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Dantrolene suppresses spontaneous Ca\(^{2+}\) release without altering excitation-contraction coupling in cardiomyocytes of aged mice

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The aged heart exhibits complex structural and functional remodeling that gives rise to altered contractile function (33). Functional changes observed at the organ level reflect underlying changes within cardiomyocytes including impaired contraction and relaxation. Cardiomyocyte excitation-contraction coupling (ECC) occurs via Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR), whereby Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) channels triggers Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) Ca\(^{2+}\) store through type 2 ryanodine receptor (RyR) channels (4). The CICR process increases the free cytosolic concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)), which acts on the myofilaments to produce cellular contraction and cardiac systole. The cardiac cycle is completed upon Ca\(^{2+}\) removal from the cytosol via the sarco-endo-sarcolemmal Na\(^+\)/Ca\(^{2+}\) exchanger, thereby decreasing myofilament interaction with cellular relaxation and cardiac diastole.

Cardiomyocyte ECC is altered with advancing age, leading to impaired cardiac function. Across animal models of aging, cardiomyocytes display variable phenotypes for Ca\(^{2+}\) signaling. For example, the amplitude of action potential-induced Ca\(^{2+}\) transients can be higher (12, 47, 62), lower (19, 65), or unchanged (9, 24) in aged compared with young mice. More consistently, age-associated impairments in Ca\(^{2+}\) homeostasis are revealed when cardiomyocytes are experimentally challenged with elevated extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_o\)) (20), increased frequency of electrical pacing (24, 25, 34), or β-adrenergic stimulation (9, 62). One source of dysfunction in the aged myocardium is the inability of the SR to appropriately sequester Ca\(^{2+}\). While this abnormality is typically attributed to diminished expression and/or function of SERCA (for review, see Janczewski and Lakatta (27)), recent findings also point to increased function of the RyR and “SR leak” during diastole (9). Lipid bilayer experiments have shown that single channel RyR activity is increased with advancing age (65), which increases the occurrence of spontaneous diastolic Ca\(^{2+}\) release events in the form of localized Ca\(^{2+}\) sparks (9, 24, 65) and global Ca\(^{2+}\) waves (9, 20). Diastolic Ca\(^{2+}\) release is detrimental to cardiac function since it may lead to diastolic contracture and SR Ca\(^{2+}\) depletion with subsequent dysfunction during systole (32). Furthermore, because the Na\(^+\)/Ca\(^{2+}\) exchanger depolarizes the membrane while extruding Ca\(^{2+}\) (3 Na\(^+\) enter per each Ca\(^{2+}\) out), diastolic Ca\(^{2+}\) release predisposes the heart to delayed afterdepolarizations and arrhythmia (7, 29, 48).

Dantrolene is a type 1 RyR inhibitor used clinically to prevent excessive Ca\(^{2+}\) release and to avoid skeletal muscle hypercontraction during episodes of malignant hyperthermia (39). Whereas dantrolene has been purported to be without effects on the cardiac type 2 RyR (14, 17, 64), recent data in cardiac disease models have shown that dantrolene may exert beneficial effects on the myocardium by stabilizing the RyR and preventing spontaneous diastolic Ca\(^{2+}\) release (6, 28, 31, 32).
38, 50, 53, 63). At the same time, dantrolene does not impair (and in some cases even enhances) Ca\(^{2+}\) release and cardiomyocyte shortening during ECC. Whether dantrolene exerts complementary effects on the heart during advanced age is unknown.

In this study we tested the hypothesis that dantrolene prevents diastolic (i.e., spontaneous) Ca\(^{2+}\) release without altering ECC in aged myocardium. For this purpose we examined Ca\(^{2+}\) handling and contractile parameters in cardiomyocytes freshly isolated from young (3 to 4 mo) and aged (24–26 mo) C57BL/6 mice. We report that dantrolene had no effect on Ca\(^{2+}\) transients or cardiomyocyte contraction during ECC in young or aged mice and had no effect on diastolic Ca\(^{2+}\) release parameters in young mice. However, in aged mice, dantrolene prevented spontaneous diastolic Ca\(^{2+}\) sparks and Ca\(^{2+}\) waves. Dantrolene may thereby represent a potential antiarrhythmic treatment strategy in the aged heart.

**MATERIALS AND METHODS**

**Animal care and use.** Male C57BL/6 mice were obtained from the National Institute on Aging Colony at Charles River Laboratories (Wilmington, MA) and housed at the animal facility at the University of Missouri for 1–4 wk before experiments. Age groups studied were 3 to 4 mo (young, \(N = 35\)) and 24–26 mo (aged, \(N = 30\)). All experimental procedures were approved by the Animal Care and Use Committee of the University of Missouri.

**Solutions and chemicals.** Reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. Standard physiological salt solution (PSS) contained (in mM) 135 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 d-glucose, and 10 HEPES (pH 7.4) with NaOH. Elevated Ca\(^{2+}\) (\([\text{Ca}^{2+}]_o\) = 10 mM) was prepared using iso-osmotic substitution of CaCl\(_2\) for NaCl. Nominally Ca\(^{2+}\)-free PSS used for cardiomyocyte isolation was prepared as above but without addition of CaCl\(_2\). PSS was supplemented with 1 mM NaHCO\(_3\) in all experiments performed at 35°C. Stock solutions of dantrolene sodium (10 mM in dimethyl sulfoxide), and isoproterenol (Iso, 100 mM in H\(_2\)O) was prepared daily before final dilution (each to 1 \(\mu\)M) in PSS. The final concentration of DMSO (included in all solutions as a control) was 0.01%. Caffeine was prepared daily and dissolved in PSS at 10 mM.

