Abnormal Cardiac Structure and Function in Mice Expressing Nonphosphorylatable Cardiac Regulatory Myosin Light Chain 2*

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Atsushi Sanbe‡, Jason G. Fewell‡, James Gulick‡, Hanna Osinska‡, John Lorenz§, D. Greg Hall†, Lisa A. Murray†, Thomas R. Kimball, Sandra A. Witt, and Jeffrey Robbins‡**

From the ‡Department of Pediatrics, Division of Molecular Cardiovascular Biology and †Department of Pediatrics, Division of Cardiology, Children's Hospital Research Foundation, Cincinnati, Ohio 45229-3039, the §Department of Pathology, University of Georgia College of Veterinary Medicine, Athens, Georgia 30605, and the ‡Department of Molecular and Cellular Physiology, University of Cincinnati, Cincinnati, Ohio 45267-576

A role for myosin phosphorylation in modulating normal cardiac function has long been suspected, and we hypothesized that changing the phosphorylation status of a cardiac myosin light chain might alter cardiac function in the whole animal. To test this directly, transgenic mice were created in which three potentially phosphorylatable serines in the ventricular isoform of the regulatory myosin light chain were mutated to alanines. Lines were obtained in which replacement of the endogenous species in the ventricle with the nonphosphorylatable, transgenically encoded protein was essentially complete. The mice show a spectrum of cardiovascular changes. As previously observed in skeletal muscle, Ca$^{2+}$ sensitivity of force development was dependent upon the phosphorylation status of the regulatory light chain. Structural abnormalities were detected by both gross histology and transmission electron microscopic analyses. Mature animals showed both atrial hypertrophy and dilatation. Echocardiographic analysis revealed that as a result of chamber enlargement, severe tricuspid valve insufficiency resulted in a detectable regurgitation jet. We conclude that regulated phosphorylation of the regulatory myosin light chains appears to play an important role in maintaining normal cardiac function over the lifetime of the animal.

The roles of the regulatory myosin light chains (RLCs)1 and the reversible post-translational modifications they undergo in striated muscle are beginning to be defined. In skeletal muscle, a serine at the amino end of the protein can be phosphorylated by a sarcoplasmic kinase (1), and it is now clear that RLCs in the different striated muscles are phosphorylated to differing degrees, leading presumably to different physiological effects. In smooth muscle, RLC phosphorylation by myosin light chain kinase (MLCK), which is activated by a Ca$^{2+}$/calmodulin-dependent pathway, is responsible for initiating muscle contraction (2, 3). However, in skeletal and cardiac muscle, in which the thin, rather than thick, filament mediates control of contraction, RLC phosphorylation does not activate contraction but appears to play a modulatory role. In skinned skeletal muscle fibers, RLC phosphorylation increases sensitivity to activating Ca$^{2+}$ such that there is a significant leftward shift in the force-Ca$^{2+}$ relationship (4–6). Increased RLC phosphorylation in skeletal muscle is also associated with potentiation of isometric twitch tension with repeated activation and inactivation of contraction (7, 8), rate of force production (9–11), and maximum Ca$^{2+}$-stimulated MgATPase activity (12).

The mechanistic basis for the effects of RLC phosphorylation in striated muscle is hypothesized to be a lessening of the weak interaction of the myosin head with the myosin backbone and is probably due to a net charge change in a critical region of the protein (13). Upon phosphorylation, the myosin heads move away from the backbone to a position closer to actin, which presumably increases the rate at which myosin-actin interactions occur. Recently, the physiological importance of RLC phosphorylation in striated muscle was addressed by examining the in vivo effects of RLC mutant proteins in the indirect flight muscle of Drosophila (14). Phosphorylatable serine residues were replaced with alanines, and the mutated proteins were introduced into a null RLC background. The resultant flies showed normal myofibrillogenesis but had reduced power output and flight ability resulting from a marked reduction in the stretch activation of the indirect flight muscle.

Little is known about the role RLC2 phosphorylation plays in maintaining normal mammalian cardiac function. In rats, increased RLC phosphorylation occurs in response to increases in beat frequency and/or LV pressure due to exercise or inotropic agents (15, 16). This response may help maintain force production and stroke volume in the face of an increased rate of relaxation. However, the response is attenuated or absent in the mouse heart after similar exercise regimens (17). Changes in RLC phosphorylation in ischemic rabbit hearts have been noted (18), and in some patients with heart failure, decreases in light chain phosphorylation occurred (19).

