td>477.3±23.1</td>
<td>434.4±23.4</td>
</tr>
<tr>
<td>$pCa_{50}$</td>
<td>5.77±0.02</td>
<td>5.75±0.01</td>
</tr>
<tr>
<td>$\eta_{1}$</td>
<td>2.60±0.13</td>
<td>2.56±0.07</td>
</tr>
<tr>
<td><strong>Tension Cost</strong></td>
<td>7.33±0.13</td>
<td>7.41±0.10</td>
</tr>
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</table>

Values are means±SEM. *P<0.05 vs WT.
The model also indicated that an isolated decrease in $d$ was associated with an increase in calcium sensitivity and slowed relaxation. Experimental data suggests T144 phosphorylation causes an increase in calcium sensitivity\(^1\) and is primarily responsible for slowed relaxation. It is therefore tempting to attribute the calcium-independent persistence of myofilament active state (ie, decreased $d$) to T144 phosphorylation. Likewise, the model indicated that an isolated decrease in $f$ was associated with a decrease in calcium sensitivity, no change in relaxation, and negative inotropy. Experimental data suggest that phosphorylation of S43 and S45 results in decreased calcium sensitivity, no change in relaxation, and negative inotropy. Experimental data suggest that phosphorylation of S43 and S45 results in decreased calcium sensitivity, no change in relaxation, and negative inotropy. Thus, it is possible that the decrease in rate constant of cross-bridge formation ($f$) is a result of S43 and S45 phosphorylation. The precise biophysical mechanisms underlying the regulation of myofilament contractile properties by PKC-mediated phosphorylation of cTnI are not presently known.

**Limitations**

(1) Although the 2D-DIGE data clearly indicate unchanged overall pattern of actual phosphorylation of cTnI in TG mice (Figure 1A and Online Table I), we cannot definitely say that cTnI phosphorylation at PKA sites was unchanged. Additional data using mass spectrometry will be needed to quantify cTnI phosphorylation at individual sites. (2) Because a single TG mouse line was used in the present study (ie, a single level of transgene expression), it is not possible to determine how the observed effects scale with the level of cTnI phosphorylation at PKC sites. Additional TG mouse lines and/or experiments using reconstituted fibers will be necessary to address this issue. (3) Although it is a commonly used technique, pseudophosphorylation by glutamate replacement may not fully recapitulate actual phosphorylation. Furthermore, the simultaneous (pseudo)phosphorylation of all 3 PKC sites may or may not be a physiologically relevant pattern of cTnI phosphorylation. However, our TG mouse data, together with data from the literature, provide new insights into the effects of PKC-mediated cTnI phosphorylation.

**Summary**

Our data show a small increase in cTnI phosphorylation at PKC sites produces significant functional changes, indicating
high sensitivity of cardiac contraction to PKC-mediated cTnI phosphorylation. Model-based analysis predicts that these functional changes are brought about by specific changes in myofilament contractile properties: decreased rate of cross-bridge formation and calcium-independent persistence of the active state. Based on our data and data from the literature, we speculate that the effects of PKC-mediated cTnI phosphorylation are site-specific (S43/S45 versus T144).

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We thank Kelly Clause and Dr Partha Roy for assistance with molecular biology–related issues and Dr Kenneth Campbell for illuminating discussions regarding the mathematical model–based analysis.

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Disclosures
None.

References
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SUPPLEMENTAL MATERIAL

Left Ventricular and Myocardial Function In Mice Expressing Constitutively Pseudo-Phosphorylated Cardiac Troponin I

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APPENDIX 1: DETAILED METHODS

BIOCHEMICAL AND MOLECULAR BIOLOGICAL METHODS AND ANALYSIS

Generation and Initial Characterization of Transgenic Mice. Serine 43 and 45 and threonine 144 residues on cTnI were replaced with glutamic acid to simulate constitutive phosphorylation. The cDNA for this mutant cTnI was placed under the control of the cardiac specific α-myosin heavy chain promoter. Steps to create the transgenic mouse were similar to the creation of the cTnIser43/45-NP mouse and have been described elsewhere. This mouse will be referred to as cTnI_{PKC-P}, or simply as the transgenic (TG) mouse.

