Troponin I protein kinase C phosphorylation sites and ventricular function

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Abstract

Objective: Cardiac Troponin I (cTnI) phosphorylation by protein kinase C (PKC) results in a reduction of maximal actomyosin ATPase activity, an effect that is more marked at higher levels of calcium (Ca\textsuperscript{2+}) and is likely to reduce active force development. We postulated that there would be greater Ca\textsuperscript{2+}-dependent changes in ventricular function in hearts of cTnI transgenic (TG) mice expressing mutant troponin I lacking PKC sites compared to wild-type (WT).

Methods: We studied left ventricular function in isolated perfused hearts over a wide range of left ventricular volumes (Frank-Starling relationships) and mechanical restitution at three levels of perfusate Ca\textsuperscript{2+} (1.5, 2.5, and 3.5 mM). Manganese-enhanced magnetic resonance imaging (MRI) was used to study \textit{in-vivo} sarcolemmal Ca\textsuperscript{2+} influx. The phosphorylation status of cTnI was examined by western blot analysis.

Results: Systolic contractile function in TG mice was altered in a calcium-dependent manner such that ventricular contractility was significantly greater in TG mice only at 3.5 mM perfusate Ca\textsuperscript{2+}. The relaxation process and passive mechanical properties were unaltered in TG mice. Mechanical restitution parameters were abnormal in TG mice only at 1.5 mM perfusate Ca\textsuperscript{2+}. In-vivo MRI data demonstrated up to 48\% reduction in Mn\textsuperscript{2+}-induced contrast enhancement, indicating reduced sarcolemmal Ca\textsuperscript{2+} influx. Western blot analysis indicated increased cTnI phosphorylation in TG mice.

Conclusions: (1) TG mice exhibit calcium-dependent positive inotropy without slowed relaxation and this phenotype is mitigated by concomitant (compensatory) changes of reduced intracellular Ca\textsuperscript{2+} and increased phosphorylation of remaining cTnI sites. (2) The contractile phenotype in TG mice can be interpreted as an amplification of the normal response to changes in cellular Ca\textsuperscript{2+} observed in WT mice. Thus, PKC phosphorylation sites on cTnI play a role in attenuating contractile responses to changes in intracellular Ca\textsuperscript{2+}.

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Keywords: Protein kinase C; Ventricular function; Transgenic animal models; Calcium (cellular); Phosphorylation; Contractile function

1. Introduction

Protein kinase C can phosphorylate several amino acid residues on troponin I, of which the most functionally important appear to be serines 43/45 [1]. Phosphorylation of these sites reduces maximal actomyosin MgATPase activity, and in-vitro substitution of these amino acids with alanine preserves actomyosin MgATPase activity in response to protein kinase C activation [1]. To understand the in-vivo physiological role of troponin I protein kinase C phosphorylation sites, we recently created a transgenic mouse expressing mutant troponin I in which serines 43/45 were replaced by alanine [2]. These mice develop normally, without any heart failure or hypertrophy. As expected, total troponin I phosphorylation was reduced 25\% in transgenic mice [3]. Alterations in protein kinase C activation or translocation to the myofilament do not account for this change [2,4]. Increased contractility related to
preserved actomyosin ATPase activity would be predicted, though in our initial studies, there was no difference in left ventricular developed pressures between transgenic and wild-type hearts. However, several compensatory responses have been observed, that might counteract the positive inotropic effect of the mutation. First, intracellular calcium transient is reduced in transgenic hearts, especially at higher levels of perfusate calcium [2], suggesting a feedback between the myofilament and the processes controlling calcium homeostasis. Second, there is an increase in troponin T basal phosphorylation in transgenic mice [3].

In the present study, we sought to further determine the effects of removing troponin I protein kinase C phosphorylation sites on global left ventricular function in isolated, perfused hearts. Our previous perfused heart studies [2] were performed at a fixed (submaximal) ventricular volume and stimulation rate of 8 Hz. Because higher levels of calcium are associated with protein kinase C activation [5,6] and because the preserved actomyosin MgATPase activity with this mutation is greater at higher levels of calcium [1,3], we predicted increased contractility at high perfusate calcium in transgenic mice compared to controls. Relaxation abnormalities have been reported with alterations in troponin I phosphorylation at protein kinase A sites [7]. We could not assess relaxation effects completely in our previous study because there is insufficient time available for complete relaxation at the pacing rate of 8 Hz in the perfused mouse heart model.