In this study, we examined the physiological role of RLC phosphorylation in the heart by transgenically overexpressing a mutated form of RLC2v (ventricular regulatory myosin light chain) that could not be phosphorylated. Multiple lines of transgenic (TG) mice were created in which the endogenous atrial and ventricular isoforms of RLC2 were replaced with a ventricular form in which alanines were substituted for the phosphorylatable serines at residues 14, 15, and 19. Analyses of these animals at the molecular, cellular, whole organ, and animal levels confirm the importance of regulated phosphoryl-

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** To whom correspondence should be addressed: Division of Molecular Cardiovascular Biology, Children’s Hospital Research Foundation, Cincinnati, Ohio 45229-3039, Tel.: 513-636-8098; Fax: 513-636-3852; E-mail: jeff.robbins@chmcc.org.

† The abbreviations used are: RLC, regulatory myosin light chain; RLC2v, ventricular regulatory myosin light chain; MLCK, myosin light chain kinase; TG, transgenic; NTG, nontransgenic; MyHC, myosin heavy chain; RV, right ventricle; LV, left ventricle; ANF, atrial natriuretic factor; PAGE, polyacrylamide gel electrophoresis; BW, body weight.

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ation of the myosin light chains in maintaining normal cardiac function.

**EXPERIMENTAL PROCEDURES**

**Transgene Construction**—A previously isolated full-length murine RLC2′ cDNA was used as a starting template (20). Primers with SacI sites engineered at the termini were made to the 3′ and 5′-untranslated regions of the cDNA. The 5′ primer also contained the mutated bases necessary to change the serines at residues 14, 15, and 19 to alanines (Fig. 1A). The polymerase chain reaction product was sequenced, and the fragment was linked to the mouse α-myosin heavy chain promoter. The final construct, RLC2′p−, was digested free of vector sequence with NotI, purified from agarose, and used to generate transgenic mice as described (20).

**Transcript Analysis**—Total ventricular RNA was prepared from freshly isolated hearts obtained from 8–16-week-old mice euthanized by CO2 asphyxiation. Samples were homogenized in Tri-Reagent (Molecular Research Center, Cincinnati, OH), and RNA was extracted according to the manufacturer’s protocol. For each analysis, 1.5 μg of total RNA was loaded onto a nicoilete membrane using a dot blotting apparatus (Bio-Rad). Hybridizations using 32P-end-labeled oligonucleotides were performed as described previously (21). Oligonucleotide sequences that were used as probes (glyceraldehyde-3-phosphate dehydrogenase, α-actinel actin, atrial natriuretic factor (ANF), β-myosin heavy chain (MyHC), and phospholamban) have been published (22). A 51-base oligonucleotide, specific for RLC2′v consisted of the sequence 5′-GGCTGTGGTTTAGGGCTGATCCCTGTCCCTTTGTCCTGTTGGAATTAATTG-3′, and the oligonucleotide 5′-AGTTGTGTGTTGATAAAACGGAGATGACCACCCGAACACTTTATTTCTGCAAAATGG-3′, specific for the sarcoplastic reticulum ATPase were also used. Hybridization signals were identified and quantitated using a STORM PhosphorImager (Molecular Dynamics, Inc.).

**Myofilament Protein Analyses**—For SDS-PAGE, the left ventricular apex and atrial flaps were obtained from euthanized RLC2′p− TG and nontransgenic (NTG) mice. Myofibrillar sample preparation, gel preparation, electrophoretic conditions, and gel staining have been described (17). Samples used for two-dimensional gel electrophoresis were prepared as described by Kirschbaum et al. (23). Cylindrical polyacrylamide gels (2 mm) for isolectric focusing were prepared as described by O’Farrell (24) using Bio-Rad ampholytes (2% (w/v) final concentration) with a restricted gradient of pH 4–6. Electrophoresis was performed at 500 V for 10 min and then at 750 V for 3.5 h. Gels were removed from the tubes and equilibrated in a solution containing Tris buffer (62.5 mm, pH 6.8), 1% (w/v) SDS, and 5% (v/v) β-mercaptoethanol for approximately 5 min. Electrophoresis in the second dimension slab gel (16% polyacrylamide, 29:1 acrylamide to bisacrylamide) was carried out in the presence of 0.1% SDS. Gels were stained with colloidal blue (Sigma). The phosphorylation state of fibers used in the mechanical studies was tested by Western blot analysis as described (17). Each sample consisted of two ventricular fiber bundles homogenized in sample buffer (8 μL urea, 18 mm Tris (pH 8.5), 20.7 mm glycine, 9.3 mm dithiothreitol, 4.6% (v/v) saturated sucrose, and 0.004% (w/v) bromphenol blue).

**Electron Microscopy and Histology**—The methods used for ultrastructural analysis have been described (17). For histological examination, hearts were briefly perfused with 4% paraformaldehyde in phosphate-buffered saline and then removed and further fixed in 4% paraformaldehyde. The methods for paraffin embedding, sectioning, and staining have been described (17).

