Inhibition of PKC phosphorylation of cTnI improves cardiac performance in vivo

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Roman, Brian B., Paul H. Goldspink, Elyse Spaiate, Dalia Urboniene, Ron McKinney, David L. Geenen, R. John Solaro, and Peter M. Buttrick. Inhibition of PKC phosphorylation of cTnI improves cardiac performance in vivo. Am J Physiol Heart Circ Physiol 286: H2089–H2095, 2004. First published January 15, 2004; 10.1152/ajpheart.00582.2003.—Protein kinase C (PKC) modulates cardiomyocyte function by phosphorylation of intracellular targets including myofilament proteins. Data generated from studies on in vitro heart preparations indicate that PKC phosphorylation of troponin I (TnI), primarily via PKC-ε, may slow the rates of cardiac contraction and relaxation (+dP/dt and −dP/dt). To explore this issue in vivo, we employed transgenic mice [mutant TnI (mTnI) mice] in which the major PKC phosphorylation sites on cardiac TnI were mutated by alanine substitutions for Ser43 and Ser45 and studied in situ hemodynamics at baseline and increased inotropy. Hearts from mTnI mice exhibited increased contractility, as shown by a 30% greater +dP/dt and 18% greater −dP/dt than FVB hearts, and had a negligible response to isoproterenol compared with FVB mice, in which +dP/dt increased by 33% and −dP/dt increased by 26%. Treatment with phenoxyphrine and propanolol gave a similar result; FVB mouse hearts demonstrated a 20% increase in developed pressure, whereas mTnI mice showed no response. Back phosphorylation of TnI from mTnI hearts demonstrated that the mutation of the PKC sites was associated with an enhanced PKA-dependent phosphorylation independent of a change in basal cAMP levels. Our results demonstrate the important role that PKC-dependent phosphorylation of TnI has on the modulation of cardiac function under basal as well as augmented states and indicate interdependence of the phosphorylation sites of TnI in hearts beating in situ.

troponin; protein kinase C; contractility; mouse

PROTEIN KINASE C (PKC)-mediated phosphorylation of cardiac troponin I (cTnI) has been shown to depress the acto-myosin interaction and may be important during the progression of heart failure (1, 3, 8, 13, 14, 28). Analyses of phosphorylation targets that are involved in excitation-contraction coupling further indicate a central role for PKC activation in the regulation of cardiac performance (9). Upon activation, PKC-ε (one of the most prominent isoforms in the heart) appears to translocate to both sarcomeric and sarcoplasmic reticular structures to phosphorylate TnI. Among PKC isoforms, PKC-ε, but not PKC-α, is a mediator for ERK activation induced by endothelin-1 and phenylephrine (PE) (5). It has been demonstrated that cardiac expression of PKC-ε in the mouse also results in cardiac hypertrophy (27), and we demonstrate here that this translocation and activation of PKC-ε is maintained in the whole heart. In vitro studies indicate that PKC-dependent TnI phosphorylation is likely to reduce tension development and impair shortening (4, 14, 18, 20). It is apparent that phosphorylation of Ser43 and Ser45 of TnI is particularly important in these effects (1, 16). Activation of PKC has also been shown to downregulate mRNA and protein expression of sarcoplasmic reticular Ca2+-ATPase 2 (SERCA2) and also to delay the relaxation phase of the calcium transient in isolated cardiocytes. Both the TnI and SERCA2 effects would be predicted to impair diastolic relaxation, the former by altering myofilament maximum tension and shortening velocity and the latter by slowing cytosolic calcium removal (2, 15). The relative role of these two regulatory mechanisms in the in situ beating heart remains unclear.

