Impaired sarcoplasmic reticulum function leads to contractile dysfunction and cardiac hypertrophy

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Impaired sarcoplasmic reticulum function leads to contractile dysfunction and cardiac hypertrophy. Am J Physiol Heart Circ Physiol 280: H2046–H2052, 2001.—Sarcoplasmic reticulum (SR)-mediated Ca2+ sequestration and release are important determinants of cardiac contractility. In end-stage heart failure SR dysfunction has been proposed to contribute to the impaired cardiac performance. In this study we tested the hypothesis that a targeted interference with SR function can be a primary cause of contractile impairment that in turn might alter cardiac gene expression and induce cardiac hypertrophy. To study this we developed a novel animal model in which ryanodine, a substance that alters SR Ca2+ release, was added to the drinking water of mice. After 1 wk of treatment, in vivo hemodynamic measurements showed a 28% reduction in the maximum speed of contraction (+dP/dtmax) and a 24% reduction in the maximum speed of relaxation (−dP/dtmax). The slowing of cardiac relaxation was confirmed in isolated papillary muscles. The late phase of relaxation expressed as the time from 50% to 90% relaxation was prolonged by 22%. After 4 wk of ryanodine administration the animals had developed a significant cardiac hypertrophy that was most prominent in both atria (right atrium +115%, left atrium +100%, right ventricle +23%, and left ventricle +13%). This was accompanied by molecular changes including a threefold increase in atrial natriuretic factor mRNA and a sixfold increase in β-myosin heavy chain mRNA. Sarcoplasmic endoplasmic reticulum Ca2+-mRNA was reduced by 18%. These data suggest that selective impairment of SR function in vivo can induce changes in cardiac gene expression and promote cardiac growth.

Ryanodine treatment. The animals in this study were handled in accordance with the animal welfare regulations of the University of California, San Diego. The experimental protocol was approved by the local Animal Subjects Committee. A ryanodine stock solution was prepared by adding 1 ml of ethanol and 1 ml of deionized, distilled H2O to 50 mg of a dehydroryanodine-ryanodine mixture (Calbiochem; San Diego, CA). The solution was stored in a light-protected glass container at room temperature for ~4 wk. The stock solution was added to the drinking water of CB6 mice in 50-ml Falcon tubes (Fisher Scientific; Springfield, NJ) closed with rubber stopcocks containing the fluid dispenser; these were refilled every second day. In a dose-response study (four animals) the concentration was increased in nine steps from 88 ng/ml (0.18 μM) to 100 μg/ml (0.2 mM). After the death of one animal at 100 μg/ml, the surviving animals were killed and the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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the organs were examined. Heart weight-to-body weight ratios were found to be increased in the three remaining animals. In a subsequent study, three concentrations of ryanodine (10, 50, and 100 \( \mu \)g/ml) were tested over a 4-wk period. At the 100 \( \mu \)g/ml dose, three of four mice died within 2 wk. At this concentration the animals showed reduced grooming and activity. At the 50 \( \mu \)g/ml dose, animals were not obviously different from untreated controls except that they appeared to be more exhausted when caught from their cages; one of four animals died during 4 wk of treatment, whereas all animals survived the 10 \( \mu \)g/ml dose. Owing to a significant increase in the heart weight-to-body weight ratio at the 50 \( \mu \)g/ml dose, we chose this as the final concentration for all subsequent experiments.

In vivo hemodynamic measurements. For 1 wk, 12 mice were treated and 10 age-matched control mice were anesthetized with a mixture of thiobutabarbital (100 mg/kg) and ketamine (100 mg/kg). Electrocardiogram and body temperature were monitored. The animals were ventilated with room air. The right jugular vein and right carotid artery were cannulated, and a bilateral vagotomy was performed. After a left-sided thoracotomy, a pressure transducer (1.8-Pr, Millar Instruments; Houston, TX) was inserted into the left atrium (LA) and advanced into the left ventricle (LV). Continuous LV pressure and aortic pressure were recorded. After measurement of the basal values, increasing doses of isoproterenol (5–1,000 ng iv) were given as a bolus. Heart rate (HR), carotid mean arterial pressure (MAP), LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), and the maximum values of LV pressure derivatives (+dP/dt max, −dP/dt max) were analyzed.