**Measurements of Fiber Function**—Standard methods for measuring force development from skinned muscle fibers were adapted for mouse cardiac fibers (25). To examine the effects of myosin light chain phosphorylation on the pCa-force relationship in vitro, skinned fibers were treated with myosin light chain kinase and calmodulin. After measurements of the pCa-force relationship (first contraction), the fiber was incubated in relaxing solution (pCa 8.0) plus 2 μM calmodulin and 0.15 μM of pCa 8.0, and Ca2+−stimulated MgATPase activity was measured. The 5-min reaction was initiated with the addition of the sample at 23 °C and stopped with 15% trichloroacetic acid. The reaction mixture was centrifuged, and inorganic phosphate was measured in the supernatant fraction. The Ca2+−stimulated MgATPase activity was estimated by subtracting the activity at pCa = 8.0.

**Intact Animal Model**—Assessment of left ventricular function was performed as follows. Mice were anesthetized with intraperitoneal injections of 50 μg/kg body weight (BW), ketamine and 100 μg/kg BW, thiobutabarbital (Inactin, Research Biochemicals International, Natick, MA) and placed on a thermally controlled surgical table. Following tracheotomy, the right femoral artery and vein were cannulated with polyethylene tubing for measuring systemic arterial pressure and for the infusion of experimental drugs. A 1.4F Millar MikroTip transducer (Millar Instruments, Houston, TX) was inserted in the right carotid artery and advanced into the left ventricle for measurement of LV pressure and dP/dt. Cardiovascular responses to increasing doses of dobutamine were determined during 3-min constant infusions of dobutamine in normal BW, and average values were determined during the final 30 s of each infusion. At the end of each dose-response protocol, a bolus dose of propranolol (100 ng/kg BW) was administered to evaluate baseline cardiac function in the absence of endogenous β-adrenergic activity. Pressure signals were recorded and analyzed using a MacLab 4/s data acquisition system at a sampling rate of 1000 samples/channel.

**Echocardiography**—Echocardiography was performed using a Hewlett Packard Sonos 5500 Ultrasound System equipped with a 12-MHz transducer (Andover, MA). In order to place the heart in the mid-field of the ultrasound sector where axial resolution is optimal, the transducer was fashioned with a latex balloon filled with warm acoustic gel producing a 1-cm standoff between the chest wall and the transducer. Two-dimensionally guided M-mode echocardiography of the left ventricle from its short axis was recorded on an optical disc and a strip chart at a speed of 100 mm/s.

**RESULTS**

**Transgenic Expression of Nonphosphorylatable RLC2′**—The object of this study was to ablate RLC2′ phosphorylation in the heart. In striated muscle, MLCK can phosphorylate serine 15 (1, 26). In *Drosophila*, serines 66 and 67 can be phosphorylated by the enzyme (27). These two residues are analogous to serines 14 and 15 in the mouse (RLC2′ has an N-terminal extension that is absent in mammalian RLC), suggesting that both residues are potential enzyme substrates. In smooth muscle, both threonine 18 and serine 19 can be phosphorylated by MLCK (28), and sequence similarities between the smooth and striated muscle RLCs suggest that serine 14 in skeletal muscle is also a target for MLCK phosphorylation. An additional consideration in the construct’s design was that other kinases such as Rho kinase and protein kinase C have the ability to phosphorylate RLC2′ at serine 15 as well as at other amino acid residues (5, 29, 30). Thus, to ensure that phosphorylation would not occur on the transgenically encoded protein, serines 14, 15, and 19 were each mutated and replaced by alanine (Fig. 1A). Multiple clones were sequenced to exclude polymerase chain reaction-induced errors. Except for the mutated residues, the consensus sequence was identical to that previously used to make TG mice (20). Three TG founders were identified and used to establish stable lines by outbreeding to NTG animals. In all cases, the analyses below were carried out on transgenic heterozygotes in order to minimize the possibility of confounding the phenotype through insertion mutagenesis. RLC2′ expression at the transcript level was quantitated by dot blot analyses with transcript-specific oligonucleotides (Fig. 1B). TG transcript levels were quite modest, ranging from 1.33- to 2.5-fold above that of the endogenous message and were constant across all four cardiac compartments.2

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2A. Sanbe, J. G. Fewell, J. Gulick, H. Osisaka, J. Lorenz, D. G. Hall, L. A. Murray, T. R. Kimball, S. A. Witt, and J. Robbins, unpublished data.
A, the wild type ventricular isoform of the regulatory myosin light chain 2 (RLC2v) was mutated such that the phosphorylatable serines at amino acid residues 14, 15, and 19 were replaced by alanines. The sequences shown correspond to amino acids 1–21. Identical nucleotide bases are indicated with a dot. The locations of the amino acid changes are numbered. The cDNA was placed in the α-MyHC promoter cassette and used to generate transgenic mice as described under “Experimental Procedures.” Three lines of mice were subsequently selected for study. B, RNAs were isolated from the ventricles, and the RNA abundance of the transgene, relative to the endogenous RLC2v (set at 1), was determined by dot blot analyses as described previously (22). Three 4-month-old males were used for each of the RNA isolations.

