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+. Cardiac hypertrophy, and heart failure in RyR2ADA/ADA mice. To address whether cardiac hypertrophy results from the elimination of CaM and S100A1 inhibition at diastolic or systolic Ca2+. A mutant mouse was generated with a single RyR2 amino acid substitution (L3591D; RyR2D). Here we report that in single-channel measurements RyR2-L3591D isolated from RyR2D hearts lost CaM inhibition at diastolic Ca2+ only, whereas S100A1 regulation was eliminated at both diastolic and systolic Ca2+. In contrast to the ~2-wk life span of RyR2ADA/ADA mice, RyR2D mice lived longer than 1 yr. Six-month-old RyR2D mice showed a 9% increase in heart weight-to-body weight ratio, modest changes in cardiac morphology, and a twofold increase in atrial natriuretic peptide mRNA levels compared with wild type. After 4-wk pressure overload with transverse aortic constriction, heart weight-to-body weight ratio and atrial natriuretic peptide mRNA levels increased and echocardiography showed changes in heart morphology of RyR2D mice compared with sham-operated mice. Collectively, the findings indicate that the single RyR2-L3591D mutation, which distinguishes the effects of diastolic and systolic Ca2+, alters heart size and cardiac function to a lesser extent in RyR2D mice than the triple mutation in RyR2ADA/ADA mice. They further suggest that CaM inhibition of RyR2 at systolic Ca2+ is important for maintaining normal cardiac function.

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 Avanti Polar Lipids. Unlabeled CaM was prepared as described previously (2). Rabbit polyclonal antibody RyR2-F9221 was prepared in single-channel measurements RyR2-L3591D isolated from cardiac ryanodine receptor; calmodulin; S100A1; cardiac hypertrophy...
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>2<sup>L</sup>L</sup>) were obtained by mating heterozygous mice (Ryr2<sup>H11001</sup>/D). 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 Hinfl.

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).

**Fig. 1.** Generation of mice with RyR2-L3591D mutation. A: schematic representation of the mouse Ryr2 gene and targeting construct. S represents SphI restriction site. Neomycin-resistant gene (neo), Cre recombinase gene driven by testis-specific angiotensin-converting enzyme promoter (tACE-cre), and thymidine kinase gene (TK) are indicated. Arrows and x indicate the position of primers for screening and a mutation site, respectively. B: Southern blot of genomic DNA. After restriction enzyme digestion of genomic DNA with SphI, 5' and 3' probes identify 13.5-kb and 11-kb fragments in the targeted allele, respectively. Both probes hybridize with 20.5-kb fragments in the wild-type (WT) allele. C: DNA sequence analysis of RyR2-L3591D. cDNA encoding the CaM binding site of Ryr2 was amplified from total RNA from homozygous (hybrid genetic background) mouse cardiac muscle and sequenced. The RyR2-L3591D mutation was confirmed. A Hinfl site created by the L3591D mutation (GACTC) was used for screening the mutant allele.
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>D/D</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 twined 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 with the ABI Prism 6700 Automated Nucleic Acid Workstation according to the manufacturer’s protocol. Forward and reverse primers and corresponding fluorogenic probes were according to the manufacturer’s protocol. Forward and reverse primers (5′- and 3′-ends) (15). RNA was isolated from the left ventricle of 4- and 6-mo-old mice, rapidly frozen in liquid nitrogen, and subsequently stored at −80°C. Total RNA was extracted from heart tissues using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions.

H88 REGULATION OF RyR2 BY CaM AND S100A1

Measurements were made with symmetrical 0.25 M CsCl, 20 mM Cs acetate, 20 mM imidazole, pH 7.0, and 0.1 mM EGTA, 1 mM potassium thiocyanate, protease inhibitors (Complete; Roche Applied Science), and 1 mM glutathione (oxidized form) with 500 mM sucrose, 0.15 M NaCl, 0.1 mM EGTA, protease inhibitors (Complete; Roche Applied Science), and 1 mM glutathione (oxidized form). Sarcoplasmic reticulum (SR) luminal side of the bilayer was defined as ground. Exogenous CaM and S100A1 were added to the cis, cytosolic solution. Electrical signals were filtered at 2 kHz, digitized at 10 kHz, and analyzed as described previously (32). Channel open probability (P<sub>O</sub>) was obtained from 2-min recordings by setting the threshold level at 50% of the current amplitude between the closed and open channel states.

