Cardiac hypertrophy associated with impaired regulation of cardiac ryanodine receptor by calmodulin and S100A1

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Yamaguchi N, Chakraborty A, Huang T, Xu L, Gomez AC, Pasek DA, Meissner G. Cardiac hypertrophy associated with impaired regulation of cardiac ryanodine receptor by calmodulin and S100A1. Am J Physiol Heart Circ Physiol 305: H86–H94, 2013. First published May 10, 2013; doi:10.1152/ajpheart.00144.2013.—The cardiac ryanodine receptor ( RyR2) is inhibited by calmodulin ( CaM) and S100A1. Simultaneous substitution of three amino acid residues (W3587A, L3591D, F3603A; RyR2ADA) in the CaM binding domain of RyR2 results in loss of CaM inhibition at submicromolar (diastolic) and micromolar (systolic) Ca2+ levels when compared to wild type. The CaM binding domain of RyR2 (W3587A, L3591D, F3603A; RyR2ADA). RyR2ADA/ADA mice die within ~2 wk after birth because of the rapid development of cardiac hypertrophy and heart failure (35). Their early death is associated with abnormal SR Ca2+ release and altered gene regulation (33, 35). S100A1 is a small Ca2+ binding protein expressed at high levels in cardiomyocytes (13). S100A1 targets multiple sites that include sarcomeric proteins, mitochondrial proteins, and Ca2+ handling proteins Ca1,2, RyR2, and sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA)2a and SERCA2a regulatory protein phospholamban (PLN) (10, 21, 24, 29). S100A1 also regulates skeletal muscle RyR1 and binds to the conserved CaM binding domain of RyR1 (25, 27). S100A1/−/− mice have normal life span and cardiac function under normal conditions (7). However, transgenic overexpression of S100A1 increases SR Ca2+ content and Ca2+ release and improves myocardial contractile performance (21). More recent studies have shown that molecular targeting of S100A1 to myocardially infarcted animals (23) and human failing cardiomyocytes (4) restores cardiac function by improving mitochondrial function, SR Ca2+ handling, and contractile performance. This suggested that S100A1 gene targeting may be a promising strategy to treat heart failure.

To further define the role of RyR2 and Ca2+ in cardiac development and function, a second mouse model was prepared with a single amino acid substitution in the CaM and S100A1 binding domain of RyR2 (L3591D; RyR2D). This mutation was selected on the basis of earlier studies showing that the RyR2-L3591D mutant is inhibited by CaM at micromolar Ca2+ but not submicromolar Ca2+ concentrations when expressed in human embryonic kidney 293 (HEK293) cells (36). Here we report that, in contrast to RyR2ADA/ADA mice, RyR2D mice live longer than 1 yr and develop a less severe impairment of cardiac function than RyR2ADA/ADA mice.

MATERIALS AND METHODS

Ethics statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the University of North Carolina at Chapel Hill and the Medical University of South Carolina Institutional Animal Care and Use Committees. Animals were anesthetized and killed with 1–4% isoflurane (drop method) or intraperitoneal injection of Avertin (160 mg/kg) followed by physical euthanasia.

Materials. [3H]ryanodine was obtained from PerkinElmer and unlabeled ryanodine from EMD Biosciences. Complete protease inhibitors were from Roche Applied Science and phospholipids from Avanti Polar Lipids. Unlabeled CaM was prepared as described previously (2). Rabbit polyclonal antibody RyR2-F9221 was prepared