**Cardiac myocyte isolation.** On the morning of an experiment, a mouse was anesthetized with pentobarbital sodium (60 mg/kg ip). Following confirmation of deep anesthesia (absence of withdrawal to toe pinch), the chest cavity was opened and hearts were rapidly (\(<30\) s) excised and placed in 4°C Ca\(^{2+}\)-free PSS. Hearts were immediately cannulated via the aorta and retrogradely perfused with Ca\(^{2+}\)-free PSS containing 2 U/ml heparin for 10 min, followed by \(<15\) min of perfusion with Eagle’s minimal essential medium (No. M0518, Sigma)-based enzymatic isolation solution supplemented with 10 mM NaHCO\(_3\), 2 mM Na-pyruvate, 10 mM NaHEPES, 10 mM HEPES, 8 mM taurine, 20 \(\mu\)M CaCl\(_2\), 50,000 U/l penicillin-streptomycin (PenStrep, Life Technologies, Grand Island, NY), and 22.5 \(\mu\)g/ml Liberase Blendzyme TH (Roche Applied Science, Indianapolis, IN) in pH 7.35 at 37°C. The left ventricle and septum were washed of bovine serum albumin, adapted to extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_o\) from 50 to 500 \(\mu\)M [Ca\(^{2+}\)]\(_o\), over 40 min), and plated on laminin-coated glass coverslips. Subsequent cardiomyocyte experimentation was performed using conditions and protocols as described below to optimize resolution of respective parameters of interest.

**Intracellular Ca\(^{2+}\) and sarcomere length measurements with epi-fluorescence microscopy.** Experiments monitoring cardiomyocyte contractile activity (Figs. 1–4) simultaneously with [Ca\(^{2+}\)]\(_o\), were performed at 35 ± 1°C to optimize mechanical activity. Plated myocytes were loaded with 5 \(\mu\)M fluo-4 AM (Molecular Probes/Life Technologies) for 10 min, followed by a 40–60-min wash, all at room temperature. Coverslips were secured in a flow chamber (RC-27N, Warner Instruments, Hamden, CT) that was placed in a temperature-controlled heating element (PH-1, Warner) and mounted on an inverted fluorescence microscope (IX71, Olympus America, Center Valley, PA). Myocytes were viewed using a \(\times 40\) oil-immersion objective (U/Apo340: 1.35 numerical aperture, Olympus America) with a recording window of 275 × 75 \(\mu\)m. Myocytes were perfused with PSS via an inline solution heater (SHM-828, Warner) at \(~2\) ml/min at 35 ± 1°C using a temperature controller (TC-344B, Warner). Solutions were changed using an electronic perfusion valve control system (VC-8, Warner). Action potentials were induced using electrical field stimulation (0.5–8 Hz; 2-ms pulse duration; voltage, \(~30\)% greater than threshold for myocyte contraction; \(~20\) volts; S48, Grass Instruments, Warwick, RI) with platinum electrodes placed at the edges of the bath. Single wavelength fluo-4 fluorescence (excitation 460–480 nm; emission 500–550 nm; sampling rate, 500 Hz) and myocyte sarcomere length (\(L_s\), recorded using transmitted light \(>695\) nm at 240 Hz with fast-Fourier transform analysis) were obtained using a calcium and contractility recording system (IonOptix, Milton, MA). To limit photodamage and photobleaching of the fluo-4 dye, the excitation shutter was opened for 10 s each min to obtain simultaneous measurements of fluorescence and \(L_s\).

The minimum duration of dantrolene treatment in all experimental protocols was 5 min. The typical protocol consisted of 5 min of recordings in PSS (\(<1\) \(\mu\)M dantrolene), followed by 3 min of recordings in the presence of 1 \(\mu\)M Iso (Iso \(\pm\) dantrolene). Ca\(^{2+}\) and \(L_s\) shortening transients stabilized after 5 min of control stimulation (Fig. 1; control/control + dantrolene) and following 3 min of Iso addition (Fig. 1; Iso/Iso + dantrolene), thus recordings at these respective time points were used for data analysis. In a subset of experiments following the 5 min of 1-Hz pacing, stimulation frequency was increased to 4 Hz for 2 min, followed by 8 Hz for 2 min. Analysis was performed from fluorescence data acquired at the end of each pacing train (\(L_s\) was not analyzed at 4 or 8 Hz because of inconsistent tracking of sarcomeres at these frequencies). To rapidly empty the SR Ca\(^{2+}\) store and thereby assess SR Ca\(^{2+}\) content under respective conditions, electrical stimulation was terminated and 10 mM caffeine was applied within 1–3 s via a gravity-fed pipette placed directly in the PSS flow stream.

**Ca\(^{2+}\) spark and Ca\(^{2+}\) wave measurements with confocal microscopy.** Experiments using confocal microscopy (Figs. 5 and 6) were performed at room temperature (22–24°C) to take advantage of improved fluo-4 dye performance at this temperature versus 35°C (52). Plated myocytes were loaded with 10 \(\mu\)M fluo-4 AM for 5 min (Ca\(^{2+}\) waves, Fig. 6) or 20 min (Ca\(^{2+}\) sparks, Fig. 5), followed by 40–60-min wash. Coverslips were placed in a custom-made chamber, mounted on a Leica SP5 confocal microscope (Leica Microsystems, Buffalo Grove, IL), and perfused with PSS at \(~0.5–1\) ml/min.