To investigate the role of PKC-dependent TnI phosphorylation and its modulatory role on cardiac function, we developed a transgenic (TG) mouse model that expresses mutant TnI (mTnI) lacking PKC phosphorylation sites at Ser43 and Ser45 (11, 12, 14, 20). These animals do not express any developmental or histological abnormalities (12). Montgomery et al. (14) examined the effect of PKC activation on papillary muscle preparations from mTnI mice. In non-TG papillary muscles, PE treatment resulted in an initial increase, followed by a steady 62% decline in force. In TG muscles, PE did not induce a transient phase, but the decline in force generation was significantly attenuated (14). Treatment of wild-type fibers with phorbol ester (TPA) induced an increase in 32P incorporation into cTnI and cTnT and a 30% decrease in maximum tension. In contrast, TPA treatment of TG preparations resulted in a diminished 32P incorporation into cTnI as well as cTnT and only a 15% reduction in maximum tension. The PKC-specific inhibitor chelerythrine inhibited these responses. These data provide evidence that specific PKC-mediated phosphorylation of Ser43/45 of TnI plays an important role in regulating force development in cardiac muscle (14). MacGowan et al. (12) reported that when hearts from these same TG animals were perfused at high calcium levels, there was a relative decrease in systolic and diastolic calcium compared with controls. Moreover, when subjected to global ischemia, mutant hearts demonstrated a twofold increase in time to ischemic contracture compared with FVB controls (11). Both of these results suggest an interaction between PKC-dependent myofilament phosphorylation and mechanisms regulating tension and cellular calcium fluxes.

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Although there were only small differences in developed pressure detected in the isolated perfused heart model (11), we hypothesized that in vivo hearts of mTnI mice would display an increase in rates of cardiac contraction and relaxation (+dP/dt and −dP/dt) under baseline conditions by virtue of an alteration of the phosphorylation state of cTnI by a lack of PKC-dependent phosphorylation of cTnI. We hypothesized that mTnI hearts would be operating at an increased level of contractility compared with FVB animals, which would not be further enhanced with inotropic stimulation. PKC-ε translocation has also been shown to occur as a result of mechanical stimulation (9), and based on these data, we suspect that a sustained increase in contractility may induce remodeling potentially through a hypertrophic process. Results of experiments reported here support these hypotheses.

METHODS

Animal care. Adult male (7–16 wk of age) FVB and mTnI TG mice were bred and maintained in a temperature-controlled room. The mice were provided food and water ad libitum. All procedures were conducted in accordance with university animal care approved protocols and national guidelines.

mTnI TG mice. The generation and characterization of these cTnI TG mice have been previously described (12). The cardiac-specific expression of the mutated cTnI cDNA lacking PKC phosphorylation sites (S43A/S45A) was driven by a mouse α-myosin heavy chain promoter in a FVB background, and FVB mice (Jackson Laboratory) served as controls. The expression of mTnI does not result in a complete replacement of native TnI in the myofilaments. Mutated TnI is ~60% of total TnI (20).

In vivo/in situ cardiac function. For in situ measurements, the animals were anesthetized with an initial dose of metofane and a plane of anesthesia was then maintained after intubation with 1% isoflurane using a Harvard small animal ventilator (respiratory rate: 120–140 breaths/min; respiratory volume: 100–300 μl). Body temperature was monitored using a rectal thermistor and maintained at 39°C using a warming table placed under the animal. A ventral incision in the neck lateral to the midline of the body was made, and the carotid artery was then exposed. The vessel was opened, and a 1.4-Fr Millar pressure transducer was passed through the carotid artery and into the left ventricle (LV). All hemodynamic measurements of wild-type and TG mice were obtained in this manner. A second incision on the medial side of the left hindlimb exposed the left femoral vein. Bolus (100 μl) injections of either isoproterenol (Iso; 1 × 10−7 M) or propranolol (Pro; 1 mg/kg) followed by PE (3.2 μg/kg) were administered to mice from both groups. To minimize β-adrenergic effects of PE, Pro was infused in combination with and before PE. These data were acquired and analyzed through a Gould/Ponemah system (Gould; Columbus, OH).