CARDIAC MUSCLE RYANODINE RECEPTORS ( RyR2s) are large, ligand-gated ion channels composed of four 560-kDa RyR2 subunits, four 12.6-kDa FK506 binding protein subunits, and various associated proteins that include protein kinases, protein phosphatases, S100A1, and calmodulin (CaM) (11, 17, 19, 30). Binding of CaM to RyR2 inhibits the release of Ca2+ from sarcoplasmic reticulum (SR) at diastolic and systolic Ca2+ levels (2, 8, 19, 31). Deletion of RyR2 amino acid residues 3583–3603 and mutations in this region eliminate CaM binding and inhibition of the recombinant RyR2 by CaM in vitro (36). An in vivo role of RyR2 regulation by CaM was established by preparing mutant mice with three amino acid substitutions in the CaM binding domain of RyR2 (W3587A, L3591D, F3603A; RyR2ADA). RyR2ADA/ADA mice die within ~2 wk after birth because of the rapid development of cardiac hypertrophy and heart failure (35). Their early death is associated with abnormal SR Ca2+ release and altered gene regulation (33, 35). S100A1 is a small Ca2+ binding protein expressed at high levels in cardiomyocytes (13). S100A1 targets multiple sites that include sarcomeric proteins, mitochondrial proteins, and Ca2+ handling proteins Ca1,2, RyR2, and sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA)2a and SERCA2a regulatory protein phospholamban (PLN) (10, 21, 24, 29). S100A1 also regulates skeletal muscle RyR1 and binds to the conserved CaM binding domain of RyR1 (25, 27). S100A1/−/− mice have normal life span and cardiac function under normal conditions (7). However, transgenic overexpression of S100A1 increases SR Ca2+ content and Ca2+ release and improves myocardial contractile performance (21). More recent studies have shown that molecular targeting of S100A1 to myocardially infarcted animals (23) and human failing cardiomyocytes (4) restores cardiac function by improving mitochondrial function, SR Ca2+ handling, and contractile performance. This suggested that S100A1 gene targeting may be a promising strategy to treat heart failure.

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Preparation of mutant mice. Mutant mice carrying the RyR2-L3591D mutation were prepared with established methods (35). Briefly, linearized targeting vector was transfected into E14 embryonic stem (ES) cells by electroporation. Cells were selected in the presence of G418 and gancyclovir and screened by both PCR and Southern blot analysis. Mutations were confirmed by DNA sequencing. Targeted ES cells were injected into the blastocysts of C57BL/6J mice in the Animal Models Core Facility of the University of North Carolina at Chapel Hill. A male chimera mouse carrying the mutation in the Ryr2 gene was mated with a 129/SvEv female mouse to obtain heterozygous offspring. The neomycin-resistant gene flanked by loxP sequences was removed by Cre recombinase driven by the testis-specific angiotensin-converting enzyme promoter (Fig. 1). Germ line transmission of the mutation to heterozygous offspring caused removal of a gene cassette flanked by loxP sequences (5).

Homozygous gene-targeted animals (Ryr2<sup>+/D</sup>) were obtained by mating heterozygous mice (Ryr2<sup>+/H</sup>). Previous experiments with Ryr2<sup>ADA/ADA</sup> mice (35) and experiments with Ryr2<sup>D/D</sup> mice were performed with animals that were backcrossed at least five times into the 129/SvEv genetic background. Offspring were genotyped by PCR followed by restriction digestion with *HinfI*.

Morphological analyses. Hearts from 6- to 7-mo-old mice were fixed with 4% (wt/vol) paraformaldehyde in PBS (pH 7.4) and dehydrated with increasing concentrations of ethanol in water. Paraffin-embedded hearts were sectioned into 6- to 9-μm thickness and stained with hematoxylin and eosin. Tetramethylrhodamine isothiocyanate (TRITC)-conjugated wheat germ agglutinin was used to visualize the cell membranes in the sections (35). TRITC fluorescence was captured by confocal microscopy (Leica TCS SP5), and cross-sectional areas of individual cells in left and right ventricles and interventricular septum were calculated with ImageJ software. Thirty cells were randomly selected from wild-type and mutant mice.

Echocardiography. Left ventricular cardiac parameters were determined by transthoracic M-mode echocardiography on restrained mice with a Vevo 2100 high-resolution imaging system (VisualSonics) with a 40-MHz probe. Unanesthetized 4- and 6-mo-old mice were restrained on a warmed mouse board (Indus Instruments for VisualSonics).
Transverse aortic constriction. Pressure overload in the left ventricle was induced by transverse aortic constriction (TAC) of 3-month-old wild-type and RyR2<sup>−/−</sup> mice as described previously (3). The aorta was ligated between the innominate and left common carotid arteries with a 7-10 silk suture and a tapered 27-gauge needle placed on top of the aorta. The tapered needle was removed, leaving the suture to produce a defined stenosis of the vessel. Control mice were subjected to a sham operation in which a band was tied around the aorta, but not ligated, and subsequently removed. The skin was closed with separate sutures, and buprenorphine was administered for analgesia. Echocardiography was performed after 4 wk of constriction.