Ca\(^{2+}\) sparks were monitored from a central region of the cell (avoiding the nucleus) in line scan (x-t) mode using the galvano scanhead of the Leica SP5 (HCX PLAN APO \(\times 63\) objective: 1.3 numerical aperture; pixel size, 0.12 \(\mu\)m; 2 ms/line) with excitation at 488 nm (1 to 2% of a 100-mW argon laser) and emission recorded from 500–560 nm using a Leica HyD GaAsP hybrid detector (Leica Microsystems). The gain of the detector was adjusted for each experiment to obtain equivalent baseline values for spark detection (typically, 350–400 gray units on a 12-bit scale). Cardiomyocytes were superfused with PSS (\(\pm\) dantrolene) for 5 min. Iso (\(\pm\) dantrolene) was then applied and cardiomyocytes were stimulated at 1 Hz for 3–5 min, followed by period of rest. The final Ca\(^{2+}\) transient of the stimulation train and \(~8\) s of the rest period were recorded, with Ca\(^{2+}\)
sparks analyzed between 1- and 7-s poststimulation. If a Ca²⁺ wave occurred within this predefined interval (preventing accurate spark detection), Ca²⁺ sparks were measured between 1-s poststimulation and the onset of the Ca²⁺ wave (e.g., Fig. 5A).

Ca²⁺ waves were monitored under Ca²⁺ overload conditions induced by elevated [Ca²⁺]₀ (10 mM) with two-dimensional imaging at 45 frames/s using the resonant scanner of the Leica SP5 (HC PL APO ×20 objective: 0.7 numerical aperture; 512 × 150 pixels; bidirectional scanning/2× line averaging; pixel size, 0.24 μm); excitation was at 488 nm and emission was recorded from 500–560 nm. Low laser intensity (~1% of a 100 mW argon laser) was used to minimize photobleaching or photodamage during extended periods of recording. Laser intensity was increased to 8% (with lower detector gain) to obtain example Ca²⁺ wave images shown in Fig. 6A.

**Spontaneous Ca²⁺ wave protocols.** Ca²⁺ waves were monitored using separate protocols.

The first protocol was used to monitor Ca²⁺ waves (and associated aftercontractions) with epifluorescence microscopy under conditions of cellular stress induced by β-adrenergic stimulation with Iso at 35 ± 1°C. Cardiomyocytes were superfused with PSS (±dantrolene) for 5 min, followed by Iso (±dantrolene) for 3 min, all with 1-Hz stimulation. Electrical stimulation ceased and the interval between the final electrical field stimulation and onset of the Ca²⁺ increase associated with the first observed spontaneous aftercontraction was defined as the time to Ca²⁺ release (Fig. 4).

The second protocol was used to monitor Ca²⁺ waves in response to Ca²⁺ overload conditions, independent of β-adrenergic stimulation. Two-dimensional imaging (performed at 22–24°C) was used to monitor cycles of spontaneous Ca²⁺ waves induced by exposure to 10 mM [Ca²⁺]₀. Myocytes were stimulated at 0.5 Hz for 60 s, followed by 60 s of rest then 60 s of imaging when spontaneous Ca²⁺ waves were monitored. After control measurements, dantrolene was applied for 3 min, and the stimulation, rest, and imaging protocol was repeated. The experimental design resulted in paired measurements of Ca²⁺ waves during Ca²⁺ overload before and 5 to 6 min following dantrolene treatment. Those myocytes not exhibiting spontaneous Ca²⁺ waves under these conditions (<10%) were excluded from analysis. To assess the SR Ca²⁺ content associated with Ca²⁺ waves (Fig. 6E), fluorescence was monitored while 10 mM caffeine was applied to completely unload the SR of Ca²⁺ before when the next Ca²⁺ wave was expected to occur in the cycle. In some trials, the caffeine reached the myocyte immediately after a spontaneous Ca²⁺ wave and were excluded to avoid underestimating the true SR Ca²⁺ content preceding wave initiation (10).

**Data analysis and statistics.** Cardiomyocyte dimensions were defined as the longest continuous cell segment perpendicular to (length) and parallel to (width) sarcomere striations. Whole cell Ca²⁺ transients and Lᵥ were analyzed off-line using IonWizard 6.3 software (IonOptix) with the following parameters assessed: 1) Ca²⁺ transient amplitude (ΔF/F₀, where F₀ is the fluorescence before the Ca²⁺ transient, and ΔF = F_peak – F₀), 2) Ca²⁺ transient recovery time (time from stimulus to 50% recovery back to baseline), 3) Ca²⁺ transient τ (an index of SERCA activity, determined using an exponential fit to the fluorescence signal decay between 80% of peak and the baseline), 4) Lᵥ and shortening amplitude, and 5) sarcomere shortening recovery time (time from stimulus to 50% recovery back to baseline). Values of 3–6 consecutive transients were averaged to obtain a single value for each myocyte in a given experimental protocol. Ca²⁺ sparks were analyzed using ImageJ software with the Sparkmaster algorithm (45) with the detection threshold set at 3.8. Spontaneous Ca²⁺ wave frequency (in waves/min) and amplitude (ΔF/F₀) were determined using a 5 μm × 15–25 μm region of interest (see Fig. 5A). SR Ca²⁺ content was assessed as the amplitude of the fluo-4 Ca²⁺ transient in response to 10 mM caffeine (ΔF/F₀, Ca²⁺free). All fluorescence values were background subtracted before analysis.

Statistical comparisons were made using t-tests, one- or two-way ANOVA depending on experimental design, with Bonferroni post hoc comparisons. Graphical and statistical analyses were performed using SigmaPlot 10/SigmaStat 3.5 (Systat, San Jose, CA). Summary data are presented as means ± SE, with statistical significance set at P < 0.05. The number of cells per protocol is indicated by n. Typically, three to six cells per animal were studied from at least three mice per experimental group.