To investigate whether variations in situ hemodynamics reflected altered LV geometry and systolic function, FVB and mTnI TG mice were assessed echocardiographically. Transhthoracic two-dimensional targeted M-mode echocardiography was performed with a 15-MHz linear array transducer attached to a commercially available Sequoia C256 system. Mice were anesthetized with metofane, intubated, and maintained on isoflurane anesthesia as described above. Images of the LV were taken from the parasternal short-axis view at the level of the papillary muscles. Interventricular septal and LV posterior wall thickness and LV internal dimensions at the end of diastole and systole were measured by the American Society of Echocardiography, leading-edge method from at least three consecutive cardiac cycles on the M-mode tracings with a paper speed of 200 mm/s. The percent fractional shortening of the LV was calculated from digital images by subtracting the LV systolic internal dimension from the LV diastolic internal dimension and dividing by the LV diastolic internal dimension.

Quantitative RT-PCR. To determine the level of expression of various genes, real-time quantitative RT-PCR with SYBR green detection was performed in a LightCycler thermocycler (Roche Diagnostics). Total RNA was extracted from the apex of the heart using TRIzol reagent, and 100 ng of total RNA were used in each RT-PCR. Quantification of the RT-PCR was based on a series of in vitro transcribed mRNA standards prepared for each gene and run in parallel as previously published (2). Melting curve analysis was performed on the standards to determine the specific temperature (the Tm of the PCR product) at which the fluorescent signal should be acquired, thereby excluding fluorescence from nonspecific products and/or primer dimers, which can be detected with the SYBR green dye.

Western blot analysis. Tissue samples were homogenized in ice-cold sucrose buffer with protease inhibitors [containing (in mM) 320 sucrose, 20 Tris-Cl, 2 EDTA, 10 EGTA, 10 BME, and 0.3 PMSF plus 20 μg/ml leupeptin]. This total homogenate was assayed spectrophotometrically [(Bradford assay) for protein content. Equal amounts of protein were isolated, TCA extracted, and then resuspended in SDS sample buffer. In addition to this total PKC sample, the homogenate was fractionated by differential centrifugation into cytosolic and particulate fractions. Protein (60–80 μg) was loaded onto 10% acrylamide gels to separate the constituent proteins. PKC-purified enzymes (positive control) and molecular weight markers were run in parallel. The proteins were transferred to nitrocellulose membranes using a semidyblot apparatus (Bio-Rad). The membranes were blocked overnight at 4°C in 5% dry milk, and the primary antibody was applied for 1–2 h at room temperature. A PKC-ε polyclonal antibody (1:300 dilution, Santa Cruz; and 1:1,000 dilution, Transduction Laboratories) was used. After a 30-min wash, the membrane was probed with secondary antibody (1:10,000–20,000 dilution) conjugated to horseradish peroxidase for 1 h at room temperature. The membrane was then washed for 30 min, exposed to chemiluminescence reagents (Pierce) for 10 min, and developed on radiographic film. Semiquantitative densitometric analysis was performed using a Bio-Rad Gel Doc system.