In order to better characterize left ventricular contraction and relaxation, studies were conducted over a wide range of both left ventricular volumes and perfusate calcium and at the pacing rate of 4 Hz. Additional studies were conducted to gain insights into mechanistic bases for the phenotype, including reduced calcium transients we have previously observed in these transgenic mice. Specifically, the mechanical restitution process was studied to assess the effects on the overall dynamics of cellular calcium handling and its contribution to the left ventricular contractile behavior. We also performed manganese-enhanced magnetic resonance imaging (MRI), a measure of in-vivo, sarcolemmal calcium influx [8]. This would assess whether the L-type calcium channel was responsible for, at least in part, the reduced calcium transients in the transgenic mice. Finally, the phosphorylation status of troponin I and levels of calcium handling proteins were examined by Western blot analysis.

2. Methods

2.1. Troponin I transgenic mice

The generation and characterization of the transgenic mice used in the present study have been previously described in detail [2]. Age matched male wild-type controls and transgenic mice were used (wild-type 19.1 ± 4.0 weeks vs. transgenic 18.8 ± 4.0 weeks, P=NS, unpaired t-test). Non-transgenic FVB mice of the same colony that the troponin I transgenic mice were generated from were used as controls.

2.2. Isolated perfused hearts

This study was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh, and conforms with 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). Anesthesia was induced with intraperitoneal injection of Avertin (2,2,2-tribromoethanol, 250 mg/kg body weight) and animals were anticoagulated with 100 units of heparin. Retrograde aortic perfusion at a constant perfusion pressure of 70–80 mm Hg was begun after aortic cannulation. The baseline perfusate solution (modified Krebs solution) consisted of (mM): NaCl 113, KCl 4.7, MgSO4 1.2, Na-EDTA 0.5, NaHCO3 28.0, Glucose 5.5, Pyruvate 5.0, CaCl2 2.5, octanoate 50 μM. The solution was oxygenated with 95% O2 and 5% CO2, and pH adjusted to 7.4. When perfusate calcium concentration was varied (to 3.5 or 1.5 mM), the concentration of NaCl was adjusted appropriately to maintain osmolality. Metoprolol (5.0 μM) (Sigma) was added to the perfusate to eliminate pacing-induced catecholamine release. A balloon made from a plastic shopping bag was placed in the left ventricle through the mitral valve, and secured with a suture through the left ventricular apex. A catheter-tip pressure transducer (MPC-500, Millar Instruments, Houston, TX) was used to measure left ventricular pressure. A screw-controlled micro-syringe (100 μl) was used to inflate the balloon. Hearts were paced at 4 Hz. In most experiments, both Frank–Starling and mechanical restitution protocols were conducted in the same heart. In all experiments, data were first acquired at baseline perfusate calcium of 2.5 mM. Thereafter, perfusate calcium was either reduced to 1.5 mM (wild-type, WT, n=7 and transgenic, TG, n=8) or increased to 3.5 mM (WT, n=8 and TG, n=11). Thus, data were acquired from each heart at two levels of perfusate calcium (2.5 and 1.5 mM or 2.5 and 3.5 mM).

2.3. Frank–Starling protocol

Steady-state isovolumic left ventricular pressure waveforms were recorded over a wide range of left ventricular volumes from an initial value of 14–16 μl and with 2 μl increments (Fig. 1). The maximal left ventricular volume (Vmax) corresponded to the volume that produced end-diastolic pressure of 30 mm Hg at baseline calcium.

2.4. Mechanical restitution protocol

Mechanical restitution data were obtained at a fixed left ventricular volume (Vref), corresponding to the end-diastolic
pressure of 3–5 mm Hg under baseline conditions (2.5 mM calcium, Fig. 2). Left ventricular pressure waveforms were recorded for a pair of contractions: a steady-state contraction at the baseline pacing interval (250 ms) and a test contraction immediately following the baseline contraction at a variable pacing interval (i.e., test pulse interval, TPI). Data from 25–30 pairs of such contractions were recorded corresponding to different values of TPI (range: 150–1200 ms).