We were interested in determining if there were changes in heart morphology in the RLC2v(P−) TG mice. When the RLC of Drosophila indirect flight muscles was replaced by a nonphosphorylatable variant, no overt changes in overall muscle organization or architecture could be detected (14). In contrast to the insect data, hearts from adult mice derived from the higher expressing lines 21 and 35 both showed hypertrophied and dilated atria when compared with line 42, which expressed only low levels of the transgene or NTG hearts (Fig. 2A). Multiple hearts (4–8 hearts) from both lines 21 and 35 showed similar gross morphology, ruling out the possibility that the observed phenotype was due to an insertional mutagenic event. Sections comparing ventricular free walls derived from wild type or NTG versus RLC2v(P−) animals indicated that some mild cardiomyocyte disarray could be detected in the latter (Fig. 2C). This mild pathology was apparent in both lines 21 and 35 but not in the line that expressed low levels of the transgene (line 42). In an attempt to define more precisely any LV hypertrophy that might be occurring, 10-week TG (line 21) and NTG hearts were dissected, and the weights of the septa and LV free walls were determined (Table I). The data show a trend toward septal hypertrophy, while the LV free wall weights are unaffected. To confirm the lack of overt hypertrophy in the ventricles, a detailed morphometric analysis was carried out on hearts from NTG and TG lines expressing either wild type RLC2v or RLC2v(P−) (lines 21 and 35). Multiple sections were cut from five blocks derived from two hearts from each line and myocyte density, diameter, and myocyte volume density determined for the LV and RV free wall, as well as the septum. No statistically significant differences between either of the TG lines, as compared with the NTG hearts, were observed.

Protein Expression—Expression of the mutated protein was first analyzed by examining myofilament proteins derived from the atria of TG animals. Previously, we overexpressed wild type RLC2v in the mouse heart, resulting in complete replacement of atrial RLC2a, while ventricular levels of RLC2v remained constant (17, 20, 31). Those studies showed that overexpression of the ventricular isoform in the atrial compartment resulted in small changes in whole heart function, with modest decreases in the rate of force development and relaxation. However, the mice exhibited no cardiac hypertrophy or chamber dilation. Protein from these mice (line 97) was included as controls in the following analyses. The ventricular and atrial isoforms of RLC2 can be resolved by SDS-PAGE, and examination of the atrial myofibrillar protein composition of juvenile and adult mice shows a progressive replacement of RLC2a with the mutated RLC2v (Fig. 3A). In young mice (3 weeks old), the highest expressing lines (21 and 35) show 50% replacement, while line 42 shows little or no replacement. However, by 3–4 months the two high expressing lines show complete replacement, while line 42 shows approximately 50% replacement. Note that for both the RLC2v and RLC2v(P−) overexpressors, no overt changes in contractile protein stoichiometry occurred (Fig. 3A). To determine ventricular replacement, a gel system capable of resolving RLC2v from RLC2v(P−) was developed. A 21% SDS-PAGE resolved the 48-dalton differences in size between the mutated and endogenous proteins (Fig. 3B). The data show that both line 35 and 21 have a nearly complete RLC2v → RLC2v(P−) shift, whereas there is little or no replacement in line 42. In all cases, the myofibrillar protein stoi-
chiometries were maintained as evidenced by no net increase in total RLC2v protein, a result consistent with previous data obtained for the RLC2v(wt) overexpressors (20). Because of the lack of a discernable gross cardiac phenotype in the low overexpressors (line 42) and essential identity observed between lines 21 and 35, both of which expressed identical levels of the

| TABLE I | Normalized heart weights for line 21 mice |
|---------|------------------------------------------|

n = 3. All values are means ± S.E. Student's t test was used to determine significant differences.

|              | Body weight | LV free wall | LV free wall/BW | Septum | Septum/BW | LA + RA/BW |
|--------------|-------------|--------------|-----------------|--------|-----------|------------|
| RLC2v(P−)    | 32 ± 1.0    | 63.9 ± 2.0   | 2.09 ± 0.06     | 26.8 ± 0.5 | 0.87 ± 0.01 | 1.28 ± 0.17 |
| NTG          | 31 ± 1.0    | 67.6 ± 0.7   | 2.1 ± 0.061     | 25.8 ± 0.5 | 0.80 ± 0.02 | 0.31 ± 0.04 |

a LA and RA, left and right atria, respectively.
b p < 0.001.