Quantitative RT-PCR. Gene expression was measured by quantitative RT-PCR with the ABI Prism 7700 Sequence Detector (Applied Biosystems) (15). RNA was isolated from the left ventricle of 4- and 6-mo-old mice, treated with a ribonuclease inhibitor, and subsequently removed. The skin was closed with 7-10 silk suture and a tapered 27-gauge needle placed on top of the aorta. The tapered needle was removed, leaving the suture to produce a defined stenosis of the vessel. Control mice were subjected to a sham operation in which a band was tied around the aorta, but not ligated, and subsequently removed. The skin was closed with separate sutures, and buprenorphine was administered for analgesia. Echocardiography was performed after 4 wk of constriction.

Preparation of heart homogenates and crude membrane fractions. Hearts were homogenized in 20 mM imidazole, pH 7.0, 0.3 M sucrose, 0.15 M NaCl, 0.1 mM EGTA, protease inhibitors (Complete; Roche Applied Science), and 1 mM glutathione (oxidized form) with a Tekmar Tissumizer twice for 30 s at 13,500 rpm (35). Homogenates were centrifuged for 45 min at 100,000 g in a type 75Ti rotor (Beckman). Pellets were suspended in the aforementioned buffer without EGTA and glutathione to obtain a crude membrane fraction. Homogenates and membranes were stored in small aliquots at −80°C.

Immunoblot analyses. Homogenates (10 µg protein/lane) were separated by 3–12% gradient SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blotted with 2% ECL Advance blocking reagent (Amersham Biosciences) in 0.5% Tween 20-Tris-buffered saline (TBS), pH 7.4 at 24°C for 1 h and probed with primary rabbit polyclonal antibody against RyR2 from rabbit. Immunoblots were developed with enhanced chemiluminescence and quantified with ImageQuantTL Analysis Software. Glyceraldehyde-3-phosphate dehydrogenase was used as reference. Levels of gene expression were quantitated relative to β-actin.

RESULTS

Mice carrying three mutations in the CaM binding domain of RyR2 (W3587A, L3591D, F3603A; RyR2 ADA) rapidly develop cardiac hypertrophy and die at ~2 wk of age (35). The triple mutation eliminated CaM-dependent inhibition of RyR2 activity at both diastolic and systolic Ca<sup>2+</sup>. To determine whether the phenotype was caused by loss of CaM inhibition at diastolic or systolic Ca<sup>2+</sup>+, another genetically modified mouse carrying a single amino acid mutation in RyR2 gene (L3591D) was prepared by gene targeting (Fig. 1). This mutation was hypothesized to eliminate CaM inhibition of RyR2 at diastolic Ca<sup>2+</sup> only (36).

Single-channel recordings measure loss of CaM and S100A1 regulation of RyR2-L3591D in mice at diastolic Ca<sup>2+</sup>. We previously showed that the RyR2-L3591D mutation caused loss of CaM-dependent regulation of channel activity at submicromolar (diastolic) but not micromolar (systolic) Ca<sup>2+</sup> when expressed in HEK293 cells (36). To determine whether CaM regulation of the RyR2-L3591D channel was maintained in mice, membranes isolated from hearts of wild-type and RyR2<sup>−/−</sup> mice were fused with a lipid bilayer (Fig. 2). RyR2 regulation by CaM, determined by changes in channel open probability (P<sub>o</sub>) was measured by changes in channel activity at submicromolar (diastolic) but not micromolar (systolic) Ca<sup>2+</sup> when expressed in HEK293 cells (36). To determine whether CaM regulation of the RyR2-L3591D channel was maintained in mice, membranes isolated from hearts of wild-type and RyR2<sup>−/−</sup> mice were fused with a lipid bilayer (Fig. 2). RyR2 regulation by CaM, determined by changes in channel P<sub>o</sub>, was recorded in the presence of 0.2 µm or 2 µm free cis cytosolic Ca<sup>2+</sup> in the absence and on the subsequent addition of cis cytosolic CaM. At 0.2 µM cytosolic Ca<sup>2+</sup>, addition of 50 nM and 1 µM cytosolic CaM resulted in significant inhibition of channel P<sub>o</sub> of wild-type RyR2, with no changes in P<sub>o</sub> for...
RyR2-L3591D (Fig. 2A). In contrast, in the presence of 2 μM Ca\(^{2+}\), \(P_o\) of both wild type and RyR2-L3591D decreased at 50 nM and 1 μM CaM (Fig. 2B). Thus CaM regulation of RyR2-L3591D was lost at low Ca\(^{2+}\) but maintained at higher Ca\(^{2+}\) levels.