Isolated papillary muscle experiments. Five mice were treated with ryanodine for 1 wk, and the contractile behavior of a LV papillary muscle preparation was compared with papillary muscle preparations of eight age-matched control mice as previously described (7). Briefly, left papillary muscles were cut from the LV in Tyrode solution containing (in mM): 136 NaCl, 5.4 KCl, 1 MgCl2, 0.33 NaH2P04, 10 HEPES, 10 glucose, 2.5 CaCl2 (pH adjusted to 7.40 at 37°C with NaOH), and 30 2,3-butanedione monoxime (BDM). The papillary muscles were transferred into the perfusion chamber containing Tyrode solution (without BDM), stimulated at 2 Hz, and gradually stretched until maximal contractility was reached (average length 2.1 ± 0.1 mm). Postrest behavior was studied by stopping stimulation (2 Hz) for intervals ranging from 0.5 to 15 s and resuming regular stimulation. For the Ca\(^{2+}\)-transient measurements the excised hearts (\( n = 4 \) in both groups) were retrograde perfused with 15 \( \mu \)M fluo 4-acetoxyethyl ester (fluor 4-AM) in oxygenated Krebs solution for 15 min at a flow rate of 5–6 ml/min before the papillary muscles were obtained. Fluorescence of fluo 4-AM was measured with \( \times 32 \) Neofluar objective (Zeiss, NY) by photon counting simultaneous with analog recording of the fluorescence intensity. A 24-bit analog-to-digital converter furnished the data to a computer where the data were analyzed using a quantitative fluorescence setup (IonOptix; Milton, MA).

Northern and Western analyses. Ventricular RNA was isolated from mice treated for different times (day 2 to day 4, \( n = 2 \) each day; day 4 wk, \( n = 6 \)) and age-matched control animals (\( n = 6 \). After electrophoresis, the RNA was transferred and hybridization was performed with restriction fragments of the cDNA of rat SERCA2a, rat ANF, mouse phospholamban, rabbit cardiac ryanodine receptor (RyR2), and an oligo specific to the rat \( \beta \)-MHC. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) message levels were determined on all blots to control for equal loading. For Western blot analysis, ventricular homogenates were prepared from mice treated for 4 wk (\( n = 8 \)) and age-matched control mice (\( n = 8 \)). The samples (15 \( \mu \)g of protein) were resolved on a 4–20% gradient gel (Novex; San Diego, CA), blotted, and exposed to monoclonal antibodies directed against \( \beta \)-MHC (J. J. Leger, Institut National de la Santé et de la Recherche Médicale; Montpellier, France), \( \alpha \)-actin (Sigma; St. Louis, MO), phospholamban (ABR; Golden, CO), and a polyclonal antibody against SERCA2 (7). After incubation with horseradish peroxidase-labeled secondary antibodies, a chemiluminescence reaction (Amersham; Arlington Heights, IL) was initiated and detected by autoradiography. All films were analyzed with NIH Image 1.61 software. Western data were normalized by \( \alpha \)-actin. Linearity of the chemiluminescence signals for the different proteins was confirmed by loading different amounts of a standard sample.

**SR 45Ca\(^{2+}\) uptake.** Oxalate-saturating SR Ca\(^{2+}\) uptake measurements were performed in ventricular homogenates from six mice treated for 4 wk and six control homogenates (450 \( \mu \)g of protein) were subjected to 15 nmol of \( ^{3} \)Hryanodine (Amersham) for 60 min at 37°C in 200 \( \mu \)l of a binding medium containing (in mM): 20 MOPS, 1 CaCl\(_2\), and 0.6 NaCl at pH 7.1 (10). Background binding was determined by the addition of 10 \( \mu \)mol of unlabeled ryanodine.