Polymerase Chain Reaction. Polymerase Chain Reaction (PCR) was performed to identify the cTnI_{PKC-P} mice. The two primers (Applied Biosystems, Foster City, CA) were: 5'-CCTTGTCTTGGATTAATCTTGGC-3' (binds to the myosin heavy chain promoter) and 5'-CTCCTGCTTGCACAATCTGAGC-3' (binds to cTnI sequence). The PCR product was then sequenced by the University of Pittsburgh DNA Sequence Core Facilities, to confirm the mutant cTnI sequence.

2D-DIGE. Myofibrils were purified from liquid nitrogen frozen mouse tissue and homogenized twice in standard relax buffer (10 mmol/L Imidazole pH 7.2, 75 mmol/L KCl, 2 mmol/L MgCl₂, 2 mmol/L EDTA, and 1 mmol/L NaN₃) with 1% (v/v) Triton X-100. For the untreated samples, the pellet was solubilized in UTC buffer (8 mol/L urea, 2 mol/L thiourea, and 4% (w/v) CHAPS). The phosphatase treated samples were washed twice in phosphatase buffer (25 mmol/L Tris-HCl pH 7.0, 0.2 mmol/L MnCl₂, 0.1 mmol/L EDTA, 5 mmol/L DTT and mammalian protease inhibitors from Sigma 1:100) then resuspended in 50 μL phosphatase buffer plus PP1A (10 units) and PP2A₁ (0.1 unit) (Calbiochem). The samples were then allowed to incubate for 4 hrs at 37°C to completely de-phosphorylate the cTnI. After the incubation period, myofibrils were spun down and the supernatant was removed. The pellet was resuspended in UTC buffer. Protein (in UTC buffer) concentration was determined with an RC-
DC assay kit (BioRad, Hercules, CA). Samples in UTC buffer (100 μg) were cleaned up with GE Healthcare’s 2D clean-up kit (Piscataway, NJ) and then re-suspended in UTC buffer.

Samples were randomly labeled with fluorescent cyanine dyes (Cy2, Cy3, and Cy5) from GE Healthcare (Piscataway, NJ) to control for any dye differences. Protein samples were labeled by adding 100 pmoles of CyDye to 50 μg of protein and then quenched with 10 mM L-lysine. The gel’s first dimension was focused using the Protean IEF cell (BioRad, Hercules, CA) with pH 4-7 or 7-11 IPG strips. The samples were actively rehydrated at 50 V in 18 cm IPG strips pH 4-7, or 7-11NL 10-16 hrs with 80-120 μg of total protein (40 μg/channel). The focusing in the first dimension was achieved using a preset linear program: 250 V rapid ramping for 15 min, 10,000 V linear ramping for 3 hours, 10,000 V rapid ramping until 60,000 V•hours. The strips were equilibrated and laid onto 12% SDS-PAGE gels. Gels were imaged with a Typhoon 9410 scanned at 100 µm and analyzed with PDQuest software (v7.1, BioRad, Hercules, CA). PDQuest software analyzes the gel images by determining the optical densities of each spot. The spot density as a percentage of total was computed by dividing the density of a particular protein spot by the total of all the spot densities for that protein. We then averaged the percent of each spot density yield the mean and standard error of the mean within each group and compared using a T-test with a level of significance set at P-value < 0.05. Gels were transferred on PVDF membrane and analyzed as previously described. The primary antibody used for cTnl was a pan monoclonal C5 1:5000 (Fitzgerald) and the secondary antibody was an anti-mouse-IgG (FAB) specific 1:100000 (Sigma).
APPENDIX 2: DETAILED METHODS

ISOLATED PERFUSED HEART METHODS AND ANALYSIS

This study was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh, and conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Isolated heart preparations were similar to those we have done in the past. Male WT FVB and cTnI_{PKC-P} mice of similar age (121 – 140 days) were used. The mice were anesthetized with an intraperitoneal injection of Avertin (2,2,2-tribromoethanol, 250 mg/kg body weight). Animals were then anticoagulated using 100 units of heparin. Once the heart was excised from the animal, the aorta was quickly cannulated. We then began retrograde aortic perfusion at a constant pressure of 70-80 mmHg. Tissue culture medium (Media 199, Invitrogen, Carlsbad, CA) was used as the perfusate (1.8 mmol/L [Ca]). To this solution the following were added: 5.0 mmol/L creatine, 4.8 mmol/L taurine, 6.0 mmol/L carnitine, 2.5 mmol/L sodium pyruvate, 8 µmol/L insulin, and 5.0 µmol/L metoprolol (to eliminate pacing-induced catecholamine release). The solution was oxygenated with 95% O₂ and 5% CO₂, pH adjusted to 7.4, and temperature maintained at 37 °C. A balloon made from high density polyethylene (HDPE) was placed in the left ventricle through the mitral valve and secured with a suture though the left ventricular apex. A catheter-tip pressure transducer (MPC-500, Millar Instruments, Houston, TX) was used to measure left ventricular pressure. The balloon volume was altered using a thumb-screw controlled 100 µL micro-syringe (Hamilton Gas Tight, Reno, NV). Platinum pacing electrodes were placed on the heart, which was then paced at 4 Hz using a Harvard stimulator (Harvard Apparatus, Holliston, MA). Pressure data were digitized on-line at 500 Hz for later offline analysis.