2.5. Analysis of perfused heart data

Left ventricular developed pressure ($P_{dev}$) was defined as the difference between peak systolic pressure ($P_{ps}$) and end-

![Fig. 1. Representative left ventricular pressure and volume data from a wild-type mouse (A and B) and a troponin I transgenic mouse (C and D) at perfusate calcium of 2.5 mM. The transgenic mouse generated greater systolic pressure over a similar range of ventricular volume.](image1)

![Fig. 2. Representative left ventricular mechanical restitution data from a wild-type mouse (A: 2.5 mM perfusate calcium, and B: 1.5 mM perfusate calcium) and a troponin I transgenic mouse (C: 2.5 mM perfusate calcium, and D: 1.5 mM perfusate calcium). Left ventricular pressure data are depicted for two beats in each panel: steady-state beat at baseline stimulation frequency (4 Hz) followed by a contraction at a variable test pulse interval (TPI). Data corresponding to several TPIs are superimposed (TPI = 150–1000 ms in this example).](image2)
diastolic pressure ($P_{ed}$). To account for small differences in both left ventricular mass and volume, a thick wall spherical model was used to convert $P_{dev}$ to developed wall stress ($\sigma_{dev}$) according to the following formula [9]:

$$\sigma_{dev} = \frac{P_{dev}}{\left(1 + \frac{M}{\rho V}\right)^{2/3}} - 1$$

where $M$, $\rho$, and $V$ are left ventricular muscle mass, muscle density (1.05 g/ml), and chamber volume, respectively. Maximal rates of stress development ($d\sigma/dt_{\text{max}}$) and decline ($d\sigma/dt_{\text{min}}$) were calculated. The relaxation time ($T_{\text{relax}}$) was defined as [9]:

$$T_{\text{relax}} = t_{25} - t_{75},$$

where $t_{25}$ and $t_{75}$ represent the time at which developed stress (pressure) falls to 25% and 75% of maximum, respectively.

To analyze data from Frank–Starling protocol, each volume was normalized ($V/V_{\text{max}}$) by maximal volume ($V_{\text{max}}$), and then regression analysis was performed to determine the several linear (e.g., $\sigma_{\text{dev}} = V/V_{\text{max}}$, $d\sigma/dt_{\text{max}} - V/V_{\text{max}}$, $d\sigma/dt_{\text{min}} - V/V_{\text{max}}$) and nonlinear (left ventricular end-diastolic pressure–volume, exponential function) relationships.

For the mechanical restitution data, the relative contractile strength of the test contraction (RCS) was calculated as the ratio $100*P_{\text{dev-test}}/P_{\text{dev-baseline}}$. The relationship between RCS and test pulse interval (TPI) represents the mechanical restitution curve. This relationship was fitted to a double exponential curve using the following formula [10]:

$$\text{RCS} = A \left[1 - e^{-\frac{(\text{TPI})}{\tau_1}}\right] + B \left[1 - e^{-\frac{(\text{TPI})}{\tau_2}}\right]$$

where $t_0$ is the TPI-axis intercept (i.e., TPI at which RCS is zero), $\tau_1$ and $\tau_2$ are the time constants of the fast and slow phases of the mechanical restitution process, respectively, and $A$ and $B$ are the corresponding amplitudes. As TPI increases, RCS plateaus to a maximum value ($\text{RCS}_{\text{max}}$), which is equal to $A + B$.

2.6. Manganese-enhanced MRI

Manganese-enhanced MRI exploits two properties of the manganese ion to noninvasively assess myocardial sarcolemmal calcium influx [11,12], and was recently described in detail by Hu et al. [8]. Mice were anaesthetized with 0.017 ml/g avertin (2,2,2-tribromoethanol) and anesthesia was maintained with 0.007 ml/g avertin injected every 30–40 min via an i.p. line. Mice were placed so that the heart was positioned above a surface coil and centered in the micro-imaging gradient set of a Bruker 7T, 15 cm Avance instrument. EKG was acquired for image gating with copper wires that were placed under the front paws of the animal with conducting gel. FVB non-transgenic mice from Hu et al. [8] were used as controls.

$\text{MnCl}_2$ (14 mM/min/g body weight) was infused via a tail venous line at a constant rate of 0.2 ml/h for 30 min. Short-axis heart images were acquired with an EKG-gated Fast Low Angle ShOt (FLASH) imaging sequence. The short-axis slices (orthogonal to the imaginary line between apex and aorta) covered the left ventricle and part of the liver. Sixteen segments were acquired, with each segment containing four-phase encodes. A water phantom was included to normalize signal intensities. The imaging parameters were as follows: matrix dimensions, 128 × 64; TE, 1.3 ms; TR, 300 ms; slice thickness, 1 mm; FOV, 2.5 cm; 8 averages. Gated images were acquired immediately after the EKG R wave at end-diastole.