**45Ca\(^{2+}\) uptake.** Oxalate-saturating SR Ca\(^{2+}\) uptake measurements were performed in ventricular homogenates from six mice treated for 4 wk and six control mice. The ventricular tissue was homogenized in solution A (25 mM of imidazol, pH 7, 1.9 tissue-to-solution ratio) with a Polytron homogenizer (3 × 20 s at a setting of 10). A 90-\( \mu \)l aliquot of the homogenate was added to 850 \( \mu \)l of solution B (final concentration in mM: 100 KCl, 25 CaCl\(_2\) (1.6 × 10\(^3\) counts⋅min\(^{-1}⋅\)nmol\(^{-1}\)), 15.5 EGTA, 4.5 MgCl\(_2\), 10 Na\(_2\)SO\(_4\), 5 potassium oxalate, and 40 imidazole at pH 7.0). After 20 min at 32°C, the uptake was started by the addition of 50 \( \mu \)l of solution C, which contained 2.5 mM of Na\(_2\)ATP. After 90, 180, and 270 s, respectively, a 100-\( \mu \)l aliquot of the reaction medium was transferred to a 0.45-\( \mu \)m filter in a filtration apparatus to terminate the Ca\(^{2+}\) uptake, and 5 ml of solution A was added to eliminate any residual reaction medium. Uptake rates were calculated from the slope of the regression line relating \( ^{45} \)Ca\(^{2+}\) uptake per milligram of protein to reaction time.

**Histological analysis.** Hearts of animals treated for 4 wk (\( n = 3 \)) and control mice (\( n = 3 \)) were quickly removed and immersion fixed in 10% formalin. The heart weight-to-body weight ratios in these hearts were increased by 24% in ryanodine-treated animals (\( P < 0.05 \)). The hearts were embedded in paraffin, and sections were cut perpendicular to the long axis of the hearts from the apex to the base of the ventricles. Sections were stained with hematoxylin-eosin and picrosirius red for connective tissue (23). The sections were analyzed for gross distribution of hypertrophy, connective tissue, myocyte density, and inflammatory cells. To investigate myocyte hypertrophy, the shortest transverse diameter in at least 85 nucleated transverse sections of myocytes per heart were measured (12).
Table 1. Organ and heart weights in mice after 1 and 4 wk of ryanodine treatment compared with age-matched controls

<table>
<thead>
<tr>
<th>BW, g</th>
<th>Liver, mg</th>
<th>Lung, mg</th>
<th>HW/BW, mg/g</th>
<th>RV/BW, mg/g</th>
<th>LV/BW, mg/g</th>
<th>RA/BW, mg/g</th>
<th>LA/BW, mg/g</th>
<th>Diameter, μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, 1 wk</td>
<td>23.5 ± 0.5</td>
<td>927 ± 12</td>
<td>158 ± 6</td>
<td>4.6 ± 0.2</td>
<td>0.69 ± 0.07</td>
<td>3.39 ± 0.09</td>
<td>0.18 ± 0.06</td>
<td>0.18 ± 0.06</td>
</tr>
<tr>
<td>n</td>
<td>14</td>
<td>4</td>
<td>4</td>
<td>11</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Ryanodine, 1 wk</td>
<td>22.7 ± 0.6*</td>
<td>872 ± 55*</td>
<td>144 ± 4*</td>
<td>4.9 ± 0.1*</td>
<td>0.73 ± 0.03*</td>
<td>3.62 ± 0.08*</td>
<td>0.15 ± 0.03*</td>
<td>0.19 ± 0.03*</td>
</tr>
<tr>
<td>n</td>
<td>14</td>
<td>3</td>
<td>3</td>
<td>14</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Control, 4 wk</td>
<td>25.3 ± 0.6</td>
<td>1,185 ± 65</td>
<td>163 ± 3</td>
<td>5.1 ± 0.1</td>
<td>0.81 ± 0.03</td>
<td>3.54 ± 0.04</td>
<td>0.13 ± 0.02</td>
<td>0.17 ± 0.04</td>
</tr>
<tr>
<td>n</td>
<td>16</td>
<td>10</td>
<td>10</td>
<td>16</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Ryanodine, 4 wk</td>
<td>24.3 ± 0.4*</td>
<td>1,099 ± 43*</td>
<td>172 ± 9*</td>
<td>6.1 ± 0.1†</td>
<td>1.00 ± 0.05‡</td>
<td>4.00 ± 0.13‡</td>
<td>0.28 ± 0.03†</td>
<td>0.34 ± 0.03†</td>
</tr>
<tr>
<td>n</td>
<td>19</td>
<td>10</td>
<td>10</td>
<td>19</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. animals studied. Dose of ryanodine was 50 μg/ml drinking water. BW, body weight; HW, total heart weight; RV, right ventricle weight; LV, left ventricle weight; RA, right atrium weight; LA, left atrium weight; n.d., not determined. Diameter, smallest transversal diameter in a nucleated transverse section of randomly analyzed myocyties (>85 per heart). *P = not significant; †P < 0.01; ‡P < 0.05.