Back phosphorylation. Hearts were flash frozen in liquid nitrogen, and the apex was removed and placed into cold isolation rigor solution [100 mM KCl, 10 mM imidazole, 1 mM MgCl2, 2 mM EGTA, and 4 mM Na2ATP (pH 7.2)] with 3% Triton X-100. The tissue was gently homogenized and incubated on ice for 10 min. Tubes were centrifuged at 4°C at 120 g for 3 min. The resultant pellet was washed twice with 4% cold isolation rigor solution (without Triton X-100). One milliliter of cold standard rigor solution (6.2 mM MgCl2, 10 mM EGTA, 10 mM phosphocreatine, 100 mM BES, 6 mM Na2ATP, 30 mM potassium propionate, pH 7.0 at 15°C, followed by 1 mM DTT, 0.1 mM PMSF, 0.1 mM leupeptin, 0.001 mM pepstatin, and 4,000 U/l creatine phosphokinase) was added to the pellet, the mixture was centrifuged at 4°C at 1,000 rpm for 2 min in an Eppendorf microcentrifuge, and ~70% of supernatant was removed, depending on the size of the apex. The back phosphorylation reaction was carried out in a final volume of 30 μl with ~15 μg protein, 40 U/reaction PKA (Sigma P-8289), 1 μCi of 7,000 Ci/mmol ATP (ICN No. 35020), and rigor solution for 20 min at room temperature. The reaction was stopped with the addition of sample buffer, followed by 5 min in water bath sonicator. The samples were then boiled for 5 min and loaded into a 10% Bio-Rad precast gel (No. 161-1155) at 20 mA until bromophenol blue dye ran off the bottom of the gel. Gels were stained for ~3 h with Coomassie blue and destained overnight. Gels were dried in a gel dryer on Bio-Rad filter paper and exposed to Kodak X-OMAT AR film for various times to determine the best exposure for analysis. Developed films along with stained myofilament gels were analyzed using Bio-Rad Gel Doc 1000 densitometry using the actin protein band as a normalization standard.
cAMP assay. Tissue samples obtained from the original hearts isolated for back phosphorylation studies (see above) and also samples from hearts stimulated with Iso were processed for cAMP analyses. cAMP levels were assayed using a cAMP assay kit (Amer sham Pharmacia No. RPN1918). Briefly, frozen tissue samples obtained from the original hearts isolated for back phosphorylation studies were thawed, and the wet weight was recorded. Samples were homogenized in 1 ml Hanks-5 mM EDTA per 100 mg wet wt. Homogenates were centrifuged, and the supernatant was applied to the Amprep columns. The elutent was freeze dried overnight and resuspended in 1.5 ml assay buffer from the Amersham Pharmacia cAMP Biotrack EIA system (No. RPN225) before being used in the nonacetylation EIA procedure outlined by the manufacturer. Additionally, tissue samples were obtained from hearts 2 min after a 100-μl bolus of Iso (see In vivo in situ cardiac function).

Statistics. The values reported are sample means ± SE unless otherwise noted. The results were compared by an unpaired or paired Student’s t-test or ANOVA (as noted) with the significance established at P < 0.05.

RESULTS

In situ hemodynamics and in vivo echocardiography. Mean hemodynamic data are shown in Table 1. There was no significant difference in heart rate between FVB and mTnI animals. The pressures measured were similar to those seen in other studies involving these mice (11, 12, 14, 20). The FVB mice had heart rates of 590 ± 30 beats/min (n = 8), and the TG mice had heart rates of 615 ± 25 beats/min (n = 5). There was a significant difference in systolic intraventricular pressure and contractility between FVB and mTnI animals. Peak intraventricular pressure was slightly elevated in TG mice (100 ± 2 mmHg, n = 12) compared with FVB mice (90 ± 3 mmHg, n = 8). FVB hearts demonstrated a +dP/dt of 9,800 ± 450 mmHg/s and −dP/dt of 9,900 ± 450 mmHg/s (n = 8) compared with TG hearts, which had a +dP/dt of 12,700 ± 470 mmHg/s and a −dP/dt of 11,700 ± 380 mmHg/s (n = 12) (P < 0.05). To investigate whether the increased contractility measured in situ reflected altered LV geometry and systolic function, FVB and mTnI TG mice were assessed echocardiographically. No significant differences in LV wall thickness or end-diastolic (FVB: 0.35 ± 0.02 cm and mTnI: 0.34 ± 0.02 cm, n = 4) and end-systolic dimensions (FVB: 0.16 ± 0.00 cm and mTnI: 0.18 ± 0.01 cm, n = 4) were seen in M-mode images. LV fractional shortening calculated from these data (FVB 47% and mTnI 54%) were not significantly different from each other.