2.7. Western blot analysis

Phosphorylation status of troponin I was examined by Western blot analysis in two ways: two-dimensional gel electrophoresis and one-dimensional gel electrophoresis using phosphorylation-sensitive antibody. Myofibrillar protein extracts for two-dimensional gel electrophoresis were prepared in 100 mM p-toluene sulfonyl fluoride [13] supplemented with sodium orthovanadate (1 mM) to block Mn$^{2+}$-dependent protein phosphatases. Samples (WT, $n = 3$; TG, $n = 3$) were electrofocused on an IPGphor Isoelectric Focusing Unit (Amersham Pharmacia Biotech) on pH 6–11 Immobiline™ DryStrips, size separated by SDS-PAGE, then probed by Western blot analysis with a cardiac TnI antibody (Santa Cruz Biotechnology). For single dimension SDS-PAGE and Western blot analysis of myofibrillar proteins, additional hearts (WT, $n = 3$; TG, $n = 3$) were extracted in RIPA buffer (135 mM NaCl, 1% IGEPAL CA-630, 1% SDS, 4.25 mM Tris, pH 8.0, 0.5% deoxycholate) without sodium orthovanadate and with or without calf intestinal alkaline phosphatase (10 units, New England Biolabs) to confirm the native phosphorylation status of cardiac troponin I. Blots were probed using a mouse monoclonal, anti-cardiac troponin I, phosphorylation-sensitive antibody (clone 5E6, 1 µg/ml, Research Diagnostic).

Protein expression of the Ca$^{1+}$,1.2a (α1c) subunit of the L type calcium channel, sarcoplasmic reticulum calcium ATPase (SERCA 2), and phospholamban was examined using Western blots (WT, $n = 5$; TG, $n = 5$) [14]. Antibodies included rabbit polyclonal anti-Ca$^{1+}$,1.2a (1 µg/ml, BD Biosciences), anti-phospholamban monoclonal antibody (1 µg/ml, Upstate Biotechnology), and rabbit polyclonal anti-SERCA antibody (5 µg/ml, Zymed).

2.8. Statistical analysis

Mechanical data obtained from the isolated, perfused heart studies corresponded to the following data structure: mouse type (WT and TG) and perfusate calcium (2.5 and 1.5 mM or 2.5 and 3.5 mM). Because data were collected only at two calcium levels in individual hearts, we subdivided our statistical analysis into two parts (2.5 vs. 1.5 mM and 2.5 vs. 3.5 mM). Thus, individual variables were analyzed using two-factor, repeated measures (perfusate calcium)
ANOVA, with post hoc pairwise comparisons performed using Newman–Keuls multiple comparison test. Parameters of continuous relationships (e.g., $\sigma_{dev} - V/V_{max}$, mechanical restitution) were compared between two datasets using the method of excess variance (or extra sum of squares) [15], which is analogous to analysis of covariance for linear relationships. Four pairwise comparisons were made here: WT vs. TG at 2.5 and 1.5 (or 3.5) mM perfusate calcium and 2.5 vs. 1.5 (or 3.5) mM perfusate calcium for WT and TG. The significance level was adjusted for multiple comparisons according to the Bonferroni correction ($P < 0.0125$ for overall $P < 0.05$).

For manganese-enhanced MRI data, mean of steady-state image contrast enhancements at the anterior wall and septum were compared between WT and TG mice by two-factor ANOVA, with post hoc pairwise comparisons performed using the Newman–Keuls test. Steady state was defined as when signal enhancement reached a plateau, and values were thereafter averaged for statistical analysis.

### 3. Results

#### 3.1. Weights and volumes

Body weight, left and right ventricular weights, and $V_{max}$ at 2.5 mM perfusate calcium are listed in Table 1.

#### 3.2. Systolic function

Examples of left ventricular pressure tracings in WT and TG mice over a similar range of chamber volumes are shown in Fig. 1. Differences in systolic function between WT and TG mice were dependent on the level of perfusate calcium. The $\sigma_{dev} - V/V_{max}$ relationship was not significantly different between the two groups at 1.5 mM perfusate calcium, and this relationship tended to be elevated in the transgenic group at higher calcium levels, reaching statistical significance at 3.5 mM perfusate calcium ($P = 0.08$ for 2.5 mM and $P < 0.05$ for 3.5 mM) (Fig. 3). No differences were observed in $\sigma_{dev}/d_{max} - \sigma_{dev}$ relationships at any perfusate calcium level (data not shown), indicating that the positive inotropy at high perfusate calcium was not associated with any changes in the shape of the pressure (stress) waveform during systole; instead, it corresponded to a simple scaling. This may indicate that troponin I protein kinase C phosphorylation sites modulate