Statistical analysis. Data are expressed as means ± SE. Statistical comparisons were made by two-way repeated-measures ANOVA (postrest behavior, force-frequency behavior, and RCCs). Student's t-test was used to analyze differences in mRNA and protein levels, [3H]ryanodine binding, 45Ca2+ SR uptake, weight and size parameters, baseline hemodynamics, twitch parameters in isolated papillary muscles, and the course of the force-frequency behavior.

RESULTS

Characteristics of ryanodine-treated animals. When ryanodine-treated animals (50 μg/ml drinking water = 0.1 mM) were anesthetized with ketamine and xylazine (300 and 15 mg/kg, respectively), some mice gradually developed a skeletal muscle rigor that was most prominent in the hindlimbs. When the abdominal cavity was opened, distended intestines were frequently present. When opening of the thoracic cavity, the atria appeared visibly enlarged, whereas the ventricles were normal in size. Total heart weight-to-body weight ratio was significantly increased by 19% after 4 wk of ryanodine treatment (6.1 ± 0.1, n = 19 vs. 5.1 ± 0.1, n = 16; P < 0.01). The separate analysis of atria and ventricles revealed a global hypertrophy with a pronounced increase in atrial weights. Normalized right atrium (RA) weight was increased 2.15-fold (0.28 ± 0.03, n = 9 vs. 0.13 ± 0.02, n = 6; P < 0.01), and normalized LA weight was increased 2.0-fold (0.34 ± 0.03, n = 9 vs. 0.17 ± 0.04, n = 6; P < 0.01). The increase in normalized right ventricular (RV) weight was 23% (1.00 ± 0.05, n = 9 vs. 0.81 ± 0.03, n = 6; P < 0.05), and the normalized LV weight was increased by 13% (4.00 ± 0.13, n = 9 vs. 3.54 ± 0.04, n = 6; P < 0.05). At 1 wk of treatment no significant difference was found (Table 1). The 4-wk mortality rate was 30%.

Hemodynamic effects of ryanodine treatment. In mice treated for 1 wk no difference in heart rate was found (Table 2). MAP under closed-chest conditions was 82 ± 5 mmHg in 10 control mice and 69 ± 4 mmHg in 12 ryanodine-treated animals (P < 0.05). The effects of isoproterenol stimulation on MAP (open chest) and LVSP did not show significant differences between ryanodine-treated animals and the control group. LVEDP was not different under either baseline conditions or isoproterenol stimulation. The maximum rise of systolic pressure in ryanodine-treated animals was reduced by 28% compared with control mice (3,903 ± 499 vs. 5,419 ± 456 mmHg/s; P < 0.05). This difference in the speed of contraction was accompanied by a 24% decrease in the maximum decline of LV pressure (−dP/dt) as an index of relaxation (−3,934 ± 277 vs. −5,204 ± 337 mmHg/s; P < 0.01).