**Fig. 3. Effects of transgene expression on myocardial protein composition.** *A*, in the left panel, myofilament proteins were extracted from the atria of 3-week-old animals of the different lines, subjected to SDS-PAGE as described under "Experimental Procedures," and stained with colloidal blue. Line 97 is a TG line expressing wild type RLC2v in the atria at levels such that the atrial isoform is completely replaced (31). The right panel shows the degree of replacement in the atria of adult (3-month) mice. Line 42 shows approximately 50% replacement, while the other two, higher expressing lines show complete replacement of RLC2a with RLC2v(P−), without altering the levels of the other sarcomeric proteins. B, quantitation of the degree of replacement in the ventricles of adult transgenic mice. A gel system was developed that allowed resolution of the RLC2v and RLC2v(P−) isoforms. In all experiments, samples were derived from two or three 3–4-month-old males. The stained gels were scanned, and the signal intensities were quantitated using NIH Image (version 1.57). The corresponding levels of the endogenous RLC2 isoform and RLC2v(P−) were determined for each line. Lines 21 and 35 show complete replacement. MIX, equal amounts of protein from line 21 and NTG mice were electrophoresed in order to confirm the resolution of the gel system. Note that the mutated form (⁎) migrates more slowly. As previously noted, in neither cardiac compartment could an overt effect on the myofilament stoichiometry of the other contractile proteins be detected.
transgene and showed bialtral hypertrophy, the analyses carried out below focused only on line 21.

To determine if detectable phosphorylatable RLC2v was left in TG ventricles, we attempted to resolve the phosphorylated and unphosphorylated forms by PAGE but were unsuccessful. Therefore, the RLC2v phosphorylated and unphosphorylated forms were separated by two-dimensional gel electrophoresis (Fig. 4). The species corresponding to RLC2v and the phosphorylated form were initially identified by Western blot analysis; multiple analyses showed that in NTG ventricles, 10–30% of the RLC2v is phosphorylated (Fig. 4, top panel). In TG animals, phosphorylated RLC2v could not be detected. These data, in conjunction with the SDS-PAGE analyses, indicate that not only is phosphorylation completely ablated in the TG RLC2v(P−), but little endogenous RLC2v remains in the adult ventricles of line 21 (and line 35) TG animals.

**Ultrastructure of Line 21 RLC2v(P−) Hearts**—In light of the histological abnormalities observed in lines 21 (Fig. 2C) and 35, we were interested in determining if there were ultrastructural changes in the RLC2v(P−) TG mice. If the RLCs of Drosophila indirect flight muscles are replaced by a nonphosphorylatable variant, no changes at the ultrastructural level take place (14). Hearts from line 21 animals were then chosen for detailed ultrastructural study using transmission electron microscopy. First, cursory screening of 1-μm epon resin sections from LV fragments of TG mice revealed irregularly shaped cardiomyocytes, the presence of vacuoles, and an irregular distribution of mitochondria (data not shown). At the ultrastructural level, cardiomyocytes from TG LVs revealed more striking abnormalities (Fig. 5). Normal NTG myocardocytes show tightly packed sarcomeric arrays and clearly defined intercalated discs (Fig. 5A), while myofibril degeneration presents in the RLV2v(P−) cardiomyocytes. In many cases, sarcomeric organization is lost and the intercalated discs are convoluted. Large interstitial spaces separate the myocytes, and there is increased collagen deposition (Fig. 5, B–D). Some myocytes, apparently in the final stages of degeneration, were filled with vacuoles, although all had intact sarcolemmal membranes.

**Molecular Markers of Hypertrophy Show a Dose Response**—Based upon the histological analyses, it appeared that the LV of the TG hearts might be mildly aberrant, although no increases in the LV free wall weights could be detected (Table I). Based upon our experience with a number of models, molecular markers of hypertrophy are often elevated in the absence of any detectable histological changes. Indices of the hypertrophic response at the molecular level were determined by transcript analyses of LVs derived from high and low expressing lines (lines 21 and 42, respectively) and compared with transcript levels in the RLC2v(wt) overexpressor as well as with NTG hearts. Transcript levels of ANF, α-skeletal actin and β-MyHC in line 21 animals were significantly elevated when compared with line 42 animals as well as the NTG and RLC2v(wt) controls (Fig. 6). Thus, both TG lines show activation of the hypertrophic markers, and a dose-dependent response is observed, in that the magnitude of the response is consistent with the degree of RLC2v replacement with the nonphosphorylatable species. Analyses of the transcripts for phospholamban and sarcoplasmic reticulum ATPase, which can be down-regulated during heart failure, reveal only very modest decreases, indicating that these hearts remained quite healthy and show no signs of decompensating into failure. No sign of a hypertrophic response at the molecular level could be discerned in the RLC2v(wt)-overexpressing line, indicating that isoform replacement in the atria and/or increased levels of total RLC2v transcript in all four cardiac compartments were not responsible for the observed phenotype.