[3H]ryanodine binding. Ryanodine binds specifically to RyRs. Hence, [3H]ryanodine binding can be used as a probe of channel activity and concentration (26). Ca<sup>2+</sup> dependence of [3H]ryanodine binding was determined by incubating homogenates with a relatively low [3H]ryanodine concentration (3 nM) at 24°C for 20 h in 0.25 M KCl, 20 mM imidazole, pH 7.0, protease inhibitors, and indicated free Ca<sup>2+</sup> concentrations. Maximum binding capacity (B<sub>M</sub>) of [3H]ryanodine binding was determined by incubating homogenates for 4–5 h at 24°C with a near-saturating concentration of 20 nM [3H]ryanodine in 20 mM imidazole, pH 7.0, 0.6 M KCl, protease inhibitors, and 0.1 mM Ca<sup>2+</sup>. Nonspecific binding was determined with a 1,000-fold excess of unlabeled ryanodine. Aliquots of samples were diluted ninefold with ice-cold water and placed on Whatman GF/B filters saturated with 2% polyethyleneimine. Filters were washed with ice-cold 0.1 M KCl, 1 mM K-PIPES, pH 7.0, and radioactivity remaining on the filters was determined by liquid scintillation counting to obtain bound [3H]ryanodine.

45Ca<sup>2+</sup> uptake rate. ATP-dependent 45Ca<sup>2+</sup> uptake rates by homogenates were determined by a filtration assay (33). 45Ca<sup>2+</sup> uptake rates were determined in 0.15 M KCl, 20 mM imidazole, pH 7.0 solution containing 5 mM ATP, 8 mM Mg<sup>2+</sup>, and 5 mM potassium oxalate, a Ca<sup>2+</sup> precipitating agent to increase Ca<sup>2+</sup> uptake capacity, 10 μM ruthenium red to inhibit RyR2, 5 mM NaN<sub>3</sub> to inhibit mitochondrial Ca<sup>2+</sup> uptake, 1 mM EGTA, and Ca<sup>2+</sup> and 45Ca<sup>2+</sup> to yield a free Ca<sup>2+</sup> concentration of 0.5 μM.

Biochemical assays and data analysis. Free Ca<sup>2+</sup> concentrations were measured with a Ca<sup>2+</sup>-selective electrode. Results are expressed as means ± SE. Differences between wild-type and mutant samples were determined by one-way ANOVA followed by Tukey’s test. P < 0.05 was considered significant.

RESULTS

Mice carrying three mutations in the CaM binding domain of RyR2 (R3587A, L3591D, F3603A; RyR2 ADA) rapidly developed 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 measurements indicate 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 sub-micromolar (diastolic) but not micromolar (systolic) Ca<sup>2+</sup> when expressed in HEK293 cells (36). To determine whether CaM regulation of the RyR2-L3591D1 channel was maintained in mice, membranes isolated from hearts of wild-type and RyR2<sup>D/D</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\(^{2+}\), \(P_0\) 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 \(P_0\) 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-
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\(^{A/ADA}\) 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\(^{+/D}\) mice and by 9% (P < 0.02) compared with
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>LVESD, mm</td>
<td>1.40 ± 0.10*</td>
<td>1.90 ± 0.09</td>
<td>1.54 ± 0.12</td>
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<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>IVSD, mm</td>
<td>1.23 ± 0.07</td>
<td>1.06 ± 0.06</td>
<td>1.22 ± 0.07</td>
</tr>
<tr>
<td>IVSS, 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; LVESD, left ventricular end-systolic dimension; FS, fractional shortening [LVEDD – LVESD]/LVEDD]; IVSD, 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.
were not altered compared with wild-type (Fig. 5). These included cardiac hypertrophy-associated genes β-MHC and BNP and genes for Ca\(^{2+}\) handling proteins Ca.1.2, RyR2, IP\(_3\)R, CSQ2, SERCA2a, and NCX1.

Cardiac muscle SR Ca\(^{2+}\) handling. The SR Ca\(^{2+}\) handling properties of wild-type and RyR2\(^{DD}\) mice were compared in heart homogenates of 6-mo-old mice. In Fig. 6A, a similar Ca\(^{2+}\) dependence of \([\text{H}]\)ryanodine binding suggested that the leucine-to-aspartic acid substitution in the CaM binding domain did not alter the Ca\(^{2+}\) sensitivity of the receptor in RyR2\(^{+/+}\) and RyR2\(^{DD}\) mice. B\(_{\text{max}}\) of \([\text{H}]\)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). \(^{45}\text{Ca}^{2+}\) uptake rates, a measure of SERCA activity by cardiac muscle isolates, were not altered (Table 2).

Pressure overload induces a modest hypertrophic response in RyR2\(^{DD}\) mice. To determine whether RyR2\(^{DD}\) mice are capable of a cardiac hypertrophic response under conditions of pressure overload, 3-mo-old RyR2\(^{DD}\) 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 RyR2\(^{DD}\) 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 RyR2\(^{DD}\) 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 RyR2\(^{DD}\) 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.

Table 2. Calcium handling by heart homogenates of 6-mo-old wild-type and RyR2\(^{DD}\) mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>B(_{\text{max}}) of ([\text{H}])ryanodine Binding, pmol/mg protein</th>
<th>(^{45}\text{Ca}^{2+}) Uptake Rate, pmol/mg protein (\cdot) 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>RyR2(^{DD})</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. B\(_{\text{max}}\), maximum binding capacity.