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Wild-type (N=15)</th>
<th>Transgenic (N=19)</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>31.6 ± 1.0</td>
<td>30.5 ± 0.6</td>
</tr>
<tr>
<td>LV weight (mg)</td>
<td>94 ± 2</td>
<td>91 ± 2</td>
</tr>
<tr>
<td>LV/body weight ratio (mg g$^{-1}$)</td>
<td>3.01 ± 0.07</td>
<td>3.00 ± 0.07</td>
</tr>
<tr>
<td>$V_{max}$ at 2.5 mM calcium (μl)</td>
<td>34 ± 2</td>
<td>33 ± 2</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. LV: left ventricle; $V_{max}$: maximal left ventricular volume.
myofilament force production without affecting the dynamics of the myofilament.

3.3. Diastolic function

There were no differences in $T_{\text{relax}}$ between WT and TG mice at any level of perfusate calcium (Fig. 4A,B,C). End-diastolic pressure–volume relationships, which are determined by the relaxation process and passive mechanical properties, were also not significantly different at any level of perfusate calcium between the two groups (Fig. 4D,E,F). Finally, there were no differences in $\sigma / \sigma_{\text{min}} - \sigma_{\text{dev}}$ relationships between the two groups at any perfusate calcium level (data not shown). This also suggests no change in the intrinsic relaxation process.

3.4. Mechanical restitution

Examples of mechanical restitution curves at two levels of perfusate calcium and the corresponding double exponential fits are illustrated in Fig. 5. Similar to the responses in systolic calcium, mechanical restitution relationships in the TG mice exhibited calcium-dependent alterations with respect to WT mice (Table 2). The analysis of data obtained at 2.5 and 3.5 mM perfusate calcium indicated that mechanical restitution parameters were not different between WT and TG mice at either calcium level and between 2.5 and 3.5 mM calcium for either mouse type (Table 2). In contrast, as the perfusate calcium was lowered from 2.5 to 1.5 mM, the amplitude of the fast component (A) decreased and the amplitude of the slow component (B) increased.

![Graphs showing diastolic function and mechanical restitution](https://academic.oup.com/cardiovascres/article-abstract/63/2/245/273673/fig4)
resulting in a net increase in $RCS_{\text{max}} (= A + B)$ for both WT and TG mice (Table 2). However, $RCS_{\text{max}}$ was significantly greater for TG mice compared to that for WT mice at 1.5 mM calcium (Table 2). Similarly, $\tau_1$ increased in both groups as calcium concentration was lowered from 2.5 to 1.5 mM and $\tau_1$ was significantly greater for transgenic mice at 1.5 mM calcium (Table 2). Thus, higher $RCS_{\text{max}}$ and $\tau_1$ at perfusate calcium 1.5 mM in TG mice was an amplification of the normal response to lowering calcium seen in WT mice.

### 3.5. Manganese-enhanced MRI

Representative single slice short-axis heart images pre-and post-manganese infusion are shown for WT (Fig. 6A and B) and TG mice (Fig. 6C and D). Clear enhancement of the left ventricular myocardium was seen with manganese infusion for both WT and TG mice, though the relative enhancement was less for the TG compared to WT mouse heart (intensity difference between Fig. 6A and B vs. intensity difference between Fig. 6C and D).

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**Fig. 5.** Representative data illustrating the relationship between % potentiation of the test contraction as a function of the test pulse interval from one wild-type (WT) and one troponin I transgenic (TG) mouse at 1.5 mM (panel A) and 2.5 mM (panel B) perfusate calcium. As perfusate calcium was lowered to 1.5 mM (panel A), maximum potentiation ($RCS_{\text{max}} = A + B$) increased and time constant of the fast component of restitution ($\tau_1$) increased (slower restitution) in both groups. However, these calcium-induced changes in mechanical restitution parameters were amplified for the TG mouse such that both $RCS_{\text{max}}$ and $\tau_1$ were greater for TG mouse compared to the WT mouse (panel A).

**Fig. 6.** MRI images pre and post manganese infusion for wild-type (A, pre and B, post) and transgenic mice (C, pre and D, post). Relative signal intensity changes were measured at the anterior (Ant.) and septal regions of the left ventricle. The relative signal change resulting from manganese in these areas was greater in the wild-type compared to transgenic mice (change from A to B vs. change from C to D), indicating reduced sarcolemmal calcium influx in transgenic mice.