Inotropic stimulation. Hearts of FVB mice elicited a positive inotropic response to Iso infusion, but this was not apparent in hearts of mTnI mice, which had increased measures of contractility at baseline. After the infusion of Iso, +dP/dt increased to 13,000 ± 650 mmHg/s and −dP/dt increased to 12,500 ± 500 mmHg/s in FVB hearts, whereas the increased measures of contractility seen in mTnI hearts at baseline were not significantly increased in response to Iso. After the Iso infusion, +dP/dt values were +12,200 ± 790 and −11,300 ± 200 mmHg/s in TnI mice (Fig. 1). These hearts were isolated and assayed for cAMP content. Although the Iso infusion resulted in an increase in cAMP, there was no significant difference between FVB and TnI mice (data not shown). Previous work by Montgomery et al. (14) and Pyle et al. (20) using these mice has demonstrated an altered response to PE treatment in isolated fiber bundles. Figure 2 and Table 2 demonstrate analogous data in vivo. In response to Pro/PE treatment, FVB mice demonstrated a significant increase in developed pressure compared with baseline conditions. In comparison, the mTnI mice exhibited a blunted response. FVB hearts demonstrated a minor decrease in developed pressure in response to Pro alone, whereas mTnI hearts were profoundly affected, as illustrated in Fig. 2. Although the mTnI heart rate decreased by 10%, the effect of Pro was most apparent in the change in contractility. For example, +dP/dt decreased approximately 2,700 mmHg/s in mTnI animals compared with 700 mmHg/s in FVB animals. Although similar in trend, this change was not as dramatic in the relaxation phase of the cardiac cycle (−dP/dt).

Hypertrophic markers. Well-described markers of cardiac hypertrophy, including several whose expression may be influenced by PKC overexpression, were assessed using quantitative RT-PCR. Atrial natriuretic factor (ANF), β-myosin heavy chain, skeletal actin, and SERCA (normalized to

Table 1. Baseline in situ hemodynamics

<table>
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<tr>
<th></th>
<th>Heart Rate, beats/min</th>
<th>LV Systolic Pressure, mmHg</th>
<th>LV Diastolic Pressure, mmHg</th>
<th>LV +dP/dt, mmHg/s</th>
<th>LV −dP/dt, mmHg/s</th>
</tr>
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<tbody>
<tr>
<td>FVB</td>
<td>590 ± 30</td>
<td>90 ± 3</td>
<td>3 ± 3</td>
<td>9,800 ± 450</td>
<td>9,900 ± 450</td>
</tr>
<tr>
<td>mTnI</td>
<td>615 ± 25</td>
<td>100 ± 2*</td>
<td>1 ± 2</td>
<td>12,700 ± 470*</td>
<td>11,700 ± 380*</td>
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Values are means ± SE; n = 8 FVB and 12 mutant troponin I (mTnI) mice. Hemodynamic data were obtained from both FVB and mTnI mice demonstrating increased contractility in mTnI hearts. LV, left ventricular; dP/dt, first derivative of LV pressure. *P < 0.05 vs. FVB mice.

Fig. 1. Mutant troponin I (mTnI) hearts have an attenuated contractility (dP/dt) response to isoproterenol (Iso) stimulation. The increased contractility observed in the FVB mice in response to an infusion of Iso was attenuated in the mTnI mice. There was no significant difference between Iso-treated FVB and mTnI hearts (n = 6, tP < 0.05). NS, not significant.
propranolol (Pro) and Pro + phenylephrine (PE) infusion. Bar graphs represent the change in control levels due to Pro and Pro + PE. mTnI hearts demonstrated a significant decrease in both heart rate and developed pressure, whereas FVB hearts had an attenuated response. FVB mice had a greater pressure response to Pro + PE than did the mTnI mice (n = 5, *P < 0.01).