**RESULTS**

Left ventricular cardiomyocytes from aged mice exhibited cellular hypertropy with an increase in both cell length (149 ± 4 μm aged vs. 130 ± 3 μm young, P < 0.001) and cell width (40 ± 1 μm aged vs. 35 ± 1 μm young, P = 0.01, n = 86–87) compared with those in young mice. We examined intracellular Ca²⁺ in cardiomyocytes loaded with fluo-4 using protocols designed to monitor the effects of dantrolene on 1) systolic Ca²⁺ transients and cell shortening during ECC and 2) spontaneous diastolic Ca²⁺ release following rest from steady-state stimulation. Spontaneous Ca²⁺ release is typically observed under conditions of elevated SR Ca²⁺ content (11, 55), and we therefore performed experiments under control conditions and in the presence of the β-adrenergic agonist Iso. Figure 1A shows example action potential (1 Hz)-induced Ca²⁺ transient (upper) and sarcomere shortening (lower) traces from cardiomyocytes of young (i and ii) and aged (iii and iv) mice in the absence (i and iii) and presence (ii and iv) of dantrolene. Measurements were made before (Fig. 1A) and 3 min following (Fig. 1B) the addition of Iso. Baseline diastolic Lᵥ during 1-Hz stimulation was similar between young and aged mice under all conditions (Table 1). Under control conditions, Ca²⁺ transient amplitude was similar between young and aged mice (Fig. 1C), yet sarcomere shortening amplitude was decreased in aged mice (Fig. 1D). Ca²⁺ transient recovery time was prolonged in aged mice (with an associated trend toward longer Ca²⁺ transient τ, P = 0.06), yet shortening recovery time (i.e., cell relaxation) was similar between young and aged mice (Table 1). In the presence of Iso, Ca²⁺ transient (Fig. 1E) and sarcomere shortening amplitude (Fig. 1F) each increased significantly compared with control conditions (Fig. 1, C and D, respectively; P < 0.001) and reached identical levels in young and aged mice. Iso thereby rescued the deficit in sarcomere shortening observed in aged mice under control conditions (compare Fig. 1, D and F). Iso significantly (P < 0.001) reduced recovery time of Ca²⁺ transients in both young and aged mice, yet Ca²⁺ transient recovery time and τ remained prolonged in aged compared with young mice (Table 1).

Cardiomyocytes challenged with increasing stimulation frequencies exhibited a decrease in Ca²⁺ transient amplitude at higher frequencies (P < 0.001, compare 1, 4, and 8 Hz in Fig. 2E), and at higher frequencies aged mice exhibited a decrease in Ca²⁺ transient amplitude compared with that in young mice (Fig. 2). SR Ca²⁺ content, measured as the amplitude of the Ca²⁺ transient in response to rapid 10 mM caffeine application, was similar between young and aged mice under control conditions (Fig. 3, A and C) and was significantly increased (P < 0.001) by Iso to an identical level in young and aged mice (Fig. 3, B and D). Dantrolene treatment had no effect on Ca²⁺ transients (Figs. 1 and 2), sarcomere shortening (Fig. 1), or SR Ca²⁺ content (Fig. 3), consistent with the drug having minimal effects on ECC.
We next examined the effects of dantrolene on spontaneous 
Ca\(^{2+}\) release following a period of rest from 1-Hz steady-state 
stimulation. Spontaneous Ca\(^{2+}\) release was infrequent and 
highly variable under control conditions (data not shown), and 
therefore release events were experimentally monitored during 
β-adrenergic stimulation with Iso when SR Ca\(^{2+}\) content was 
elevated (Fig. 3). Figure 4 illustrates examples of spontaneous 
SR Ca\(^{2+}\) release (top, marked with arrow) and aftercontractions 
(bottom, marked with arrowhead), following rest from 
stimulation in cardiomyocytes from young (Fig. 4A) and aged
DANTROLENE SUPPRESSES Ca\textsuperscript{2+} SPARKS AND Ca\textsuperscript{2+} WAVES

Table 1. Parameters of action potential-induced Ca\textsuperscript{2+} transients and cell shortening in cardiomyocytes from young and aged mice

<table>
<thead>
<tr>
<th></th>
<th>Ca\textsuperscript{2+} Transient Time to Peak, ms</th>
<th>Ca\textsuperscript{2+} Transient Recovery (50%), ms</th>
<th>Ca\textsuperscript{2+} Transient Recovery (τ), ms</th>
<th>Sarcomere Length (1 Hz, Diastolic, μm)</th>
<th>Shortening Time to Peak, ms</th>
<th>Shortening Recovery (50%), ms</th>
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<tr>
<td><strong>Young</strong></td>
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<td>Control</td>
<td>19 ± 1</td>
<td>133 ± 6</td>
<td>125 ± 13</td>
<td>1.73 ± 0.01</td>
<td>58 ± 4</td>
<td>91 ± 5</td>
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<tr>
<td>Dant</td>
<td>19 ± 1</td>
<td>122 ± 6</td>
<td>110 ± 12</td>
<td>1.73 ± 0.01</td>
<td>57 ± 3</td>
<td>94 ± 5</td>
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<tr>
<td>Iso</td>
<td>18 ± 1</td>
<td>87 ± 2#</td>
<td>56 ± 2#</td>
<td>1.69 ± 0.02</td>
<td>47 ± 4</td>
<td>86 ± 5</td>
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<tr>
<td>Iso + Dant</td>
<td>16 ± 1</td>
<td>85 ± 1#</td>
<td>55 ± 2#</td>
<td>1.71 ± 0.02</td>
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<td><strong>Aged</strong></td>
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<tr>
<td>Control</td>
<td>19 ± 1</td>
<td>138 ± 5#</td>
<td>148 ± 14</td>
<td>1.71 ± 0.02</td>
<td>45 ± 3</td>
<td>94 ± 8</td>
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<tr>
<td>Dant</td>
<td>23 ± 2</td>
<td>145 ± 7#</td>
<td>139 ± 12</td>
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<td>Iso</td>
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<tr>
<td>Iso + Dant</td>
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<td>91 ± 2#</td>
<td>65 ± 4#</td>
<td>1.70 ± 0.02</td>
<td>62 ± 4</td>
<td>101 ± 5</td>
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Parameters shown are means ± SE from n = 14–19 individual cells per group from hearts of 12 young and 16 aged mice. *P < 0.05, aged vs. young, 2-way ANOVA [factors: age and dantrolene (Dant) treatment]. Bonferroni correction post hoc. #P < 0.05, isoproterenol (Iso) vs. control; paired t-test.