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**Table 2**

Left ventricular mechanical restitution parameters for wild-type and transgenic mice at various perfusate calcium levels

<table>
<thead>
<tr>
<th>Perfusate calcium (mM)</th>
<th>$t_0$ (ms)</th>
<th>$\tau_1$ (ms)</th>
<th>$A$ (%)</th>
<th>$\tau_2$ (ms)</th>
<th>$B$ (%)</th>
<th>$RCS_{\text{max}}$ (%)</th>
</tr>
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<tbody>
<tr>
<td><strong>1.5 mM</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>WT 138 ± 2</td>
<td>42 ± 12</td>
<td>70 ± 1</td>
<td>424 ± 54</td>
<td>160 ± 32</td>
<td>230 ± 32</td>
<td></td>
</tr>
<tr>
<td>TG 136 ± 8</td>
<td>62 ± 7*</td>
<td>74 ± 8</td>
<td>634 ± 78</td>
<td>213 ± 10*</td>
<td>287 ± 7*</td>
<td></td>
</tr>
<tr>
<td><strong>2.5 mM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT 135 ± 3</td>
<td>27 ± 2</td>
<td>87 ± 2</td>
<td>594 ± 42</td>
<td>81 ± 7</td>
<td>168 ± 6</td>
<td></td>
</tr>
<tr>
<td>TG 131 ± 3</td>
<td>25 ± 2</td>
<td>86 ± 2</td>
<td>524 ± 55</td>
<td>74 ± 11</td>
<td>160 ± 10</td>
<td></td>
</tr>
<tr>
<td><strong>3.5 mM</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT 136 ± 4</td>
<td>29 ± 3</td>
<td>91 ± 1</td>
<td>510 ± 52</td>
<td>55 ± 6</td>
<td>146 ± 7</td>
<td></td>
</tr>
<tr>
<td>TG 140 ± 2</td>
<td>26 ± 2</td>
<td>88 ± 3</td>
<td>492 ± 131</td>
<td>68 ± 16</td>
<td>156 ± 15</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. WT: wild-type mice; TG: transgenic mice; $t_0$: test pulse interval-axis intercept; $\tau_1$, $A$: time constant and amplitude of the fast component of mechanical restitution, respectively; $\tau_2$, $B$: time constant and amplitude of the slow component of mechanical restitution, respectively; $RCS_{\text{max}}$: maximum potentiation or relative contractile state ($= A + B$). Because data were collected only at two perfusate calcium levels in individual hearts (2.5 mM and 1.5 or 3.5 mM), the statistical analysis (method of excess variance) was subdivided into two parts by comparing parameters at perfusate 2.5 vs. 1.5 mM and 2.5 vs. 3.5 mM (see text). Data from all hearts are included in the averages for 2.5 mM perfusate calcium. Symbols denote overall $P<0.05$ (adjusted for multiple pairwise comparisons).

1 $P<0.05$ 1.5 vs. 2.5 mM perfusate calcium for a given mouse type (WT or TG).

* TG. vs. WT at 1.5 mM perfusate calcium.

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* *G.A. MacGowan et al. / Cardiovascular Research 63 (2004) 245–255*
A plot of the relative signal intensity for the left ventricular anterior wall over time for WT and TG mice is shown in Fig. 7. By 50 min, TG mice showed significantly reduced enhancement in signal intensity, which persisted for approximately 20 more minutes. Table 3 summarizes the signal enhancement data for the left ventricular septum, anterior wall, and also the chest wall and liver.

3.6. Western blot analysis of troponin I phosphorylation and calcium handling proteins

Results of two-dimensional gel electrophoresis of troponin I are shown in Fig. 8A. This procedure separates highly phosphorylated troponin I from the less phosphorylated form by focusing the former closer to the positive pole (left side of Fig. 8A) and the latter closer to the negative pole (right side of Fig. 8A). The gels were aligned at the center (open triangle), which corresponds to an intermediate level of cardiac troponin I phosphorylation that was present in all WT and TG samples. Intermediate forms of phosphorylated troponin I were markedly reduced in TG mice relative to WT mice (arrows immediately to the right and left of the triangle, Fig. 8A). In addition, TG mice showed an increased presence of highly phosphorylated troponin I species (arrow near positive pole, Fig. 8A).