Table 2. Hemodynamic data from FVB and mTnI propranolol- and PE-treated mice

<table>
<thead>
<tr>
<th></th>
<th>Heart Rate, beats/min</th>
<th>LV Developed Pressure, mmHg</th>
<th>LV +dP/dt, mmHg/s</th>
<th>LV –dP/dt, mmHg/s</th>
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<tr>
<td></td>
<td>FVB</td>
<td>mTnI</td>
<td>FVB</td>
<td>mTnI</td>
</tr>
<tr>
<td>Baseline</td>
<td>590 ± 30</td>
<td>615 ± 25</td>
<td>87 ± 3</td>
<td>92 ± 2</td>
</tr>
<tr>
<td>Propranolol</td>
<td>570 ± 20</td>
<td>550 ± 10†</td>
<td>86 ± 2</td>
<td>88 ± 2*</td>
</tr>
<tr>
<td>Propranolol + PE</td>
<td>540 ± 25</td>
<td>540 ± 10†</td>
<td>108 ± 6*</td>
<td>93 ± 4*</td>
</tr>
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</table>

Values are means ± SE; n = 6 FVB and mTnI mice. PE, phenylephrine. *P < 0.05 compared with the change in FVB mice; †P < 0.05 compared with baseline.

DISCUSSION

Our results are the first to demonstrate a significant effect of PKC-ε phosphorylation at Ser43 and Ser45 of cTnI in the in situ beating heart. In the absence of an adrenergic agonist, mice defective in the primary PKC phosphorylation sites of TnI (Ser43 and Ser45) evidenced a significant increase in both +dP/dt and –dP/dt without cardiac hypertrophy or ventricular dilation. The increases in rates of contraction and relaxation were analogous to those seen in wild-type mice in response to the adrenergic agonists Iso and PE. Furthermore, the presence of the mutated TnI phosphorylation sites appeared to recapitulate the functional effects of adrenergic stimulation in that we saw no further enhancement of contractility when the mTnI mice were exposed to adrenergic stimulation.

Some biochemical and molecular characteristics of these mice have been published previously (4, 11–14, 20), so these measurements were not repeated in the present study; however, they are important to recapitulate. Specifically, PKC back conditions, PKC-ε translocates to the membrane fraction when activated with PE (data not shown). This result reinforces in the whole heart what was observed in the isolated papillary muscle.

Back phosphorylation of TnI. To examine potential mechanisms for the hemodynamic response to Pro treatment, we measured the phosphorylation state of TnI. Results of back phosphorylation of TnI by PKA are illustrated in Fig. 3. Uniform loading of isolated protein and the identification of TnI was confirmed in the mTnI baseline Coomassie-stained gel (data not shown). A relative increase in band density was interpreted as a relative increase of in vitro phosphorylation by PKA. This implies a relatively lower phosphorylated state in vivo. These densitometric data illustrate that under control conditions, there was an increase in in vivo TnI phosphorylation in TG hearts compared with control hearts. Upon treatment with Pro, there was a decrease in in vivo TnI phosphorylation that reached the baseline phosphorylation state of control hearts. This effect was unique to TnI in that densitometric analysis of the C-protein band, which was also PKA phosphorylated, showed no differences between FVB and mTnI groups (data not shown).

cAMP assay. To determine whether the increased level of mTnI phosphorylation reflects a functional adaptation of the β-adrenergic system in TG mice, cAMP levels were determined. Figure 4 illustrates that, although there was a decrease in cAMP levels due to Pro treatment in both FVB and mTnI hearts, there were no significant differences in cAMP levels at either baseline (6.0 ± 0.28 vs. 5.5 ± 0.29 pmol/mg tissue, n = 6) or after Pro treatment (3.7 ± 0.14 vs. 3.7 ± 0.31 pmol/mg tissue, n = 6).