(Fig. 4B) mice in the absence (i) and presence (ii) of dantrolene. Across experiments, the onset of spontaneous Ca\textsuperscript{2+} release preceded the aftercontraction by 253 ± 3 ms (n = 61). The time to Ca\textsuperscript{2+} release was similar between young and aged mice (Fig. 4C). However, dantrolene application significantly prolonged this time in aged mice with no effect in young mice. The postrest Ca\textsuperscript{2+} release exhibited a delayed time from onset to peak (371 ± 3 ms, n = 50) and in some cases the initial elevation in Ca\textsuperscript{2+} preceded a secondary rapid elevation in Ca\textsuperscript{2+} and contraction (see star in Fig. 4B, i). This likely reflects the initial Ca\textsuperscript{2+} wave triggering a delayed afterdepolarization, “triggered” action potential, and subsequent synchronized increase in global [Ca\textsuperscript{2+}]

The Ca\textsuperscript{2+} spark is the elementary Ca\textsuperscript{2+} release event through a cluster of RyR channels and is visualized as a spatially restricted increase in cellular fluorescence (8). Ca\textsuperscript{2+} sparks were monitored in the presence of Iso during a period of rest following 1-Hz stimulation. Shown in Fig. 5A is an example line scan image of an electrically stimulated Ca\textsuperscript{2+} transient (vertical increase in fluorescence near beginning of line scan image) followed by rest where Ca\textsuperscript{2+} sparks were detected before the spontaneous initiation of a Ca\textsuperscript{2+} wave (angular increase in fluorescence in line scan image). In this example, the Ca\textsuperscript{2+} wave propagated along the scan line with time until triggered Ca\textsuperscript{2+} transient occurred (i.e., triggered by electrical activity of the cardiomyocyte). Presented in Fig. 5, B and C, are pseudocolored line scan images of Ca\textsuperscript{2+} sparks from young (Fig. 5B) and aged (Fig. 5C) mice treated with Iso in the absence (left) and presence (right) of dantrolene. Ca\textsuperscript{2+} sparks were of higher frequency (Fig. 5D) and amplitude (Fig. 5E) in aged versus young mice, with a similar full width at half maximum (Fig. 5F) and full duration at half maximum (Fig. 5G). Treatment with dantrolene reduced Ca\textsuperscript{2+} spark frequency in aged mice with no effect on additional Ca\textsuperscript{2+} spark properties. Dantrolene treatment was without effect on any measured Ca\textsuperscript{2+} spark parameter in young mice (Fig. 5, D–G).

We next designed experiments to monitor the effect of dantrolene on properties of spontaneous Ca\textsuperscript{2+} waves under Ca\textsuperscript{2+} overload conditions in the absence of β-adrenergic stimulation. Cardiomyocytes from young and aged mice were exposed to elevated [Ca\textsuperscript{2+}]o (10 mM) to facilitate SR Ca\textsuperscript{2+} loading. Under these experimental conditions, the SR accumulates Ca\textsuperscript{2+} until SR Ca\textsuperscript{2+} content reaches a threshold level for initiation of spontaneous Ca\textsuperscript{2+} waves (49). Two-dimensional imaging was used to examine Ca\textsuperscript{2+} waves before and 5 min following dantrolene treatment using a paired experimental design. Presented in Fig. 6A are pseudocolored images of a fluo-4-loaded cardiomyocyte immediately after (top) and 1 s following (bottom) Ca\textsuperscript{2+} wave initiation, illustrating the non-uniform Ca\textsuperscript{2+} signal associated with wave propagation. Shown in Fig. 6, B and C, are example traces of spontaneous Ca\textsuperscript{2+} waves from young (Fig. 6B) and aged (Fig. 6C) cardiomyocytes in the absence (i) and presence (ii) of dantrolene. Ca\textsuperscript{2+} waves were of similar frequency in young (8.7 ± 2.5 waves/min; n = 6) and aged (10.2 ± 1.9 waves/min; n = 7) mice, yet Ca\textsuperscript{2+} waves were of diminished amplitude in aged mice (Fig. 6D). Dantrolene significantly reduced Ca\textsuperscript{2+} wave frequency in aged mice (to 7.4 ± 1.8 waves/min; P = 0.01) with a similar but non-statistically significant effect in young mice (to 5.7 ± 1.8 waves/min; P = 0.12). A similar nonsignificant reduction in frequency was observed in time control experiments (10.0 ± 2.1 waves/min at t = 0 vs. 8.7 ± 2.6 waves/min at t = 5 min, P = 0.27), suggesting mild rundown of this variable under our experimental conditions. Dantrolene treatment was associated with an increase in Ca\textsuperscript{2+} wave amplitude in aged mice (see dashed line in Fig. 6C) with no effect on young mice (Fig. 6, B and D). We next assessed the SR Ca\textsuperscript{2+} content associated with Ca\textsuperscript{2+} waves by applying 10 mM caffeine at the approximate time where a Ca\textsuperscript{2+} wave was expected to occur in the Ca\textsuperscript{2+} wave cycle. Similar to differences in Ca\textsuperscript{2+} wave amplitude, the SR Ca\textsuperscript{2+} content associated with Ca\textsuperscript{2+} waves was lower in aged than in young mice (Fig. 6E). Dantrolene treatment significantly increased the SR Ca\textsuperscript{2+} content in aged mice with no effect in young (Fig. 6E). Time control measurements showed that Ca\textsuperscript{2+} wave amplitude (∆F/Fluo) 3.87 ± 0.36 at t = 0 and 4.15 ± 0.30 at t = 5 min, n = 7) and associated SR Ca\textsuperscript{2+} content (∆F/Fluo) 4.74 ± 0.40 at t = 0 and 4.83 ± 0.34 at t = 5 min, n = 7) were reproducible for the duration of the experimental protocol. Taken together, these data show that in cardiomyocytes from aged mice, dantrolene prevents spontaneous diastolic Ca\textsuperscript{2+} release without altering Ca\textsuperscript{2+} transients or contraction during ECC.