**Calcium Sensitivity of Force Development**—In skeletal muscle, RLC2v phosphorylation results in increased sensitivity to activating Ca2+ levels, leading to a leftward shift in the force–Ca2+ curve. In line 21 mice, RLC2v cannot be phosphorylated. To test the effect of this change on fiber Ca2+ sensitivity, skinned fiber bundles from LV papillary muscles were isolated from 8-week-old TG (line 21) and NTG hearts. At this stage of development, the pathological and histological changes have not yet occurred. Under normal isolation conditions, the fibers as isolated are completely dephosphorylated. However, treatment with MLCK results in approximately 50% of RLC2v being phosphorylated. This was confirmed by performing Western blots on proteins derived from the NTG fiber bundles before and after kinase treatment, using antibody specific for RLC2v. Kinase treatment resulted in approximately 50% phosphorylation of endogenous RLC2v, and no phosphorylation could be observed in the RLC2v(P−) fibers.2

Force production in response to increasing Ca2+ levels was measured under the dephosphorylated conditions and also following MLCK treatment (Fig. 7). There were no significant differences in pCa-force relationships between the first and the second contraction in the control protocol without any calmodulin or MLCK (see “Experimental Procedures”), indicating that the kinase protocol itself did not alter fiber response to Ca2+ (data not shown). When TG and NTG fibers were compared before MLCK treatment (dephosphorylated) the curves were identical, indicating that TG fibers behave normally under dephosphorylated conditions. After MLCK treatment, when the degree of RLC2v phosphorylation would differ between the NTG and TG animals, NTG animals show increased Ca2+ sensitivity as indicated by a leftward shift in the force–Ca2+ curve (Fig. 7, A and C). The magnitude of this response is similar to data obtained using skinned fibers isolated from rabbit ventricle (7). In contrast, in the TG fibers, a leftward shift in the force–Ca2+ curve after kinase treatment did not occur (Fig. 7, B and D). Similar differences were seen in the pCa-Mg2+-ATPase activity curves, with a leftward shift (increase in sensitivity) occurring in the NTG fibers (Fig. 7E) and
no changes presenting in the RLC2\(v(P^-)\) (Fig. 7F). These data confirm that the RLC2\(v(P^-)\) fibers cannot be phosphorylated and, by this mechanism, cannot exhibit the shifts in force development or Mg\(2^+\)-ATPase activity as a function of Ca\(2^+\).

**Responses to an Inotropic Stimulus**—What is the mechanistic basis of the development of the phenotype seen in the RLC2\(v(P^-)\) mice? The fiber data indicate probable changes, in vivo, of tension development as a function of Ca\(2^+\) concentration. Thus, the end stage phenotype might reflect a functional deficit that could arise as a result of the heart being unable to increase contractile force in response to increased loading conditions or chronotropic stimulation with increases in RLC2\(v\) phosphorylation throughout the animal’s life span. To test this hypothesis, we measured increases in cardiac performance in
response to β-adrenergic stimulation. Cohorts of RLC2v(wt) and RLC2v(P−) as well as NTG animals were examined by utilizing the intact closed chest mouse model (32, 33). β-Adrenergic stimulation was carried out by infusing increasing concentrations of dobutamine (1–32 ng/min/g of body weight) over a 3-min period, with peak responses measured during the final 30 s of the infusion period (Fig. 8). At the end of the experiment, a propranolol bolus (100 ng/g of body weight) was infused and cardiac function reevaluated in the absence of β-adrenergic stimulation.