![Graph showing response to β-adrenergic and α-adrenergic stimulation via propranolol (Pro) and pro + phenylephrine (PE) infusion.](http://www.ajpheart.org)
phosphorylation and phosphorylation profiling experiments indicated that the TG mouse hearts have 60% replacement of wild-type TnI with the mutant protein and that there is the predicted reduction in endogenous phosphorylation of TnI in isolated muscle fibers under both baseline conditions and in response to PKC stimulation. Papillary muscle preparations show blunted force generation in response to TPA, which was paralleled by a reduction in TnI phosphorylation. Montgomery et al. (14) demonstrated that the mutation of Ser43 and Ser45 to Ala resulted in a 10–15% decrease in maximum tension developed by skinned fiber preparations. It is evident from our data on the in situ beating heart that this relatively small decrease in maximum tension was overwhelmed by other differences between the non-TG and TG myofilaments. Montgomery et al. (14) demonstrated that Ser43 and Ser45 play a role in the transient positive and steady-state negative inotropic effects of PE on papillary muscles. Notably, there was no transient increase in force in PE-treated fibers from mTnI hearts with the Ser43A/Ser45A mutation (as is seen in FVB mice), a finding that mirrors our in situ data using Iso. Additional characteristics of the mTnI mice not previously reported, include gene profiling of the adult TG animals, did not reveal any changes in mRNA for sarcomeric or calcium-handling proteins that might have been predicted to influence in situ contraction and relaxation due to changes in protein phosphorylation (24, 27, 32). In addition, there were no changes in adrenergic receptor function as indicated by the cAMP levels at baseline and after Iso treatment.

While these results confirm and extend earlier data derived from reconstituted preparations and isolated muscle studies that suggest the presence of phosphorylation at Ser43 and Ser45 on cTnI significantly influences strong cross-bridge binding to the thin filament (11, 14), they also provide new insights into the mechanisms of cardiac muscle regulation in vivo versus in vitro and the mechanical correlates. The differences in baseline hemodynamics, particularly contractility (± dP/dt), observed between mTnI and wild-type measurements obtained in these studies differ slightly from the in vitro isovolumic studies conducted by MacGowan et al. (11) and is likely attributed to the difference in models. Grupp et al. (6) examined the difference in models by comparing the in vitro isolated working heart with the in situ mouse heart. They demonstrated that mechanical performance indexes that were highly significant between experimental groups using the working heart preparation were less significant when obtained using the in situ model. In fact, some parameters, such as LV pressure, were no longer statistically significant when assayed in TG mice in situ (6). The dynamic range also increases in the in situ preparation. This was illustrated by measurement results (dP/dt) being over 200% greater than those in the working heart preparation.

The finding that contraction and relaxation are augmented in the basal state in mTnI mice is consistent with previous studies in isolated fibers showing that PKC-dependent phosphorylation of TnI (at Ser43 and Ser45) decreases the calcium sensitivity of the myofilaments and depresses peak Mg-ATPase activity (16, 18, 20) as well as produces a depression in the cross-bridge cycling rate (4, 7). Thus our data indicate that Ser43 and Ser45 are at least partially phosphorylated in the basal state in FVB mice and, as a consequence, induce a partially inhibited state with regard to the dynamics of contraction and relaxation. In the case of mTnI mice, the lack of phosphorylation at cTnI Ser43 and Ser45 releases the hearts from this inhibited state, inducing the heart to function at near-maximum kinetics of contraction and relaxation. That this is seen in the absence of a pathological load (where the mTnI mice would be predicted to have preserved function) is of interest because it has generally been presumed that the PKC effect on myofilaments...
ment function is predominantly a maladaptive one seen in failing cardiac muscle in which endogenous PKC activities are increased (25, 26). Our data speak to the concept that basal contractility represents a balanced circumstance that reflects a number of contradictory influences impacting the thin filament.