**DISCUSSION**

The RyR intracellular Ca\textsuperscript{2+} release channel is a critical component of ECC in the myocardium with defective RyR regulation frequently observed with heart disease (3) and
advancing age (9, 24, 65). Dantrolene is a type 1 RyR inhibitor clinically used to treat excessive skeletal muscle contracture associated with malignant hyperthermia (39). However, recent data have shown that dantrolene may also exert beneficial effects on the myocardium by stabilizing the cardiac type 2 RyR and preventing spontaneous diastolic SR Ca²⁺ release (6, 28, 31, 38, 50, 53, 63). We hypothesized that dantrolene would prevent spontaneous Ca²⁺ release and aftercontractions without compromising Ca²⁺ release or contractile activity during ECC in the aged myocardium. The major findings of this investigation are 1) dantrolene prolonged the time to spontaneous Ca²⁺ release and aftercontraction in aged without effect in young mice, 2) dantrolene decreased the frequency of Ca²⁺ sparks and altered Ca²⁺ wave properties in aged mice without effect in young, and 3) dantrolene did not alter any component of ECC in young or aged mice.

**Cardiomyocyte ECC in young and aged mice.** In cardiomyocytes isolated from aged mice, Ca²⁺ transients were similar in amplitude to those in young mice both under control conditions and following β-adrenergic stimulation with Iso (Fig. 1). Consistent with previous findings in mice (24, 25, 34), differences in Ca²⁺ transient amplitude were revealed in aged cardiomyocytes when challenged with increased stimulation frequency (Fig. 2). Impaired Ca²⁺ transient recovery, observed as a prolonged Ca²⁺ transient recovery and τ (Table 1), likely reflects changes in SERCA expression or function commonly observed with advancing age (25, 34, 65). However, under our experimental conditions, this did not result in differences in SR Ca²⁺ loading (Fig. 3), consistent with cardiomyocytes acutely treated with the SERCA inhibitor thapsigargin, where a reduced rate of Ca²⁺ reuptake was associated with a disproportionately small decrease in SR Ca²⁺ content (5). Under control conditions, whereas Ca²⁺ transients were similar between the young and aged, cell shortening was decreased in aged mice (Fig. 1), suggesting decreased Ca²⁺ sensitivity of the myofibers (19, 65). Subsequent treatment with the β-adrenergic agonist Iso at 1 μM, a concentration designed to produce near-maximum positive inotropic effects in both the young and aged (16), rescued the deficit in shortening observed in aged mice with both Ca²⁺ transient and shortening reaching similar amplitudes in young and aged mice (Fig. 1).

**Spontaneous diastolic Ca²⁺ release in young and aged mice.** Diastolic Ca²⁺ release is typically observed under conditions of elevated SR Ca²⁺ content (56), and evidence supports an increased prevalence of spontaneous Ca²⁺ release events with advancing age (9, 20, 24, 65). When SR Ca²⁺ content was elevated following β-adrenergic stimulation, we typically did not observe Ca²⁺ release during the diastolic interval at 1-Hz steady-state stimulation in young or aged mice (see traces in Fig. 1), which is consistent with the >3-s latency of Ca²⁺ release determined following rest from stimulation (Fig. 4C). Furthermore, during rest following stimulation, the time to Ca²⁺ release (Fig. 4C) and SR Ca²⁺ content associated with release (Fig. 3D) were both similar between young and aged mice. The postrest Ca²⁺ release observed using epifluorescence microscopy (Fig. 4, A and B) was confirmed to be due to the initiation and propagation of Ca²⁺ waves (Fig. 5A). In agreement with the similar time to Ca²⁺ wave initiation (i.e., time to Ca²⁺ release in Fig. 4C), Ca²⁺ wave propagation velocity was also similar between young and aged mice (93 ± 6 μm/s young vs. 99 ± 9 μm/s aged, see Fig. 5 legend). Thus