Isolated Heart Protocols. A Frank Starling protocol was performed on all of the isolated hearts. A reference LV volume (V_{ref}, typically 10–12 µl) was set by adjusting the balloon volume to yield a LV end diastolic pressure of 5 mmHg. Pressure data were continuously recorded over...
a 2-min period. The first 20 seconds corresponded to data at the reference volume, followed by a period wherein LV volume was increased in 2 µl increments to the point when developed pressure had reached its maximum value ($V_{\text{max}}$). The Frank Starling protocol alone was performed on one subset of experiments (wild-type, WT: $n = 6$, transgenic, cTnI PKC-P: $n = 6$).

In the second subset of the experiments (WT: $n = 6$, cTnI PKC-P: $n = 6$), the Frank Starling protocol was performed, and then isolated hearts were treated with 1 µmol/L isoproterenol for ten minutes. A second Frank-Starling protocol was conducted on the heart post isoproterenol treatment.

**Data Analysis.** In isolated heart experiments, individual cardiac cycles were first identified by using the spike in the pacer signal. Signal averaging over the cardiac cycle was then performed to reduce high-frequency random noise. Pressure data from five to seven steady state contractions at a given LV volume were averaged to yield a representative cycle. LV pressure waveforms were characterized by several amplitude and rate indices. Amplitude indices included end diastolic pressure ($P_{\text{ed}}$), peak systolic pressure ($P_{\text{sys}}$), and developed pressure ($P_{\text{dev}} = P_{\text{sys}} - P_{\text{dia}}$). Rate indices included maximal rate of rise ($dP/dt_{\text{max}}$), maximal rate of fall ($dP/dt_{\text{min}}$), rise time ($T_{\text{rise}}$), and relaxation time ($T_{\text{relax}}$). $T_{\text{relax}}$ was defined as the time taken for developed pressure to decay from 75% to 25% of its maximum value. Similarly, $T_{\text{rise}}$ was defined as the time taken for pressure to rise from 25% to 75% of its maximum value. Calculations of $T_{\text{relax}}$ and $T_{\text{rise}}$ were performed after the diastolic value of the pressure waveform was subtracted, such that each waveform rose from and declined to zero.

Wall stress ($\sigma$) was estimated using a thick-walled spherical model from equation A2.1:

$$\sigma = \frac{P}{\left(1 + \frac{M}{\rho V}\right)^{\frac{3}{2}} - 1}$$

where $P$ is pressure, $M$ is the mass of the left ventricle, $\rho$ is the density of muscle, which was assumed to be 1.05 g/ml, and $V$ is the volume of the ventricle. Equation A2.1 was used to
calculate $\sigma_{\text{dev}}$, $\sigma_{\text{ed}}$, $d\sigma/dt_{\max}$, and $d\sigma/dt_{\min}$ by using $P_{\text{dev}}$, $P_{\text{ed}}$, $dP/dt_{\max}$ and $dP/dt_{\min}$ in place of $P$, respectively.

Statistical Analysis. For the isolated heart data, regression analysis with dummy variables was used to identify differences in various relationships between wild-type and transgenic groups: $\sigma_{\text{dev}}$–volume, $d\sigma/dt_{\max}$–volume, $T_{\text{relax}}$–volume, $d\sigma/dt_{\min}$–volume.
APPENDIX 3: DETAILED METHODS