Two separate attempts to measure the hemodynamic function after 4 wk of treatment failed due to the death of all animals shortly after injection of the anesthetic. However, functional papillary muscles could be recovered from these hearts by quickly excising and submerging them in BDM-Tyrode solution.

Papillary muscle function and Ca2+ handling. To characterize the contractile effects of ryanodine treatment on LV papillary muscle preparations, we studied isometric force development, postrest behavior, Ca2+ transients, RCCs, and the force-frequency relation. Papillary muscle preparations were obtained from mice treated for 1 wk. Developed stress at a stimulation frequency of 2 Hz was found to be significantly reduced in papillary muscle preparations from ryanodine-treated mice (6.4 ± 1.3 mmN/mm2, n = 8 vs. 2.3 ± 0.7 mmN/mm2, n = 10; P < 0.05).

Table 2. Basal hemodynamic characteristics after 1 wk of ryanodine treatment compared with control mice

<table>
<thead>
<tr>
<th>Control (n = 10)</th>
<th>Ryanodine (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>23.5 ± 0.7</td>
</tr>
<tr>
<td>Heart weight, mg</td>
<td>107 ± 5</td>
</tr>
<tr>
<td>Heart rate (closed chest), beats/min</td>
<td>451 ± 20</td>
</tr>
<tr>
<td>MAP (closed chest), mmHg</td>
<td>82 ± 5</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>71 ± 5</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>+dP/dt max, mmHg/s</td>
<td>5,419 ± 456</td>
</tr>
<tr>
<td>−dP/dt max, mmHg/s</td>
<td>−5,204 ± 337</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. Ryanodine dose was 50 μg/ml drinking water. MAP, mean arterial pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt max, maximal rise of left ventricular pressure; −dP/dt max, maximal decline of left ventricular pressure. *P = not significant; †P < 0.01; ‡P < 0.05.
0.4 mN/mm², n = 5; P < 0.05). At higher stimulation frequencies the difference in developed stress was not statistically significant. In four muscle strips obtained from ryanodine-treated animals additional ryanodine (10 µM) was added to the isometrically contracting preparation in vitro. Developed stress decreased by 86 ± 2% (2.6 ± 0.4 vs. 0.4 ± 0.1 mN/mm²; P < 0.01).

As shown in Fig. 1, time to peak tension was slightly but significantly reduced by 11% in papillary muscles from five ryanodine-treated mice compared with eight papillary muscles from control animals (41.8 ± 1.8 vs. 47.0 ± 1.8 ms; P < 0.05). The first phase of relaxation (time from peak tension to 50% relaxation) was unaffected by the ryanodine treatment. However, the late phase of relaxation (time from 50% relaxation to 90% relaxation) was markedly prolonged by 29% (45.0 ± 2.9 vs. 35.0 ± 1.4 ms; P < 0.01). Postrest behavior of isolated papillary muscles was completely inverted by the ryanodine treatment (P < 0.01). In papillary muscles from untreated control mice, a short rest interval from 0.5 to 15 s was always followed by a postrest beat that was larger than the previous steady-state beats. In sharp contrast to this postrest potentiation, all papillary muscles from ryanodine-treated mice displayed rapid postrest decay (Fig. 2, A and B). This observation, which indicates a time-dependent loss of SR Ca²⁺, was confirmed by fluo 4 measurements and RCCs. In papillary muscles from control mice both fluo transients and RCCs increased with prolonged rest intervals, whereas these values were always reduced in ryanodine-treated animals (Fig. 2, B and C). Single-contraction recordings of force and Ca²⁺ transients under steady-state conditions (2 Hz) and postrest contractions after rest intervals of 1 and 15 s are shown in Fig. 3.

In addition the force-frequency relation was affected by treatment with ryanodine (P < 0.01). In papillary muscles obtained from control animals, developed stress decreased with each subsequent increase in frequency (negative force-frequency relation). In compar-

**Fig. 1.** Basal time indices of isometric contractions in left ventricular (LV) papillary muscles stimulated at 2 Hz after 1 and 4 wk of ryanodine (Rya) treatment (50 µg ryanodine per ml drinking water) compared with papillary muscles from untreated control animals. Time to peak tension (TPT) and the times from peak tension to 50% and 90% relaxation (RT₅₀ and RT₉₀, respectively) were determined. Time from 50% relaxation to 90% relaxation (RT₅₀–₉₀) was used as a parameter for the late phase of relaxation. All data depict means ± SE; *P < 0.05.