S100A1 is a Ca\(^{2+}\) binding protein reported to bind to the conserved CaM binding region of RyR1 (25) and regulate RyR2 activity (10, 21, 24, 29). Single-channel measurements in Fig. 3A show that at 0.2 μM Ca\(^{2+}\) the addition of 0.3 μM and 1 μM cytosolic S100A1 reduced \(P_o\) of wild type. At 2 μM Ca\(^{2+}\), addition of 1 μM S100A1 was required for significant wild-type inhibition (Fig. 3B). No inhibition of RyR2-L3591D channels by S100A1 was observed under any of these conditions. The single-channel data suggest that the L3591D muta-

Fig. 2. Effects of calmodulin (CaM) on wild-type and RyR2-L3591D single-channel activities. Membranes isolated from the hearts of wild-type and RyR2\(^{D/D}\) mice were fused with a lipid bilayer. Top: single-channel currents were recorded at −35 mV in the presence of 0.2 μM (A) and 2 μM (B) cis cytosolic Ca\(^{2+}\) as described in MATERIALS AND METHODS in the absence of CaM (top) and on the subsequent addition of 50 nM (middle) and 1 μM (bottom) cis CaM. \(P_o\) open channel probability. Bottom: mean ± SE \(P_o\) of number of recordings indicated in parentheses, normalized to control. *\(P < 0.05, **P < 0.001\) compared with control (no CaM added). Mean \(P_o\) in absence of CaM were 0.08 ± 0.02 (\(n = 10\)) and 0.10 ± 0.07 (\(n = 8\)) at 0.2 μM Ca\(^{2+}\) and 0.55 ± 0.05 (\(n = 15\)) and 0.47 ± 0.12 (\(n = 8\)) at 2 μM Ca\(^{2+}\) for wild-type and RyR2-L3591D channels, respectively.
tion impairs CaM and S100A1 regulation of RyR2 at 0.2 μM Ca\(^{2+}\). At 2 μM Ca\(^{2+}\), RyR2-L3591D inhibition by CaM was retained whereas that by S100A1 was lost. One possible explanation is that, in contrast to RyR1 (34), S100A1 and CaM do not compete for the same CaM binding site in RyR2. Nitu et al. (22), using fluorescence resonance energy transfer, recently reported that S100A1 likely interacts allosterically with the CaM binding domain on RyR2 rather than by a direct interaction.

Ryr2\(^{D/D}\) mice show modest signs of cardiac hypertrophy. Unlike Ryr2\(^{AdA/AdA}\) mice (35), Ryr2\(^{D/D}\) mice live longer than 1 yr without a significant difference in cardiac performance compared with Ryr2\(^{+/+}\) mice, as determined by echocardiography (n = 3, not shown). No major changes in cardiac gross morphology were observed for 6- to 7-mo-old homozygous mutant mice (Fig. 4A). In homozygous mice, heart weight-to-body weight ratio increased by 11% (P < 0.003) compared with Ryr2\(^{+/+}\) mice and by 9% (P < 0.02) compared with
wild-type mice (Fig. 4B). Histological examination showed modest but significant changes in right ventricular wall thickness and cross-sectional area in homozygous mutant mice compared with heterozygous mice (Fig. 4, C and D). However, the three groups of mice showed no significant changes in septal and left ventricular wall thickness and cross-sectional area.

To determine the effect of the RyR2-L3591D mutation on cardiac function, echocardiography was performed on conscious 6-mo-old animals (Table 1). Significant increases in left ventricular end-diastolic and end-systolic wall thicknesses were observed in homozygous mice compared with wild-type mice. Left ventricular posterior diastolic and systolic wall thicknesses were increased in homozygous mice compared with heterozygous mice. Heart rate, fractional shortening, interventricular septum, and left ventricular diastolic and systolic posterior wall thicknesses were all similar among the three genotypes, suggesting limited alterations in cardiac morphology and contractility of the mutant mice.