ISOLATED PAPILLARY MUSCLE METHODS AND ANALYSIS

Male FVB mice of similar age and body mass were anesthetized with inhaled isoflurane, and then cervically dislocated. The heart was quickly excised and placed in modified Krebs-Henseleit (KH) solution containing 30 mmol/L 2,3-butadione monoxime (BDM). The KH solution contained 141 mmol/L NaCl, 50 mmol/L Dextrose, 25 mmol/L NaHCO₃, 5 mmol/L HEPES, 5 mmol/L KCl, 1.2 mmol/L NaH₂PO₄, 1 mmol/L MgSO₄ and 2.0 mmol/L CaCl₂, pH adjusted to 7.35, and bubbled with 95% O₂, 5% CO₂. Thin papillary muscle strips of similar shape and size were dissected from the right ventricle and mounted by suturing the valve and ventricular tissue to a force transducer (Aurora Scientific Inc., Aurora, Ontario, Canada) and motor arm (Aurora Scientific Inc., Aurora, Ontario, Canada). The muscle was lowered into a constantly perfused bath containing KH solution and maintained at 37 °C, and paced at 2 Hz. Stimulation voltage and muscle length (Lmax) were adjusted to obtain maximum developed force. The muscle was allowed to equilibrate for at least 30 minutes before performing the experimental protocol. Muscle diameter was recorded using a digital video microscopy system (Ionoptix, Milton, MA), and cross-sectional area (mm²) was calculated assuming cylindrical geometry.

Calcium measurements using fura-5F required a system capable of dual excitation, single emission measurements. An IonOptix Hyperswitch (Ionoptix) alternated between excitation frequencies of 340 and 380 nm at 1000 Hz. The setup also included an inverted microscope, a filter, and a pair of photomultiplier tubes. Background fluorescence was recorded before dye loading. Fura-5F AM (50 μg) was dissolved in 25 μL dimethyl sulfoxide (DMSO) and 25 μL Pluronic. To this solution, 2.755 mL KH solution, 4.3 mg/L TPEN, and 5.0 mg/L cremophor were added. The muscle was loaded with this solution for 30 minutes. The ratio of fluorescence emission from 340 nm excitation and 380 nm excitation, \( R \), was calculated after subtracting background fluorescence.

Data Analysis. In isolated papillary muscle experiments, individual cycles were first
identified by using the spike in the pacer signal. Signal averaging over the cycle was then performed to reduce high-frequency random noise. Force and fluorescence data from five to seven steady state contractions at a given length were averaged to yield a representative cycle. Force waveforms were characterized by several amplitude and rate indices. Amplitude indices included end diastolic force ($F_{ed}$), peak systolic force ($F_{sys}$), and developed force ($F_{dev} = F_{sys} - F_{dia}$). Rate indices included maximal rate of rise ($dF/dt_{max}$), maximal rate of fall ($dF/dt_{min}$). The calcium transient was characterized in terms of $R/R_{ed}$ ($R_{ed}$: fluorescence ratio at end-diastole), $R_{sys}/R_{ed}$ ($R_{sys}$: peak systolic fluorescence) and $dR/dt_{min}$.

Muscle length was normalized to $L_{max}$ and 70% $L_{max}$, representing the full range of force producing lengths (sarcomere lengths of 2.4 µm and 1.7 µm).

**Calcium Calibration.** We did perform the standard calibration measurements after every papillary muscle experiment (i.e., collecting $R_{min}$ and $R_{max}$ values corresponding to zero (or near-zero) and saturating intracellular calcium). However, we found that in approximately half of the experiments, the measured calibration values did not make physical sense (e.g., $R_{min}$ measured at the end of the experiment below the background value measured at the beginning of the experiment). Therefore, we decided to report our calcium measurements in terms of uncalibrated fluorescence ratios, which should be valid for relative comparisons, if not for absolute values of calcium. In about half of the experiments, the calibration values did make sense (n: WT = 3, TG = 3). Upon reviewing these subset of data, we found no significant difference in diastolic calcium between the WT and TG groups (WT = 401 ± 142 nM, TG = 524 ± 152 nM, $P = 0.58$). These values are similar to calibrated calcium levels reported in the literature.