**Fig. 2.** Mouse papillary muscle experiments after 1 wk of ryanodine treatment (●) compared with age-matched control animals (○). A: postrest (PR) contractile behavior of isolated papillary muscles. Regular stimulation at a 2-Hz stimulation frequency was suspended for rest intervals ranging from 0.5–15 s. Data show the stress of the first PR beat when regular stimulation was resumed. All papillary muscles from control mice displayed PR potentiation. In contrast, PR decay was observed in all papillary muscles obtained from mice treated with ryanodine for 1 wk. B: PR data at 1- and 15-s rest intervals in four fluo 4-acetoxymethyl ester (fluo 4-AM)-loaded papillary muscles. Isometric contraction of PR beat and fluo-4 Ca²⁺ transients are shown. C: rapid cooling contractures (RCCs) were induced in papillary muscles from control animals and ryanodine-treated animals. All preparations from control animals showed a potentiation of the RCCs from 1–15 s, whereas all papillary muscles of ryanodine-treated animals showed greatly abolished RCCs, indicating a depletion of the sarcoplasmic reticulum (SR) Ca²⁺ stores. D: force-frequency behavior of isolated papillary muscles from ryanodine-treated animals and control animals. The frequency was stepwise increased from 2 to 6 Hz. Actively developed stress was measured at each frequency after steady state had been reached. All data depict means ± SE.
ison, papillary muscles from ryanodine-treated animals displayed an increase in developed stress up to 4 Hz (positive force-frequency relation) followed by a small decrease in developed stress to 6 Hz (Fig. 3D). These differences were statistically substantiated by comparing the normalized force-frequency behavior (3–6 Hz all \( P < 0.01 \)).

In four animals treated for 4 wk, we obtained papillary muscles and analyzed the time parameters at 2 Hz (Fig. 1). Time to peak tension was not significantly altered. The time from peak tension to 50% relaxation was significantly prolonged by 30% (49.1 \( \pm \) 3.5 vs. 37.9 \( \pm \) 0.9 ms; \( P < 0.05 \)). The late phase of the relaxation expressed as the time from 50% to 90% relaxation, which was increased by 29% after 1 wk of treatment was increased by 134% after 4 wk (82.0 \( \pm \) 9.6 vs. 35.0 \( \pm \) 1.4 ms; \( P < 0.01 \)) indicating a much more pronounced disturbance of cardiac relaxation. Postrest potentiation and the force-frequency behavior remained inverted after 4 wk of treatment but could be restored to normal after removing the ryanodine from the drinking water (data not shown).

Molecular changes. In animals treated with 50 \( \mu \)g/l ryanodine in drinking water for 4 wk, steady-state message levels for several mRNAs were analyzed. GAPDH normalized ANF, and \( \beta \)-MHC message levels were increased threefold (\( n = 6 \) in both groups; \( P < 0.01 \)) and sixfold (\( P < 0.01 \)), respectively. Normalized SERCA2a mRNA levels were downregulated by 18% (\( P < 0.01 \)). No significant differences in gene expression of phospholamban and RyR2 were detected. Representative blots are shown in Fig. 4A. To test whether changes in expression levels occur already in the first days of ryanodine administration, we obtained hearts at days 2, 3, and 4. Compared with untreated control animals, ANF mRNA was unaltered at day 2, increased by 33% at day 3, and increased by 67% at day 4, whereas SERCA2a levels decreased by 10% at day 2, 22% at day 3, and 28% at day 4. Because changes of mRNA do not always translate into changes in protein levels, Western blots of ventricular tissue after 4 wk of