Fig. 6. Ca\(^{2+}\) dependence of \([\text{H}]\)ryanodine binding and RyR2 expression in hearts of wild-type and mutant mice. A: specific \([\text{H}]\)ryanodine binding to homogenates containing wild-type and mutant RyR2s was carried out at the indicated free Ca\(^{2+}\) 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 wild-type and RyR2\(^{DD}\) mice. Right: levels of RyR2 expression, quantitated relative to GAPDH, were normalized to RyR2\(^{+/+}\). Data are means ± SE of 3 preparations done in triplicate.

Table 3. Echocardiography of RyR2\(^{+/+}\) and RyR2\(^{DD}\) mice after TAC

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham ((n = 8))</th>
<th>RyR2(^{DD}) ((n = 6))</th>
<th>RyR2(^{+/+}) ((n = 8))</th>
</tr>
</thead>
<tbody>
<tr>
<td>HW/BW, %</td>
<td>0.55 ± 0.01</td>
<td>0.56 ± 0.01</td>
<td>0.63 ± 0.03</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>583 ± 33</td>
<td>573 ± 27</td>
<td>650 ± 10</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>2.67 ± 0.15</td>
<td>2.18 ± 0.09</td>
<td>2.59 ± 0.10</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>1.25 ± 0.17</td>
<td>0.67 ± 0.07</td>
<td>1.12 ± 0.09</td>
</tr>
<tr>
<td>FS, %</td>
<td>55.0 ± 5.1</td>
<td>69.2 ± 2.4</td>
<td>57.1 ± 2.1</td>
</tr>
<tr>
<td>IVSD, mm</td>
<td>1.12 ± 0.13</td>
<td>1.37 ± 0.06</td>
<td>1.36 ± 0.08</td>
</tr>
<tr>
<td>IVSS, mm</td>
<td>1.75 ± 0.18</td>
<td>2.10 ± 0.07</td>
<td>2.04 ± 0.09</td>
</tr>
<tr>
<td>LVPWD, mm</td>
<td>1.07 ± 0.14</td>
<td>1.32 ± 0.06</td>
<td>1.39 ± 0.11</td>
</tr>
<tr>
<td>LVPWS, mm</td>
<td>1.62 ± 0.21</td>
<td>1.89 ± 0.09</td>
<td>1.88 ± 0.13</td>
</tr>
</tbody>
</table>

Data are means ± SE for n mice. HW/BW, heart weight-to-body weight ratio; Sham, sham operated; TAC, transverse aortic constriction. *P < 0.05 compared with Sham RyR2\(^{+/+}\); †P < 0.05 compared with Sham RyR2\(^{DD}\).
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/D</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><sup>+</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>DD</sup>) was generated. In contrast to Ryr2<sup>ADA/ADA</sup> mice, Ryr2<sup>DD/D</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> channels 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/D</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/D</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 in 10-day-old Ryr2<sup>DD</sup> mice compared with an <i>Ryr2<sup>DD/DD</sup></i> mutation eliminated the inhibitory effect of <i>S100A1</i> on RyR2 at both Ca<sup>2+</sup> concentrations. Nevertheless, Ryr2<sup>DD/D</sup> mice did not demonstrate major changes in cardiac function and growth. Together, the results suggest that <i>S100A1</i> has a limited role in regulating RyR2 in vivo under normal conditions.

In conclusion, our studies with Ryr2<sup>DD/D</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 <i>S100A1</i> and CaM share a single regulatory domain in proteins such as RyR2, voltage-dependent L-type Ca<sup>2+</sup> channel, and the SERCA2a-PLN complex (10, 21, 24, 29). <sup>[3H]Ry</sup>yanodine binding and Ca<sup>2+</sup> spark measurements indicated that <i>S100A1</i> has multiple effects on RyR2 channel activity. In <sup>[3H]Ry</sup>yanodine binding studies, low concentrations of <i>S100A1</i> increased RyR2 activity at diastolic 0.1 μM Ca<sup>2+</sup> but high concentrations decreased activity. At systolic Ca<sup>2+</sup>, <i>S100A1</i> activated RyR2 (14, 20). In permeabilized cardiomyocytes, Ca<sup>2+</sup> spark frequency decreased in response to exogenously added <i>S100A1</i> (28). Similar to previous reports, we observed <i>S100A1</i> effects on RyR2 function. However, this is the first study that describes a genetically modified mouse with a loss of <i>S100A1</i> 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 <i>S100A1</i> inhibited wild-type RyR2 at 0.2 and 2 μM Ca<sup>2+</sup>. The RyR2-L3591D mutation eliminated the inhibitory effect of <i>S100A1</i> on RyR2 at both Ca<sup>2+</sup> concentrations. Nevertheless, Ryr2<sup>DD/D</sup> mice did not demonstrate major changes in cardiac function and growth. Together, the results suggest that <i>S100A1</i> has a limited role in regulating RyR2 in vivo under normal conditions.

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