**Statistical Analysis.** For the papillary muscle data, regression analysis with dummy variables was used to identify differences in various relationships between wild-type and transgenic groups: $F_{dev}$-length, $dF/dt_{min}$-length, $R/R_{ed}$-length, and $dR/dt_{min}$-length.
APPENDIX 4: DETAILED METHODS

SKINNED FIBER METHODS AND ANALYSIS

This study was approved by the Institutional Animal Care and Use Committee of Washington State University, and conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Both the wild-type and the transgenic mice were anesthetized with pentobarbital sodium (50 mg/kg body wt), and hearts were rapidly excised and immediately placed into ice-cold high relaxing (HR) solution containing (in mM) 20 MOPS, 53 KCl, 10 EGTA, 6.81 MgCl₂, 5.35 Na₂ATP, and 1.0 DTT. The pH was adjusted to 7.0 with KOH and a fresh cocktail of protease inhibitors (in μM: 4 benzamidine-HCl, 5 bestatin, 2 E-64, 10 leupeptin, 1 pepstatin, and 200 PMSF) were added to the fresh ice-cold buffered solutions. Papillary muscle fiber bundles were then carefully cut from the wall of the left ventricles of wild-type and transgenic mouse hearts. Thin muscle fiber bundles (~200 μm in width and ~2.0 mm in length) were dissected and were detergent skinned overnight using 1% TritonX-100 to chemically permeabilize the muscle fiber preparations.

Simultaneous Measurement of Isometric Force and ATPase Activity. The detergent-skinned muscle fiber bundles were attached to the arm of a displacement motor (model 308B, Aurora Scientific Inc) on one end and to a force transducer (AE 801, Sensor One Technologies Corporation) on the other end using aluminum T-clips. The sarcomere length of the muscle fiber bundle was adjusted to 2.2μm using a He-Ne laser diffraction system. The sarcomere length was readjusted to 2.2μm after two cycles of full activation and relaxation to stabilize the resting sarcomere length throughout the later stages of the experiment. The elliptical cross-sectional area of the muscle fiber was determined by measuring the width and thickness at three points along the length of the muscle fiber bundle. The muscle fiber bundle was immersed in a 15-μL bath containing activating solutions with different pCa (−log₁₀([Ca²⁺])). Activating solution in the
bath was constantly stirred by means of a motor-driven vibration of the membrane positioned on the underside of the bath. Maximum activating solution (pCa 4.3) contained (in mM) 31 potassium propionate, 5.95 Na₂ATP, 6.61 MgCl₂, 10 EGTA, 10.11 CaCl₂, 50 BES (pH 7.0), 10 NaN₃, 0.9 NADH, and 10 phosphoenolpyruvate as well as 4 mg/ml pyruvate kinase (500 U/mg), 0.24 mg/ml lactate dehydrogenase (870 U/mg), and 20 μM A₂P₅, and a cocktail of protease inhibitors. The Ca²⁺ concentration of the activating solution was changed to produce steady-state force-generating activations at pCa 4.3, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, and 9.0¹⁵.

Isometric force and ATPase activity were simultaneously measured at 20°C as described previously¹³, ¹⁴. Briefly, the force developed at steady-state was measured by the force transducer and recorded using LabView software. ATPase activity of the detergent-skinned muscle fiber bundles was measured by projecting near UV light through the muscle chamber. Light was split via a beam splitter (50/50) and intensities measured at 340 nm (sensitive to changes in NADH concentration) and at 410 nm (insensitive to changes in NADH concentration), which served as a reference signal. An analog divider and a log amplifier produced a signal proportional to the amount of ATP consumed in the muscle chamber solution. ATP regeneration from ADP was enzymatically coupled to the breakdown of phosphoenolpyruvate (PEP) by pyruvate kinase to pyruvate with a concomitant regeneration of ATP from ADP. This reaction is coupled to the synthesis of lactate from pyruvate catalyzed by lactate dehydrogenase during which process there is a breakdown of NADH. This breakdown of NADH is proportional to the amount of ATP consumed and was measured online by UV absorbance at 340 nm¹⁴.

Data Analysis. For skinned fiber experiments, normalized force or MgATPase activity was calculated as the ratio of the measured quantity at a given pCa and the maximally activated value (i.e., at pCa = 4.33). Normalized force-pCa and MgATPase-pCa data were fitted to a modified Hill equation (equation A3.1) using a nonlinear regression algorithm (Prism, GraphPad Software, Inc., San Diego, CA):
\[ \frac{X}{X_{\text{max}}} = \frac{1}{1 + 10^{n_H (p\text{Ca}_{50} - p\text{Ca})}} \]  

(A4.1)

where \( X \) is a measured quantity at a given pCa and \( X_{\text{max}} \) is the maximally activated value (i.e. at pCa = 4.33). Two parameters were estimated from normalized force-pCa data for each fiber: \( p\text{Ca}_{50} \) (pCa required to produce normalized value of 50%) and the Hill coefficient \( (n_H, \text{a measure of the steepness of the normalized force-pCa or MgATPase-pCa curve, which characterizes the cooperative phenomena}) \).
APPENDIX 5: DETAILED METHODS