Fig. 3. Representative force and Ca\(^{2+}\) tracings (fluor 4-AM) in papillary muscles obtained from a ryanodine-treated mouse and an untreated mouse (control). First tracings show steady-state contractions at a stimulation frequency of 2 Hz followed by two tracings from postrest contractions obtained after rest intervals of 1 and 15 s. Note the greatly diminished contraction and Ca\(^{2+}\) transient after 15 s of rest (postrest decay = SR Ca\(^{2+}\) loss) in the papillary muscle obtained from the ryanodine-treated mouse compared with increased contraction and Ca\(^{2+}\) transient (postrest potentiation = SR Ca\(^{2+}\) accumulation) in the control mouse.

Fig. 4. Representative Northern and Western blots depicting changes in cardiac gene expression in animals treated with ryanodine over a 4-wk period. A: cardiac ventricular RNA samples were obtained from ryanodine-treated mice after 4 wk of treatment (+, solid bars) and from age-matched control mice (–, open bars). Blots were sequentially hybridized to \(^{32\text{P}}\)-labeled probes corresponding to cardiac sarcoplasmic (endo)plasmic reticulum Ca\(^{2+}\) (SERCA2a), phospholamban (PLB), \( \beta \)-myosin heavy chain (MHC-\( \beta \)), atrial natriuretic factor (ANF), and cardiac SR Ca\(^{2+}\) channel (RyR2). Bar graphs present means (not glyceraldehyde-3-phosphate dehydrogenase corrected) \( \pm \) SE; \( n = 6 \) in both groups. B: Western blots of SERCA2a, PLB5 (pentamer), and MHC-\( \beta \). Bar graphs represent means (not actin corrected) \( \pm \) SE; \( n = 8 \) in both groups; * \( P < 0.05 \).
Table 3. Specific [3H]ryanodine binding and 45Ca2+ uptake in ventricular homogenates after 4 wk of ryanodine treatment compared with untreated controls

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (50 μg/ml drinking water)</th>
<th>Ryanodine</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]ryanodine binding, fmol/μg</td>
<td>395 ± 25</td>
<td>412 ± 25*</td>
</tr>
<tr>
<td>protein</td>
<td>n</td>
<td>6</td>
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<tr>
<td>Sarcoplasmic reticulum 45Ca2+ uptake, nM·mg⁻¹·s⁻¹</td>
<td>5.36 ± 0.26</td>
<td>4.65 ± 0.43*</td>
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<td>n</td>
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Values are means ± SE; n, no. of animals. Ryanodine binding was calculated from the binding of 15 nmol/l of [3H]ryanodine to ventricular homogenates minus background binding in the presence of 10 μmol/l of unlabeled ryanodine (1 h, 37°C). 45Ca2+ uptake was performed in ventricular homogenates at 32°C. *P < not significant.

To study SERCA2 activity, a Ca2+-uptake study was performed in ventricular homogenates (Table 3). Although there was a trend toward decreased uptake levels, it did not reach statistical significance.

Cardiac histology. No obvious differences between control hearts and hearts from treated animals (such as myocyte density and presence of connective tissue and inflammatory cells) were found. The ventricles appeared to be diffusely hypertrophied without signs of regional hypertrophy. The shortest transverse diameter of nucleated sections of LV myocytes was increased by 16% in three ryanodine-treated animals compared with three normal hearts (31.9 ± 0.6 vs. 27.6 ± 1.3 μm; P < 0.01) providing direct evidence for myocyte hypertrophy.