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**Fig. 2. Frequency dependence of Ca²⁺ transients in cardiomyocytes from young and aged mice.** A–D: example fluo-4 Ca²⁺ transients (ΔF/F₀) in response to 1, 4, and 8-Hz electrical field stimulation (35°C, stimuli denoted by diamonds) in cardiomyocytes from young (A and B) and aged (C and D) mice in the absence (A and C) and presence (B and D) of 1 μM Dant. E: summary data of Ca²⁺ transient amplitude vs. stimulation frequency in young (black) and aged (white) in the absence (circles) and presence (triangles) of Dant. Ca²⁺ transient data are presented as ΔF/F₀, where ΔF = F – F₀. *P < 0.05, aged vs. young, 2-way ANOVA (factors: age and Dant treatment). Bonferroni correction post hoc. Ca²⁺ transient amplitudes at the respective stimulation frequencies were significantly (P < 0.001) different from one another (one-way ANOVA); n = 7–14 individual cells from hearts of 4 young and 5 aged animals.
Ca$^{2+}$ wave properties were similar between the young and aged even though the frequency and amplitude of Ca$^{2+}$ sparks were elevated in aged mice, which should promote Ca$^{2+}$ wave initiation and propagation. These findings suggest that $\beta$-adrenergic stimulation of aged mice may produce protective effects (16) that counteract the increase in RyR activity, thereby preventing Ca$^{2+}$ sparks from transitioning into Ca$^{2+}$ waves. Consistent with this interpretation, when SR Ca$^{2+}$ content was elevated using 10 mM [Ca$^{2+}$]o (i.e., independently of $\beta$-adrenergic stimulation), spontaneous Ca$^{2+}$ waves in aged mice were of diminished amplitude (Fig. 6D) and associated with a lower SR Ca$^{2+}$ content (Fig. 6E).

The mechanisms of Ca$^{2+}$ wave initiation and propagation are complex and include factors such as the open probability of the RyR (activity assayed here as Ca$^{2+}$ sparks), RyR release cluster spacing (26), and SERCA-mediated Ca$^{2+}$ uptake (30, 37), along with cytosolic and intra-SR Ca$^{2+}$ buffering and diffusion (46). RyR release clusters localize near the Z-line in cardiac myocytes, and our data show no differences in diastolic Z-line spacing before Ca$^{2+}$ wave initiation (as monitored by cellular $L_s$, Table 1). SERCA-mediated Ca$^{2+}$ uptake was slower in aged mice (τ of Ca$^{2+}$ transient, Table 1), yet the contribution of SERCA to Ca$^{2+}$ wave generation and propagation (independent of SR Ca$^{2+}$ content) remains a matter of debate (1, 30, 35, 37, 40, 46) given the complex role of SERCA in regulating SR Ca$^{2+}$ refilling (which may enhance Ca$^{2+}$ wave propagation), cytosolic Ca$^{2+}$ removal, and Ca$^{2+}$ buffering between RyR release clusters (which may hinder Ca$^{2+}$ wave propagation). Thus it remains unclear why Ca$^{2+}$ wave properties were similar between the young and aged in the presence of $\beta$-adrenergic stimulation when Ca$^{2+}$ spark frequency was restored to levels found in young mice (Fig. 5D).

**Fig. 3.** SR Ca$^{2+}$ content in cardiomyocytes from young and aged mice. A and B: example traces of the fluo-4 cytosolic Ca$^{2+}$ transient ($\Delta F/F_0$) in response to 10 mM caffeine (Caff) application (black bars marked Caff). Caff was applied immediately (~1–3 s) following cessation of 1-Hz (35°C) electrical field stimulation to assess sarcoplasmic reticulum (SR) Ca$^{2+}$ content. Shown are examples of cardiomyocytes from young (i and ii) and aged (iii and iv) mice in the absence (i and iii) and presence (ii and iv) of 1 μM Dant. Experiments were performed in the absence (A) and presence (B) of Iso (1 μM for 3 min). C and D: summary data of SR Ca$^{2+}$ content under control conditions (C) and in the presence of Iso (D) in cardiomyocytes from young (white bars) and aged (gray bars). Hatched bars in C and D represent cells treated with Dant. SR Ca$^{2+}$ content (presented as $\Delta F/F_0$ Caffeine, where $F_0$ is the fluorescence before the caffeine-induced response and $\Delta F = F_{peak} - F_0$) was similar between young and aged and was not affected by Dant. Two-way ANOVA (factors: age and Dant treatment), Bonferroni correction post hoc. $# P < 0.001$ Iso vs. control, unpaired t-test; $n = 8–15$ individual cells from hearts of 16 young and 15 aged animals.

Dantrolene and cardiac Ca$^{2+}$ homeostasis. Dantrolene typically does not affect the healthy myocardium (14, 17, 64). The cardiac RyR2 contains the purported binding site for dantrolene.  

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Peptides that destabilize interdomain interactions within the RyR lead to a gain in channel function producing abnormal diastolic Ca²⁺ release (31, 50, 53). Such effects can be explained by these peptides “unzipping” the RyR and thereby exposing the dantrolene binding site with subsequent dantrolene treatment restoring normal channel function. Similar destabilization of interdomain interactions within the RyR is a proposed mechanism of Ca²⁺ handling abnormalities with cardiac disease and dantrolene has been shown to prevent excessive spontaneous Ca²⁺ release in heart failure (31, 38), catecholaminergic polymorphic ventricular tachycardia (6, 28, 50, 53), and following ventricular fibrillation (63). Our findings in aged mice are consistent with dantrolene reducing excessive diastolic Ca²⁺ release through the RyR (6, 28, 31, 38, 50, 53, 63). The RyR is a large tetrameric protein that serves as a macromolecular signaling platform, and as such its activity is subject to complex regulation by post-translational modification and association with accessory proteins. Key phosphorylation residues on the RyR are serine-2030 (61), serine-2808 (36, 60), and serine-2814 (59), although considerable controversy surrounds the relative importance and physiological/pathological role of phosphorylation at these respective sites [see recent reviews, Dobrev and Wehrens (13) and Houser (23)]. Oxidative stress is elevated with cardiac disease and advancing age with redox modification of the RyR associated with dysfunctional SR Ca²⁺ release (2, 9, 18, 51). Cellular redox modifications may also facilitate RyR phosphorylation and increase SR Ca²⁺ release (15, 22). We therefore speculate that in aged mice, redox modification or phosphorylation of the RyR destabilizes interdomain interactions within the channel, thereby increasing SR Ca²⁺ release. These modifications may also expose the dantrolene binding site within the RyR, with subsequent dantrolene treatment stabilizing the channel and restoring normal SR Ca²⁺ release.