MODELING METHODS

The four-state model schematic is shown in Figure 6B, and the model is described by the following set of ordinary differential equations (Equations A5.1-A5.5):

\[
\dot{A} = -k_1[A][A] + k_3[Ca-A] + d[A-M] \tag{A5.1}
\]

\[
\dot{M} = d[A-M] - f[Ca-A][M] + g[Ca-A-M] \tag{A5.2}
\]

\[
[Ca-A] = k_1[Ca][A] - k_3[Ca-A] - f[Ca-A][M] + g[Ca-A-M] \tag{A5.3}
\]

\[
[Ca-A-M] = f[Ca-A][M] + k_2[Ca][A-M] - g[Ca-A-M] - k_4[Ca-A-M] \tag{A5.4}
\]

\[
[A-M] = k_4[Ca-A-M] - d[A-M] - k_2[Ca][A-M] \tag{A5.5}
\]

Initial conditions represent the concentrations (per unit volume of sarcoplasm) at t=0 of each state prior to introduction of free calcium\textsuperscript{16,17}: 

\[ [A]_{(t=0)} = 70 \text{ µM}; \quad [M]_{(t=0)} = 20 \text{ µM}; \quad [Ca-A]_{(t=0)} = 0 \muM; \quad [Ca-A-M]_{(t=0)} = 0 \muM; \quad [A-M]_{(t=0)} = 0 \muM. \]

The force generating states (Equations A5.4 and A5.5) exert a feedback simultaneously on two of the rate constants. This feedback is described by Equations A5.6-A5.7:

\[
k_1(t) = \alpha_1([Ca-A-M] + [A-M])^{1.25} + \beta_1 \tag{A5.6}
\]

\[
f(t) = \alpha_f([Ca-A-M] + [A-M]) + \beta_f \tag{A5.7}
\]

The model output is force, and is the sum of the two force-generating states (Equations A5.4 and A5.5) times a scaling factor, and is shown in Equation A5.8:

\[
F(t) = \gamma([Ca-A-M] + [A-M]) \tag{A5.8}
\]

The following parameters were used for the baseline: \( \alpha_1 (\muM^{-2}\cdot s^{-1}) = 2.0 \), \( \beta_1 \), \( \alpha_f (\muM^{-2}\cdot s^{-1}) = 0.15 \), \( \beta_f (\muM^{-2}\cdot s^{-1}) = 0.7 \), \( k_2 (\muM^{-1}\cdot s^{-1}) = 25 \), \( k_3 (s^{-1}) = 84 \), \( k_4 (s^{-1}) = 84 \), \( g (s^{-1}) = 205.3 \), \( \beta_d (s^{-1}) = 44.6 \).

The model input is calcium. For the steady state data, constant levels of calcium are used as input. For the dynamic data, a time-varying calcium is used, described by Equation A5.9:
\[
[Ca]^i(t) = A(1 - e^{-Bt})(e^{-Ct})
\] (A5.9)

Where \( A = 1.739 \, \mu\text{M} \), \( B = 205.89 \, \text{s}^{-1} \), and \( C = 30.52 \, \text{s}^{-1} \).

The MgATPase activity was calculated from the model-predicted steady-state data using equation A5.10:

\[
\text{MgATPase} = g \cdot [\text{Ca-A-M}] + d \cdot [\text{A-M}]
\] (A5.10)

The parameter \( d \) has very little effect on the value, however, as the \([A-M]\) state is much less populated than the \([\text{Ca-A-M}]\) state. Thus, \( g \) and force production are the primary effectors of MgATPase activity.
Values are expressed as mean ± SEM. TM-P: phosphorylated tropomyosin, RLC-P: phosphorylated myosin regulatory light chain, cTnT-P: phosphorylated troponin T, cTnl-P – cTnl-P5: cTnl with PTMs (1 – 5), S1-S11-MyBP-C: myosin binding protein-C. Quantification of percent phosphorylation of the total indicated by P (n=4). The percent replacement of the mutant troponin I is labeled as replacement (n=9). The percent of myosin binding protein C spot of the total myosin binding protein C spots labeled as S1 through S11 (n=4). Note: There were no significant differences between WT and TG.
Online Table II. Isolated heart and papillary muscle data