Relevant gene expression in 6-mo-old wild-type and RyR2D/D mice was determined by quantitative RT-PCR. ANP mRNA increased 1.8-fold in the hearts of homozygous mice compared with wild type (Fig. 5). This suggests that the RyR2-L3591D mutation elicited a modest hypertrophic response in RyR2D/D mice (16). However, mRNA levels of other genes associated with cardiac remodeling and Ca\(^{2+}\) handling

![Fig. 4](http://ajpheart.physiology.org/)

**Fig. 4.** Phenotypic characterization of wild-type and mutant hearts. A: hematoxylin- and eosin-stained heart sections from 6- to 7-mo-old wild-type and RyR2D/D mice. B: body weights and heart weight-to-body weight ratios of wild-type and mutant mice. Data are means ± SE for number of mice shown in parentheses. *P < 0.05 compared with RyR2+/− and RyR2−/− mice. C: wall thickness of ventricles of 6- to 7-mo-old wild-type and mutant mice. RV, right ventricle; LV, left ventricle. D: cross-sectional areas of individual cardiomyocytes in ventricles were measured as described in MATERIALS AND METHODS. C and D: data are means ± SE for number of mice shown in parentheses. *P < 0.05 compared with RyR2−/− mice.

![Fig. 5](http://ajpheart.physiology.org/)

**Fig. 5.** Quantitative RT-PCR of 6-mo-old wild-type and RyR2D/D mice. RTPCR was performed with total RNA from left ventricles of 6-mo-old mice. RNA levels were normalized to those of wild type for each gene. α-MHC, α-myosin heavy chain; β-MHC, β-myosin heavy chain; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; Ca\(_{1.2}\), L-type Ca\(^{2+}\) channel; RyR2, cardiac ryanodine receptor; IP-3R, inositol trisphosphate receptor; CSQ2, calsequestrin; SERCA2a, sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase 2a; NCX1, sodium-calcium exchanger 1. Data are means ± SE of 9–16 samples. *P < 0.05 compared with RyR2+/− mice.

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### Table 1. Echocardiography in 6-mo-old wild-type and mutant mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>RyR2+/− (n = 13)</th>
<th>RyR2−/− (n = 15)</th>
<th>RyR2D/D (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>542 ± 30</td>
<td>449 ± 30</td>
<td>491 ± 23</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>2.82 ± 0.12*</td>
<td>3.34 ± 0.08</td>
<td>3.00 ± 0.12</td>
</tr>
<tr>
<td>LVEDS, mm</td>
<td>1.40 ± 0.10*</td>
<td>1.90 ± 0.09</td>
<td>1.54 ± 0.12</td>
</tr>
<tr>
<td>FS, %</td>
<td>50.6 ± 2.6</td>
<td>43.2 ± 1.8</td>
<td>49.3 ± 2.4</td>
</tr>
<tr>
<td>IVSS, mm</td>
<td>1.23 ± 0.07</td>
<td>1.06 ± 0.06</td>
<td>1.22 ± 0.07</td>
</tr>
<tr>
<td>LV, mm</td>
<td>1.77 ± 0.10</td>
<td>1.54 ± 0.06</td>
<td>1.83 ± 0.10</td>
</tr>
<tr>
<td>LVPWD, mm</td>
<td>1.10 ± 0.07</td>
<td>0.97 ± 0.04</td>
<td>1.34 ± 0.11*</td>
</tr>
<tr>
<td>LVPWS, mm</td>
<td>1.69 ± 0.09</td>
<td>1.47 ± 0.04</td>
<td>1.79 ± 0.13*</td>
</tr>
</tbody>
</table>

Data are means ± SE for n mice. HR, heart rate; LVEDD, left ventricular end-diastolic dimension; LVEDS, left ventricular end-systolic dimension; FS, fractional shortening (LVEDD − LVEDS)/LVEDD; IVSS, interventricular septum diastolic thickness; IVSS, interventricular septum systolic thickness; LVPWD, left ventricular posterior wall diastolic thickness; LVPWS, left ventricular posterior wall systolic thickness. *P < 0.05 compared with heterozygous mice.
Ca²⁺ main did not alter the Ca²⁺ properties of wild-type and mutant mice. Ryr2D/D mice are capable of a cardiac hypertrophic response under conditions of pressure overload, 3-mo-old Ryr2D/D and wild-type mice were subjected to TAC for 4 wk. TAC induced a significant 1.4-fold increase in heart weight-to-body weight ratio in Ryr2D/D mice compared with 1.1-fold increase in wild-type mice (Table 3). Echocardiography indicated that TAC increased interventricular septum dimensions and left ventricular posterior wall diastolic thickness in homozygous mice compared with sham-operated wild-type mice, without significantly affecting overall cardiac performance.