Results of one-dimensional gel electrophoresis of troponin I using anti-cardiac troponin I, phosphorylation-sensitive antibody are shown in Fig. 8B. This antibody is

![Image](https://example.com/image.png)

**Fig. 8.** (A) Representative gels from two-dimensional gel electrophoresis of cardiac troponin I (cTnI) from wild-type (WT) and transgenic (TG) mice (three examples in each group). Highly phosphorylated cTnI protein focuses at the positive pole (left, +) and less phosphorylated protein closer to the negative pole (right, −). The gels were aligned at the center (open triangle), which corresponds to an intermediate level of cTnI phosphorylation that was present in all wild-type and transgenic samples. Intermediate forms of phosphorylated troponin I were markedly reduced in TG mice relative to WT mice (arrows immediately to the right and left of the triangle, Fig. 8A). In addition, TG mice showed an increased presence of highly phosphorylated cTnI species (arrow near positive pole). (B) One-dimensional Western blots with anti-cTnI, phosphorylation-sensitive antibody (preferentially sensitive to dephosphorylated protein) showed reduced band intensity in TG mice at baseline compared to WT. This difference in band intensity was no longer present after exposure to phosphatase (PPase). This observation indicates that there is increased phosphorylation of remaining sites on cTnI in TG mice and is consistent with the two-dimensional gel electrophoresis data in panel A.

**Table 3**
Steady-state MRI signal enhancement (% change from baseline) with manganese infusion in wild-type and transgenic mice at the ventricular septum, left ventricular (LV) anterior wall, chest wall, and liver

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>TG</th>
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<tbody>
<tr>
<td>Ventricular septum</td>
<td>41.1 ± 6.2</td>
<td>23.6 ± 7.6*</td>
</tr>
<tr>
<td>LV anterior free wall</td>
<td>47.0 ± 7.1</td>
<td>24.5 ± 4.5*</td>
</tr>
<tr>
<td>Chest wall</td>
<td>18.7 ± 7.1</td>
<td>−7.6 ± 3.0</td>
</tr>
<tr>
<td>Liver</td>
<td>39.9 ± 13.3</td>
<td>58.2 ± 19.3</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. WT: wild-type mice (n = 6); TG: transgenic mice (n = 4).

*P = 0.05 WT vs. TG.

† P < 0.06 WT vs. TG.
preferentially sensitive to the unphosphorylated form of cardiac troponin I. Band intensity in TG mice was reduced in TG mice compared to WT mice (Fig. 8B). Pretreatment with phosphatase resulted in increased band intensity in TG mice such that there was no longer any difference between WT and TG mice. These two observations further confirm that troponin I phosphorylation at the remaining phosphorylation sites is elevated in TG mice.

Western blots of the Ca,1.2a (α1c) subunit of the L type calcium channel, sarcoplasmic reticulum calcium ATPase (SERCA 2) and phospholamban showed no significant differences between WT and TG mice (Fig. 9).

4. Discussion

4.1. Ventricular function phenotype

The present study demonstrates that expression of mutant troponin I lacking protein kinase C phosphorylation sites affects left ventricular function in a calcium-dependent manner. Compared to WT mice, systolic contractile function is enhanced in TG mice at higher levels of perfusate calcium (e.g., 3.5 mM) and this positive inotropic effect is no longer seen at lower levels of calcium (e.g., 1.5 mM). In contrast to the changes in contractile function, the relaxation process and end-diastolic (passive) mechanical properties are unaltered in TG mice. These calcium-dependent changes in contractility with minimal changes in relaxation appear unique compared to other pharmacological or genetically engineered changes in inotropy.

4.2. Concomitant changes in other processes

Manganese-enhanced MRI provides a measure of L-type calcium channel ion transport [8,12], and therefore demonstrates in-vivo reduction in sarcolemmal calcium influx in transgenic mice. These data substantiate the reduced calcium transients in these transgenic mice that we have previously demonstrated using a fluorescent dye [2]. One potential explanation for the reduced calcium transients was that there was increased troponin C calcium binding that reduced the availability of free intracellular calcium to bind to the fluorescent dye. However, as the MRI data reflect sarcolemmal calcium influx, we can now state that the reduced calcium transients are not predominantly related to altered troponin C calcium binding, and that L-type calcium channel function (though not protein expression) is clearly implicated in this process. Overall, these findings are consistent with other recent studies demonstrating that changes in myofilamental proteins can affect calcium handling. For example, a mouse model of familial hypertrophic cardiomyopathy with a missense mutation in the α-myosin heavy chain (αMHC403V) has abnormal calcium handling with reduced sarcoplasmic reticulum calcium storage, and reversal of this abnormality by treatment with the calcium channel blocker diltiazem [16]. Similarly, a troponin T mouse mutant (I79N) exhibits prolonged calcium transients with increased diastolic levels in response to isoprenaline [17].