Values are mean ± SEM at $V_{max}$ (volume which produced maximum pressure) for isolated hearts and $L_{max}$ (length which produced maximum force) for isolated papillary muscles. WT, wildtype; TG, transgenic; $P_{dev}$, peak developed pressure; $P_{ed}$, end-diastolic pressure; $\sigma_{dev}$, developed stress (using a thick-walled spherical model); $d\sigma/dt_{max}$, maximum time-derivative of stress; $T_{rise}$, rise time of the pressure waveform (25% to 75% of $P_{dev}$); $T_{relax}$, relaxation time of the pressure waveform (75% to 25% of $P_{dev}$); $d\sigma/dt_{min}$, minimum time-derivative of stress; $F_{dev}$, maximum developed force; $dF/dt_{min}$, minimum time-derivative of stress; $R_{sys}$, peak systolic fluorescence ratio; $R_{ed}$, end-diastolic fluorescence ratio; $dR/dt_{min}$, minimum time-derivative of the fluorescence ratio. *$P<0.05$ by dummy analysis over the entire volume/length-range.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>TG</th>
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<tbody>
<tr>
<td><strong>Isolated Heart</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>($data at V_{max}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P_{dev}$ (mmHg)</td>
<td>110.6 ± 7.0</td>
<td>87.8 ± 5.9 *</td>
</tr>
<tr>
<td>$P_{ed}$ (mmHg)</td>
<td>30.7 ± 4.3</td>
<td>25.8 ± 1.6</td>
</tr>
<tr>
<td>$\sigma_{dev}$ (mmHg)</td>
<td>76.8 ± 6.8</td>
<td>61.9 ± 7.5 *</td>
</tr>
<tr>
<td>$d\sigma/dt_{max}$ (mmHg ms⁻¹)</td>
<td>2.32 ± 0.17</td>
<td>1.90 ± 0.23 *</td>
</tr>
<tr>
<td>$T_{rise}$ (ms)</td>
<td>26 ± 3</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>$T_{relax}$ (ms)</td>
<td>36 ± 4</td>
<td>41 ± 5 *</td>
</tr>
<tr>
<td>$d\sigma/dt_{min}$ (mmHg ms⁻¹)</td>
<td>-1.76 ± 0.16</td>
<td>-1.48 ± 0.14 *</td>
</tr>
<tr>
<td><strong>Isolated Papillary Muscle</strong></td>
<td></td>
<td></td>
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<tr>
<td>($data at L_{max}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$F_{dev}$ (mN mm⁻²)</td>
<td>9.4 ± 1.5</td>
<td>4.4 ± 0.6 *</td>
</tr>
<tr>
<td>$dF/dt_{min}$ (mN mm⁻² s⁻¹)</td>
<td>-144 ± 16</td>
<td>-81 ± 13 *</td>
</tr>
<tr>
<td>$R_{sys}/R_{ed}$</td>
<td>1.6 ± 0.1</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>$dR/dt_{min}$ (s⁻¹)</td>
<td>-96 ± 17</td>
<td>-64 ± 12</td>
</tr>
</tbody>
</table>
Online Figure I. Western blot of 2D gel probed with cardiac TnI specific antibody either treated with phosphatase (PP1A and PP2A1) or not treated. U, unmodified protein and Px, post translational modified with at least one phosphorylation site associated with the spot (where x= number of possible modifications). TG spot, transgenic mutant cTnI. (A) Transgenic sample probed with a cTnI specific antibody indicating a faint transgenic spot and a dominant unphosphorylated spot. (B) Same blot as in Figure 1C, except for a longer exposure to show all 6 spots. Wild type sample probed with cTnI specific antibody indicating 6 spots reacting with the antibody. Note: Enhanced chemiluminescence (ECL) is similar to photographic development in that the linear range is limited. This results in saturated and exaggerated spots corresponding to abundant proteins as compared to spots corresponding to proteins that are orders of magnitude less abundant. We did not use the Western blot analysis to quantify the differences; it only served the purpose of identifying the spots as cTnI.
Online Figure II. Passive mechanical properties for (A) isolated hearts and (B) intact papillary muscles. (A) The end-diastolic stress ($\sigma_{ed}$) – volume relationship. (B) The end-diastolic force ($F_{ed}$) – normalized length relationship. There were no differences between the WT (closed) or cTnl$_{PKC-P}$ mice (open) in either the isolated heart at any volume or intact papillary muscle at any length.