Under nonstimulating conditions, there were no differences in the heart rate (Fig. 8A), mean arterial pressure (Fig. 8B), left ventricular end diastolic pressure (Fig. 8E) and dP/dt max (Fig. 8C) among RLC2v(P−), RLC2v(wt), and NTG animals. In NTG and RLC2v(wt) animals, dobutamine increased the heart rate, dP/dt max, and dP/dt min in a dose-dependent fashion (Fig. 8, A, C, and D). In the RLC2v(P−) animals, the response of dP/dt max to β-adrenergic stimulation was significantly reduced when compared with the first two cohorts (Fig. 8C), although the chronotropic response (heart rate) was essentially identical (Fig. 8A). The absolute values of dP/dt min were significantly lower in RLC2v(P−) animals under nonstimulated conditions and at all levels of β-adrenergic stimulation when compared with NTG and RLC2v(wt) animals (Fig. 8D). There were no differences in mean arterial pressure and LV end diastolic pressure under nonstimulated conditions and at all levels of β-adrenergic stimulation in the three groups. Thus, the inotropic responses of RLC2v(P−) animals are not due to alterations in afterload and preload during β-adrenergic stimulation or to differences in heart rate. The myocardial contractile response to β-adrenergic stimulation in nonphosphorylatable RLC2v animals is reduced, indicating that RLC2v phosphorylation can play a role in the positive inotropic effect in response to β-adrenergic stimulation.

Cardiac Remodeling in Older Mice—To assess the contractile and structural consequences the above changes might have on whole heart function, mice (line 21) aged 16 weeks were subjected to echocardiography (34, 35). In RLC2v(P−) TG mice, shortening fractions were nearly the same. Consistent with the molecular and histologic evidence, the mean left ventricular free wall thickness at end diastole was increased in control animals compared with TGs, although the difference did not reach statistical significance (0.085 ± 0.010 versus 0.104 ± 0.016 mm, respectively). No differences could be discerned between the two groups’ heart rates as measured in resting, conscious animals using a computerized tail cuff apparatus (581 ± 2 versus 592 ± 25 beats/min for the TG and NTG mice, respectively). Apical four-chamber views using two-dimensional guided color Doppler echocardiography were carried out in RLC2v(P−) TG mice and compared with the RLC2v(wt) transgenics (Fig. 9). No abnormalities were observed in the RLC2v(wt) transgenics, while the RLC2v(P−) mice show that altered cardiac function is associated with severe tricuspid valve insufficiency as evidenced by the regurgitation jet (Fig. 9A). It is likely that the tricuspid valve itself is not the primary defect but that insufficiency stems from the leaflets becoming stretched apart as a result of RV dilation. In addition, thrombi were identified in the RV.

DISCUSSION

The physiological effects of RLC2v phosphorylation in the mouse heart were examined by overexpressing a nonphosphorylatable variant of RLC2v. Previous reports suggested that light chain phosphorylation can play a modulatory role in cardiac function in response to changing loads and/or heart rate (6, 19, 36). In skeletal muscle there is clear evidence that the degree of RLC phosphorylation plays a critical role in determining Ca2+-sensitivity cross-bridge transitions (37). Recently, insight into the structural basis for the observed kinetic differences was obtained by examining negatively stained skeletal thick filament preparations using a combination of electron microscopy and optical diffraction techniques (38). These studies showed that RLC phosphorylation probably increased the population of the myosin heads that could form productive interactions with actin, either by increasing the mobility of the head region or changing its conformation. Multiple biochemical studies are consistent with the idea that phosphorylation can potentiate productive interactions of the head with the thin filament by physical movement away from the thick filament “backbone” and closer to actin (4, 13, 39). We show here that, over the early lifetime of the animal, the role of RLC phosphorylation is critical for the maintenance of normal heart function under sedentary conditions and that without the ability to phosphorylate RLC2v, severe hypertrophy and dilation of the atria occur, leading to poor cardiac performance. Previously, we overexpressed wild type RLC2v in the mouse heart, resulting in complete replacement of atrial RLC2a (17, 20, 31). That study showed that overexpression of the ventricular isoform in the atrial compartment was relatively benign. No overt changes in chamber size or overall architecture of the heart could be discerned in those studies or the studies above. Thus, the phenotype reported in the RLC2v(P−) mice is due to expression of the mutated protein and not merely to overexpression of RLC2v in the atrial compartment. It is interesting that the most sensitive assay, the appearance of molecular markers of hypertrophy, shows a dose response; i.e., line 42, in which only low levels of transgene replacement occurs, shows much less up-regulation of the transcripts measured, although the changes are significant (Fig. 6). These data are consistent with the lack of a detectable phenotype in these mice at the gross histological level (Fig. 2). However, it may be that subtle histopathology will develop in these mice over time, particularly if
they are subjected to conditions in which cardiovascular load is increased, such as defined exercise regimens (17).

The best characterized effect of RLC phosphorylation in striated muscle is a leftward shift of the force-Ca\(^{2+}\) curves in skinned fibers. When TG and NTG mice were analyzed, the NTG mice responded as expected to the kinase treatment with a leftward shift of the curve. There was no response to kinase treatment in the TG fibers as would be expected with a phosphorylation-deficient protein. Importantly, there were no significant differences between NTG and TG hearts under dephosphorylated conditions, although it should be noted that the degree of phosphorylation in the intact TG and NTG myocardia are not identical. This observation suggests that myofibers from TG animals have the capacity to behave normally and that the mutated protein does not, by itself, structurally alter the basic fiber, leading to changes in fiber function independent of its inability to become phosphorylated.