Two-dimensional gel electrophoresis data provide qualitative information about troponin I phosphorylation, clearly indicating a shift in the proportion of molecules from intermediate to higher levels of phosphorylation in TG mice. These data are further confirmed by one-dimensional gel electrophoresis data wherein a phosphorylation-sensitive antibody to troponin I was utilized. Thus, there appears to be increased phosphorylation on remaining troponin I sites in TG mice. The interpretation of these data requires an integration of several findings in these TG mice: (1) Total amounts of troponin I protein (wild-type plus mutant) is not different compared to WT mice [2]. (2) There is a 25% reduction in total (maximal) troponin I phosphorylation [3]. (3) Protein kinase C translocation to myofilament (both α and ε isoforms) is unchanged in the TG mice [4]. Thus, there is a reduced pool of available phosphorylation sites in TG mice (points 1 and 2), with unchanged phosphorylation stimulus (point 3). Consequently, a greater proportion of troponin I molecules in TG mice can exist in the highly phosphorylated form, even though total (maximal) troponin I phosphorylation is reduced. The exact sites of increased phosphorylation are presently unknown.

Both reduced intracellular calcium and increased phosphorylation of remaining troponin I phosphorylation sites in TG mice mitigate the phenotypic expression: positive inotropy in troponin I TG mice would have been more marked if these concomitant changes in cellular calcium and troponin I phosphorylation were absent. In this regard, one can consider these two alterations in other processes as compensatory changes.

4.3. Potential mechanisms underlying the observed ventricular function phenotype

Increased contractility at 3.5 mM perfusate calcium in TG mice cannot be attributed to increased cellular calcium. On the contrary, we have previously shown that peaks of calcium transients are reduced at 2.5 and 3.5 mM perfusate...
calcium in this transgenic mouse model, and we have confirmed the reduced calcium in-vivo in the present study.

Mechanical restitution process is an index of the overall dynamics of cellular calcium handling and is one of the determinants of cardiac contractile force. For example, even with total intracellular calcium and myofilamental properties (calcium sensitivity, crossbridge kinetics, cooperativity) held constant, cardiac contractile force at a given stimulus interval will be greater if the restitution process is faster (i.e., smaller time constants). Given that none of the parameters of mechanical restitution were different between WT and TG mice at 3.5 mM calcium (Table 2), this process was not responsible for the higher left ventricular contractility in TG mice at 3.5 mM perfusate calcium.

Johannsson and Åsgrimsson [10] have reported that as perfusate calcium was decreased in guinea pig atrial muscles, mechanical restitution was slower (i.e., both fast and slow time constants increased) and amplitudes of fast and slow components decreased and increased, respectively. Our results are in line with these previous observations, except for the time constant of the slow component, which did not change significantly with calcium (Table 2). These calcium-induced changes in mechanical restitution were present and directionally similar in WT and TG mice. However, the quantitative changes for TG mice, especially at 1.5 mM calcium, are an amplification of the responses observed in the WT mice (Table 2).

The increased troponin I phosphorylation in TG mice is expected to affect ventricular mechanical function. For instance, increased phosphorylation of protein kinase A sites (serines 23/24) is known to result in enhanced relaxation [7], and may contribute to preserved relaxation despite increased contractility seen in the TG mice. This is in contrast to another troponin I transgenic mouse model wherein cardiac form of troponin I was completely replaced by the slow skeletal form [18]. Here increased cardiac contractility was associated with slower relaxation [18,19]. Phosphorylation of remaining protein kinase C phosphorylation sites (serines 43/45 on wild-type protein or at threonine 144) would mitigate the expected increase in contractility [1].

The contractile phenotype in TG mice can be interpreted as an amplification of the response to changes in cellular calcium observed in WT mice. A possible explanation for this may be as follows. An increase in cellular calcium is known activate protein kinase C [5,6], which would limit the calcium-induced positive inotropy via increased phosphorylation of protein kinase C sites on troponin I. This negative feedback is attenuated in TG mice because of reduced number of protein kinase C phosphorylation sites, resulting in greater contractility in TG mice at high calcium. These results suggest that protein kinase C phosphorylation sites on troponin I play a role in attenuating contractile responses to changes in intracellular calcium.

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