DISCUSSION

To test whether a SR-induced cardiac dysfunction can induce cardiac hypertrophy, mice were treated with ryanodine, a highly specific inhibitor of the SR Ca2+ channel. An animal model was developed in which mice were treated by adding ryanodine to the drinking water. As early as 3 days after the ryanodine treatment was started, cardiac ANP expression was markedly increased and SERCA2a mRNA was decreased. These changes were accompanied by impaired cardiac performance as seen in hemodynamic studies and papillary muscle experiments. Ryanodine-treated animals displayed a marked slowing of the maximum speeds of contraction and relaxation in vivo and isolated papillary muscle preparations from treated animals had a decrease in actively developed stress and a prolongation of the late phase of relaxation. Analysis of postrest behavior of contractile force and Ca2+ transients in isolated tissue revealed that the postrest potentiation in control papillary muscles was inverted to a postrest decay in ryanodine-treated animals. This suggested that ryanodine had rendered some cardiac SR Ca2+ channels to an open locked state and allowed Ca2+ to leak out of the SR during prolonged rest periods. This finding, which has been reported under in vitro conditions at nanomolar concentrations of ryanodine (22), was further confirmed by RCCs. This experiment showed that the SR Ca2+ stores in ryanodine-treated animals could be depleted during prolonged rest periods. This leak-induced reduction in SR Ca2+ load presents the most likely explanation for the observed prolongation of cardiac contractions both in vitro and in vivo. Interestingly, we did not detect a decreased binding of [3H]ryanodine in myocardium of treated animals, which suggests that the fraction of occupied ryanodine binding sites in the treated animals is very small. This indicates, however, that even a small number of defective Ca2+ channels can disturb cardiac function profoundly. Because the ryanodine-induced SR dysfunction depends on the length of the time interval between two contractions, a lower stimulation rate facilitates SR Ca2+ depletion. We found this reflected in the inversion of the force-frequency relation. With prolonged rest intervals (low frequencies) the Ca2+ from the SR leaks from the SR back into the cytoplasm from where it is eliminated to the extracellular space via alternative Ca2+ transport mechanisms such as the sarcolemmal Na+/Ca2+ exchanger, resulting in lower steady-state forces (1, 5).

Attempts to measure hemodynamic function in animals that were treated for 4 wk failed due to the death of all animals shortly after the initiation of anesthesia. This finding suggests an increase in the severity of the cardiac dysfunction after prolonged periods of ryanodine treatment and was confirmed in the papillary muscles obtained from these animals. Compared with the changes in papillary muscle function after 1 wk of treatment, we found a greatly increased delay in cardiac relaxation, which indicates a further deterioration of diastolic function. In the ventricular tissue obtained from animals treated for 4 wk, ANP and β-MHC mRNA remained markedly induced, whereas SERCA2a mRNA was significantly reduced. Gene expression of phospholamban and cardiac ryanodine receptors were not altered. Although SERCA2a mRNA levels were depressed, protein levels and Ca2+ transport activity were not significantly reduced. In contrast, β-MHC protein levels were markedly induced in the treated animals. These findings were accompanied by a global cardiac hypertrophy with dilated atria and increased normalized LV and RV weights. Analysis of histological sections of the hearts from ryanodine-treated animals showed increased cross-sectional diameters in LV
myocytes with otherwise normal findings. No ventricular dilatation was observed. Interestingly transgenic mice overexpressing the SR Ca\(^{2+}\)-storage protein calsequestrin also show disturbed Ca\(^{2+}\) handling accompanied by cardiac hypertrophy (10); this supports the concept that a dysfunctional SR can promote cardiac growth. From these findings it may be speculated that depressed cardiac performance leads to a compensatory hypertrophy. This could promote a vicious circle because some of the molecular changes in hypertrophy are detrimental to cardiac contractility. In the ryanodine model, for example, increased expression of β-MHC may further slow cardiac contraction. In other animal models of heart failure and in human disease, reduced SERCA2 activity and/or depressed levels of the SR Ca\(^{2+}\) channel could accelerate the progression of failure and hypertrophy by further reducing cardiac performance. Although some evidence indicates an altered behavior of the SR Ca\(^{2+}\) channels in human end-stage heart failure (3, 19), single-channel recordings in human end-stage heart failure are reported to be normal (8). Our study, however, provides some evidence that even a small number of affected SR Ca\(^{2+}\) channels can profoundly alter cardiac function. From a significant number of unexplained cardiomyopathies, impaired SR function could present a potential mechanism of disease that could lead to contractile dysfunction, heart failure, and hypertrophy.

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