In animal models of cardiac disease, dantrolene exerts positive inotropic effects on the heart by increasing the amplitude of cardiomyocyte Ca²⁺ transients and the strength of contraction (31). The counterintuitive finding of a RyR inhibitor leading to positive inotropic effects is explained by restoration of SR Ca²⁺ content. Because diastolic Ca²⁺ release leads to depletion of the SR Ca²⁺ store (10) and impaired cardiac ECC, preventing SR Ca²⁺ leak by stabilizing the RyR shifts Ca²⁺ flux balance toward SR Ca²⁺ accumulation, restoring SR Ca²⁺ content and increasing CICR in response to the action potential. The effect of RyR inhibitors preserving SR Ca²⁺ content is strongest when Ca²⁺ release is observed between transients (54), and our finding that dantrolene did not appreciably alter SR Ca²⁺ content during ECC (Fig. 3) is consistent with minimal Ca²⁺ release following action potential stimulation (i.e., extended diastolic interval, dantrolene increased SR Ca²⁺ content in aged mice with no effect in young mice (Fig. 6E)).

Different mechanisms of RyR inhibition can create distinct effects on cellular Ca²⁺ homeostasis and cellular SR Ca²⁺ content (21, 58). The local anesthetic tetracaine blocks the RyR in its closed state to produce inhibition by decreasing channel opening. During ECC, tetracaine produces a transient decrease in cellular contraction that recovers partially when Ca²⁺ flux balance shifts toward SR Ca²⁺ accumulation, elevating SR

![Graph A: Young](image)

![Graph B: Aged](image)

![Graph C: Summary Data](image)

**Fig. 4.** Spontaneous Ca²⁺ release and aftercontractions in cardiomyocytes from young and aged mice. A and B: cardiomyocytes from young (A) and aged (B) mice were treated with Iso (1 μM for 3 min) during 1-Hz stimulation (35°C) in the absence (i) and presence (ii) of 1 μM Dant. Shown in example traces are 3 electrically-stimulated (stimulation marked by diamonds) Ca²⁺ transients (ΔF/ΔF₀, top) and corresponding sarcomere length changes (L/L₀; bottom) followed by cessation of stimulation and a period of rest. During the rest period, spontaneous Ca²⁺ release (top, marked by arrow) and aftercontractions (bottom, marked by arrowhead) were observed with the interval between final stimulus and onset of the Ca²⁺ release defined as the time to Ca²⁺ release (marked with gray highlight). Ca²⁺ waves transitioning into spontaneous Ca²⁺ transients (B,ii, marked with star) were observed in the following ratios (transients/waves): young (1/19), young Dant (2/20), aged (5/14), aged Dant (2/12). C: summary data of time to Ca²⁺ release in cardiomyocytes from young (white bars) and aged (gray bars) treated with Iso. Hatched bars represent cells treated with Dant. *P < 0.05 vs. young Iso, young Dant plus Iso, and aged Iso. Two-way ANOVA (factors: age and Dant treatment), Bonferroni correction post hoc; n = 12–20 individual cells from 11 young and 7 aged animals.
Ca\textsuperscript{2+} content and counteracting the effect of RyR inhibition (43). In contrast, the antiarrhythmic agent flecainide blocks the open state of RyR, which decreases Ca\textsuperscript{2+} spark duration and mass (a product of spark amplitude and full width at half maximum), consequently preventing Ca\textsuperscript{2+} sparks from transitioning into arrhythmogenic Ca\textsuperscript{2+} waves (21, 58). A unique feature of flecainide is that it produces an increase in Ca\textsuperscript{2+} spark frequency which offsets the reduction in Ca\textsuperscript{2+} spark mass, with the net result of unchanged SR Ca\textsuperscript{2+} content. The effects of dantrolene on Ca\textsuperscript{2+} sparks observed here in aged mice (Fig. 5) are similar to effects of tetracaine with reduced Ca\textsuperscript{2+} spark frequency (31, 41, 50, 53) and no change in additional Ca\textsuperscript{2+} spark properties (38). Furthermore, similar to tetracaine and unlike flecainide (21, 42), dantrolene increased SR Ca\textsuperscript{2+} content associated with Ca\textsuperscript{2+} waves (Fig. 6) (31), indicative of an increase in the intra-SR Ca\textsuperscript{2+} threshold for Ca\textsuperscript{2+} wave generation (38). However, contrasting with both tetracaine and flecainide, dantrolene affects Ca\textsuperscript{2+} sparks only when Ca\textsuperscript{2+} spark...
activity is excessive (i.e., only in aged mice, Fig. 5D). These data support a unique mode of action, consistent with evidence that dantrolene stabilizes aberrant interdomain interactions within the RyR with subsequent restoration of normal channel function (31).

In conclusion, we find that aged mouse cardiomyocytes are sensitive to dantrolene which exerts beneficial effects by attenuating proarrhythmic spontaneous diastolic Ca\(^{2+}\) sparks, Ca\(^{2+}\) waves, and aftercontractions. Dantrolene selectively altered diastolic modes of Ca\(^{2+}\) release without effects on Ca\(^{2+}\) transients or cell shortening in response to ECC. Dantrolene may thus represent a therapeutic strategy for cardiac dysfunction observed with advancing age.

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AUTHOR CONTRIBUTIONS


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