To further investigate the response of Ryr2D/D mice to pressure overload, mRNA levels of cardiac hypertrophy-related genes were measured by quantitative RT-PCR. No significant changes in α-MHC, β-MHC, and BNP mRNA levels were observed (Fig. 7). However, there was a significant two- to threefold increase in ANP levels of sham-operated Ryr2D/D animals compared with sham-operated wild-type mice. In pressure-overloaded mice, 1.3- and 2.1-fold increases in ANP mRNA levels were observed in wild-type and homozygous mutant mice.

![Figure 6. Ca²⁺ dependence of [³H]ryanodine binding and RyR2 expression in hearts of wild-type and mutant mice. A: specific [³H]ryanodine binding to homogenates containing wild-type and mutant RyR2s was carried out at the indicated free Ca²⁺ concentrations as described in MATERIALS AND METHODS. Data are means ± SE of 4 samples each. B, left: immunoblots of RyR2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of heart homogenates from 6-mo-old Ryr2⁺/+ , Ryr2⁺/- , and Ryr2D/D mice. Right: levels of RyR2 expression, quantitated relative to GAPDH, were normalized to Ryr2⁺/+ . Data are means ± SE of 3 preparations done in triplicate.](http://ajpheart.physiology.org/)

Genotypes were not altered compared with wild-type (Fig. 5). These included cardiac hypertrophy-associated genes β-MHC and BNP and genes for Ca²⁺ handling proteins Ca.1.2, RyR2, IP₃R, CSQ2, SERCA2a, and NCX1.

Cardiac muscle SR Ca²⁺ handling. The SR Ca²⁺ handling properties of wild-type and Ryr2D/D mice were compared in heart homogenates of 6-mo-old mice. In Fig. 6A, a similar Ca²⁺ dependence of [³H]ryanodine binding suggested that the leucine-to-aspartic acid substitution in the CaM binding domain did not alter the Ca²⁺ sensitivity of the receptor in Ryr2⁺/+ and Ryr2D/D mice. Bₘₐₓ of [³H]ryanodine binding, a measure of RyR protein concentration, indicated a modest decrease (not significant) in RyR2 concentration in hearts of homozygous mice compared with heterozygous and wild-type mice (Table 2). Likewise, immunoblot analysis did not reveal significant differences in RyR2 expression among the three genotypes (Fig. 6B).

![Figure 7. Quantitative RT-PCR analysis of marker genes for cardiac hypertrophy in mice subjected to transverse aortic constriction (TAC). RT-PCR was performed with total RNA from left ventricles of 4-mo-old mice. RNA levels were normalized to β-actin. Abbreviations are as in Fig. 5. Data are means ± SE of 6–8 samples. *P < 0.05 compared with sham-operated (Sham) wild type; †P < 0.05 compared with Sham Ryr2D/D.](http://ajpheart.physiology.org/)

### Table 2. Calcium handling by heart homogenates of 6-mo-old wild-type and Ryr2D/D mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Bₘₐₓ of [³H]Ryanodine Binding, pmol/mg protein</th>
<th>⁴⁵Ca²⁺ Uptake Rate, pmol·mg protein⁻¹·min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ryr2⁺/+</td>
<td>0.16 ± 0.02 (10)</td>
<td>3.67 ± 0.26 (6)</td>
</tr>
<tr>
<td>Ryr2⁺/-</td>
<td>0.17 ± 0.02 (6)</td>
<td>3.44 ± 0.49 (4)</td>
</tr>
<tr>
<td>Ryr2D/D</td>
<td>0.13 ± 0.02 (13)</td>
<td>3.76 ± 0.34 (6)</td>
</tr>
</tbody>
</table>

Values are means ± SE for number of heart preparations indicated in parentheses. Bₘₐₓ, maximum binding capacity.
respectively, compared with sham-operated animals. Together, the data suggest that small differences between mutant and wild-type mice under baseline conditions become more prominent during pressure overload of RyR2<sup>DD</sup>/mice.