The long term effects associated with an inability to phosphorylate RLC\(_{2v}\) are profound. Dilation and hypertrophy of the atrial compartments are common characteristics of the two high expressing lines. Remodeling of the ventricles is subtler. Although the cellular indicators of hypertrophy are present at the molecular level (activation of ANF, \(\alpha\)-skeletal actin, and \(\beta\)-MyHC), careful measurement of the LV free wall/body weight showed no changes, although septal weights are increased. Morphometric analyses of multiple fields in multiple sections derived from three TG hearts revealed no statistical differences.\(^2\) However, ultrastructural changes in both high expressing

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**Fig. 7.** Calcium activation of isolated ventricular fibers. The ability of NTG (A, C, and E) and line 21 TG fibers (B, D, and F) to develop force as a function of [Ca\(^{2+}\)] and treatment with MLCK was measured in isolated skinned fibers as described under “Experimental Procedures.” The NTG fibers, when treated with MLCK, exhibit increased sensitivity to Ca\(^{2+}\). Force (A, B), normalized force (C, D), and Mg\(^{2+}\)-ATPase activity (E, F) are graphed as a function of pCa. Line 21 TG fibers show no changes in Ca\(^{2+}\) sensitivity as a result of MLCK treatment. Each fiber set \((n = 4)\) was derived from four 8–9-week-old animals, and statistical significance was determined using the paired \(t\) test except for the Mg\(^{2+}\)-ATPase activity, where statistical significance was determined using Student’s \(t\) test. *, \(p < 0.05\); **, \(p < 0.01\).
lines were obvious with widely scattered, degenerating myocytes apparent in multiple fields, changes in T-tubule organization, and interstitial abnormalities. In addition to the obvious changes in atrial morphology, we detected by histological analysis aspects of myocyte degeneration, although no extensive fibrosis occurred. This is probably due to the fact that the sarcolemmal membranes remain intact, and there is a lack of necrosis; therefore, no obvious substantive fibrosis results.

By ablating the ability of RLC2v to be phosphorylated, the manner in which the contractile apparatus responds to Ca\(^{2+}\), particularly under conditions of increased load, was significantly altered. Obviously, there are other ways in which the cardiac myofilament can modulate its sensitivity to Ca\(^{2+}\) in response to stress. Indeed, β-adrenergic stimulation, which is thought to be the physiologic pathway mediating RLC2v phosphorylation, can result in the phosphorylation of such proteins as troponin I, which can also have major effects on the myofilament's contractile response to Ca\(^{2+}\). The lack of the fibers' ability to "fine tune" the heart's force production by phosphorylating RLC appears to be critical over the lifetime of the

Fig. 8. Dobutamine dose-response relationships. The closed chest intact mouse model and the dobutamine infusion protocols have been described in detail (33). Each experimental set \(n=5\) consisted of 8–9-week-old animals. Cardiovascular function was measured under control conditions (no dobutamine) and under increasing β-adrenergic stimulation. Three cohorts, NTG, RLC2v(wt), and line 21 RLC2v(P\(^{-}\)) were all subjected to the same regimen, which included a series of 3-min infusions of increasing concentrations of dobutamine and measuring the cardiovascular indices in the last 30 s of each period in order to obtain peak response. A propranolol bolus was used to terminate the protocol so that function could be measured in the absence of β-adrenergic stimulation. Pressure signals from both the COBE and Millar transducers were recorded using a MacLab 4/s data acquisition system (AD Instruments, Milford, MA). The software directly determines arterial systolic and diastolic pressure, mean arterial pressure, heart rate, left ventricular systolic pressure, developed pressure, and both positive and negative \(dP/dt\) data. Data were analyzed using a mixed, two-factor analysis of variance with repeated measures on the second factor. When necessary, post hoc comparisons were performed by single degree-of-freedom contrasts. D, * \(p<0.05\); #, \(p<0.005\).
intracellular Ca\(^{2+}\) levels. Consistent with this hypothesis, increasing MLCK with decreasing RLC phosphorylation is implicated in the hypertrophic response that occurs due to experimentally induced myocardial infarction in rats (36). As the heart continues to remodel, it may be that alterations in Ca\(^{2+}\) transients occur in order to maximize the ability to produce force. This, in turn, might then activate the pathways leading to the end stage phenotype observed in the mice. These are all testable hypotheses with this mouse model.

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