The inability of the mTnI mice to augment cardiac function further in response to an adrenergic agonist is not easily explained, and our data would suggest that this result coupled with the enhanced basal contractility is reflective of a relative increase in basal phosphorylation of the cTnI PKA sites and a lack of phosphorylation at the PKC sites at Ser23 and Ser45. Adrenergic agonists influence cardiac thin filament function largely via the cAMP-dependent (PKA) phosphorylation of cTnI at the unique NH2-terminal sites, Ser22 and Ser23, which leads to a decreased affinity of cTnI for TnC, a decreased affinity of TnC for calcium, and a resultant increased off rate for calcium exchange and augmented muscle relaxation (30). If the only effect of the S43A/S45A mutation was to block PKC phosphorylation at this site, then the PKA effect should have been superimposable. The fact that it was not suggests that PKC can potentially subserve a hierarchy of phosphorylation sites (and functions) in vivo, including phosphorylation at the putative PKA site. This phenomenon has been observed in reconstituted myofilaments containing TnI (S43A/S45A) in which isoforms of PKC, in particular PKC-ε, were shown to phosphorylate Ser22 and Ser23 (16). Our data from the back phosphorylation studies confirm these findings in vivo. Basal PKA-dependent phosphorylation (only permitted at Ser23 and Ser25) was increased in the mTnI mice relative to wild-type FVB mice. Furthermore, the recent work of Turnbull et al. (29) and Kentish et al. (10) demonstrating that TnI phosphorylation mediated by cAMP-dependent PKA hastens relaxation by increasing the off rate for Ca2+ exchange with cTnC and the cross-bridge cycling rate supports a functional link between the increased contractility and increased PKA phosphorylation of TnI. With the use of TG mouse hearts with nonphosphorylatable TnI, Kentish et al. (10) reported that this effect occurs independent of C-protein phosphorylation. Thus, even though the differences in cTnI phosphorylation at the PKA site in the mTnI mice were relatively small, it is plausible that this cross phosphorylation of PKA sites by PKC had a significant functional impact.

The increase in basal contractility seen in the mTnI mice coupled with their inability to augment function further in response to Iso (suggesting that the TG hearts are operating at or near maximal contractility) underscores an important clinical caveat. MacGowan et al. (12) reported that mice lacking Ser23 and Ser45 are more sensitive to ischemic contracture after global ischemia. Iso is known to increase contractility but with a greater demand for oxygen (23). As these hearts are operating at a higher level under baseline conditions, it is possible the oxygen dependency of Iso limits its ability to induce a further change in contractility and/or renders the heart more vulnerable.

Truly unexplained is the marked effect of Pro to differentially blunt the enhanced contraction and relaxation kinetics in the mTnI mice. This effect does not appear to be due to a major inhibition of adrenergic signaling through adenyly cyclase as cAMP levels did not differ in the two groups of animals. The differential effects are likely due to the highly coordinated regulation of the thin filament system. The model used in these experiments has PKC phosphorylation sites 43 and 45 mutated, leaving intact 144 as well as PKA phosphorylation sites. The important interaction between PKC and PKA in regulating cTnI has recently been demonstrated by Pi et al. (19) using several TG models with PKC and/or PKA phosphorylation sites removed. Their data provide evidence that phosphorylation of cTnI at either PKC or PKA sites regulates in distinct and opposite ways myofilament Ca2+ sensitivity and the ATP hydrolysis rate. We suggest that the β-blockade provided by Pro plus the absence of PKC phosphorylation increases the required Ca2+, which is illustrated by a decrease in contractility compared with the wild type. Another plausible and testable hypothesis to explain this is that Pro, a nonselective β-blocking agent, might affect other downstream pathways such as those involving ERK and MAPK (7) and p38 (22), which might have been differentially activated in mTnI mice. Alternatively, Pro might have changed the energy balance of contraction differentially in FVB and mTnI mice.

In conclusion, the complexity of in vivo regulation of thin filament interactions, and by extension muscle function, is critically important to appreciate, as novel pharmacological therapies are developed to treat cardiac muscle dysfunction. For example, our data would suggest that blocking the PKC phosphorylation site of TnI would be beneficial in treating the mechanical maladaptation in clinical heart failure; however, the unanticipated effect of such therapy might be enhanced phosphorylation of the putative PKA sites (Ser23 and Ser25) with an attendant decrease in calcium sensitivity of the myofilaments and perhaps increased vulnerability to ischemic contracture.

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