DISCUSSION

Accumulating evidence suggests that a dysfunctional RyR2 calcium channel has a major role in the genesis of cardiomyopathy. Aberrant phosphorylation, oxidation, and S-nitrosylation of RyR2, which cause leaky Ca<sup>2+</sup> channels with an increased activity at diastolic Ca<sup>2+</sup>, have been implicated in arrhythmias and heart failure (1, 6, 18, 30, 37). Three simultaneous mutations in the CaM binding site of RyR2 (W3587A, L3591D, F3603A; RyR2<sup>ADA</sup>) attenuated CaM regulation of RyR2 at diastolic and systolic Ca<sup>2+</sup> concentrations (35). RyR2<sup>ADA/ADA</sup> knockin mice carrying the three mutations have severe cardiac hypertrophy and early death. Cardiomyocytes derived from homozygous RyR2<sup>ADA/ADA</sup> mice showed prolonged Ca<sup>2+</sup> release. This suggests that CaM inhibition of RyR2 likely contributes to the termination of intracellular Ca<sup>2+</sup> transients. To determine at which Ca<sup>2+</sup> concentration, at diastolic or systolic Ca<sup>2+</sup>, CaM inhibition of RyR2 is of physiological significance, an additional knockin mouse carrying a single mutation (L3591D; RyR2<sup>W3587A</sup>) was generated. In contrast to RyR2<sup>ADA/ADA</sup> mice, RyR2<sup>DD</sup> mice showed only modest changes in heart size and function. Single RyR2<sup>ADA</sup> and RyR2<sup>DD</sup> channels lost CaM inhibition at diastolic Ca<sup>2+</sup>; however, unlike RyR2<sup>ADA</sup> channels, RyR2<sup>DD</sup> maintained CaM inhibition at systolic Ca<sup>2+</sup>. The results suggest that loss of CaM regulation does not have as much an impact in RyR2<sup>DD</sup> mice as in RyR2<sup>ADA/ADA</sup> mice, hence implying that CaM inhibition of RyR2 at systolic Ca<sup>2+</sup> is necessary for normal cardiac growth and function.

RyR2<sup>DD</sup> mice showed modest changes in heart contractility, morphology, and gene expression compared with RyR2<sup>ADA/ADA</sup> mice. Heart weight-to-body weight ratio of 6-mo-old RyR2<sup>DD</sup> mice increased 9% compared with wild-type mice. In comparison, a more than twofold increase in heart weight-to-body weight ratio was observed for 10-day-old RyR2<sup>DD/ADA</sup> mice compared with wild-type mice (35). ANP gene expression during stress such as exercise. However, it should be noted that even under pressure overload of RyR2<sup>DD</sup> mice, changes in heart growth and function were smaller than those in 10-day-old RyR2<sup>ADA/ADA</sup> mice not subjected to TAC.

S100A1 is an EF hand Ca<sup>2+</sup> binding protein expressed at high levels in the heart (13). Evidence indicates that S100A1 regulates intracellular Ca<sup>2+</sup> homeostasis and cardiac contraction. S100A1, like CaM, regulates cardiac Ca<sup>2+</sup> handling proteins such as RyR2, voltage-dependent L-type Ca<sup>2+</sup> channel, and the SERCA2a-PLN complex (10, 21, 24, 29). In permeabilized cardiomyocytes, Ca<sup>2+</sup> spark frequency decreased in response to exogenously added S100A1 (28). Similar to previous reports, we observed S100A1 effects on RyR2 function. However, this is the first study that describes a genetically modified mouse with a loss of S100A1 binding to RyR2, thus avoiding the multiple effects of gene delivery such as altering the regulation of SERCA and L-type Ca<sup>2+</sup> channel. Single-channel recordings showed that 1 μM S100A1 inhibited wild-type RyR2 at 0.2 and 2 μM Ca<sup>2+</sup>. The RyR2-L3591D mutation eliminated the inhibitory effect of S100A1 on RyR2 at both Ca<sup>2+</sup> concentrations. Nevertheless, RyR2<sup>DD</sup> mice did not demonstrate major changes in cardiac function and growth. Together, the results suggest that S100A1 has a limited role in regulating RyR2 in vivo under normal conditions.

In conclusion, our studies with RyR2<sup>DD</sup> mice show that severe cardiac hypertrophy and early death in RyR2<sup>ADA/ADA</sup> mice (33, 35) can be ascribed to impaired RyR2 inhibition by CaM at systolic Ca<sup>2+</sup>. Single-channel measurements showed that S100A1 and CaM share a single regulatory domain in RyR2. Loss of inhibition of RyR2 by CaM at diastolic Ca<sup>2+</sup> and by S100A1 at diastolic and systolic Ca<sup>2+</sup> in mice had only modest effects on cardiac morphology and function.

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AUTHOR CONTRIBUTIONS

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