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Reduced threshold for store overload-induced Ca^{2+} release is a common defect of RyR1 mutations associated with malignant hyperthermia and central core disease

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Abstract

Mutations in the skeletal muscle ryanodine receptor (RyR1) cause malignant hyperthermia (MH) and central core disease (CCD), whereas mutations in the cardiac ryanodine receptor (RyR2) lead to catecholaminergic polymorphic ventricular tachycardia (CPVT). Most disease-associated RyR1 and RyR2 mutations are located in the N-terminal, central, and C-terminal regions of the corresponding ryanodine receptor (RyR) isoform. An increasing body of evidence demonstrates that CPVT-associated RyR2 mutations enhance the propensity for spontaneous Ca^{2+} release during store Ca^{2+} overload, a process known as store overload-induced Ca^{2+} release (SOICR). Considering the similar locations of disease-associated RyR1 and RyR2 mutations in the RyR structure, we hypothesize that like CPVT-associated RyR2 mutations, MH/CCD-associated RyR1 mutations also enhance SOICR. To test this hypothesis, we determined the impact on SOICR of 12 MH/CCD-associated RyR1 mutations E2347-del, R2163H, G2434R, R2435L, R2435H, and R2454H located in the central region, and Y4796C, T4826I, L4838V, A4940T, G4943V, and P4973L located in the C-terminal region of the channel. We found that all these RyR1 mutations reduced the threshold for SOICR. Dantrolene, an acute treatment for MH, suppressed SOICR in HEK293 cells expressing the RyR1 mutants R164C, Y523S, R2136H, R2435H, and Y4796C. Interestingly, carvedilol, a commonly used β -blocker that suppresses RyR2-mediated SOICR, also inhibits SOICR in these RyR1 mutant HEK293 cells. Therefore, these results indicate that a reduced SOICR threshold is a common defect of MH/CCD-associated RyR1 mutations, and that

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Author Contribution

W.C., A.K., Y.L., R.W., D.H.M., R.T.D., and S.R.W.C. designed the research. W.C., A.K., Y.L., W.G., J.W., and R.W. performed the research. W.C., A.K., Y.L., W.G., J.W., R.W., and S.R.W.C. analyzed data. W.C., R.W., R.T.D., and S.R.W.C. wrote the paper.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

carvedilol, like dantrolene, can suppress RyR1-mediated SOICR. Clinical studies of the effectiveness of carvedilol as a long-term treatment for MH/CCD or other RyR1-associated disorders may be warranted.

Introduction

Ryanodine receptors (RyRs) are a family of intracellular Ca^{2+} release channels essential for a variety of physiological processes, such as muscle contraction and Ca^{2+} signaling [1–3]. RyRs also play a pivotal role in the pathophysiology of many muscle diseases. Naturally occurring mutations in the skeletal muscle RyR (RyR1) are associated with malignant hyperthermia (MH) and central core disease (CCD) [4–7], whereas naturally occurring mutations in the cardiac RyR (RyR2) are linked to catecholaminergic polymorphic ventricular tachycardia (CPVT) [8,9]. To date, a large number of disease-associated RyR1 and RyR2 mutations have been identified. Interestingly, most of these RyR1 and RyR2 mutations are located within three mutation ‘hot spots’ in the linear sequence of the corresponding channel isoforms: the N-terminal, central, and C-terminal regions [6,8–11]. This similarity in mutation location and distribution suggests that disease-associated RyR1 and RyR2 mutations are likely to possess similar molecular defects and affect the same intrinsic properties of the channel, although they cause different muscle disorders.

Recent studies on the mechanisms of RyR2-associated CPVT have provided some important insights into the molecular defects that may be common not only to RyR2 mutations, but also to RyR1 mutations. It is now well established that CPVT is caused by delayed afterdepolarizations (DADs) [9,12,13], which are triggered by spontaneous Ca^{2+} release from the sarcoplasmic reticulum (SR) under conditions of SR Ca^{2+} overload [14–16]. This SR Ca^{2+} overload-induced spontaneous Ca^{2+} release, also known as store overload-induced Ca^{2+} release (SOICR), is governed by the RyR2 channel [9,17,18]. We recently reported that many disease-associated RyR2 mutations located in different regions of the channel enhance the propensity for SOICR, suggesting that enhanced SOICR is a common defect of RyR2 mutations associated with CPVT [9,12,17–23]. Given their similar locations in the RyR structure, it is reasonable to propose that MH/CCD-associated RyR1 mutations, like CPVT-associated RyR2 mutations, may also enhance the propensity for spontaneous SR Ca^{2+} release or SOICR. Consistent with this hypothesis, spontaneous muscle contracture as a consequence of spontaneous SR Ca^{2+} release in skeletal muscle has long been recognized as a cause of porcine MH that is linked to a single RyR1 mutation R615C [24–28]. Furthermore, we previously demonstrated that this porcine MH mutation (R615C) enhances RyR1-mediated SOICR by reducing the SOICR threshold [29]. Importantly, MH-triggering agents (halothane and caffeine) also reduce the threshold for SOICR, whereas dantrolene, an effective treatment for MH, suppresses RyR1-mediated SOICR [29]. These observations led us to propose that the porcine MH RyR1 mutation confers MH susceptibility by reducing the threshold for SOICR, and that halothane and caffeine trigger MH by further reducing the already lowered SOICR threshold, leading to SOICR and spontaneous muscle contracture [29].

Unlike one single porcine MH RyR1 mutation, there are hundreds of MH-associated human RyR1 mutations located in different regions of the channel [5,6,11,30]. An important unresolved question is whether enhanced SOICR is a common feature of human MH RyR1 mutations. To address this question, in the present study, we systematically determined the impact of 12 human MH/CCD RyR1 mutations located in different regions of the channel on SOICR following stable, inducible expression in HEK293 cells. Our data indicate that a reduced threshold for SOICR is a common defect of MH/CCD-associated RyR1 mutations. Furthermore, we demonstrate for the first time that like dantrolene (an acute treatment for MH), carvedilol, a commonly used β -blocker that inhibits RyR2-mediated SOICR [31], is also capable of suppressing enhanced SOICR resulting from MH/CCD-associated mutations in RyR1. These findings suggest that carvedilol may potentially be used as a long-term treatment for MH/CCD and other muscle disorders associated with enhanced RyR1-mediated SOICR.

Experimental procedures

Materials

Restriction endonucleases and DNA-modifying enzymes were purchased from New England BioLabs, Inc. (Ipswich, MA). Soybean phosphatidylcholine was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate and other reagents were purchased from Sigma (St Louis, MO).

Site-directed mutagenesis

Point mutations in the rabbit RyR1 were generated by using the overlap extension method with PCR [32]. Briefly, an HpaI/BsiwI fragment containing the R2454H mutation was generated by overlapping PCR. This fragment was then used to replace the corresponding wild-type (WT) fragment in the full-length rabbit RyR1 cDNA in pcDNA5. A ClaI/XbaI fragment containing the Y4796C, T4826I, L4838V, A4940T, G4943V, or P4973L mutation was obtained by overlapping PCR. This fragment was then used to replace the corresponding WT fragment in the full-length rabbit RyR1 cDNA in pcDNA5. All point mutations and deletions were confirmed by DNA sequencing. The construction of point mutations R164C, Y523S, R2163H, E2347-del, G2434R, R2435L, and R2435H has been described previously [33–36].

Generation of stable, inducible HEK293 cell lines and cell culture

Stable, inducible HEK293 cell lines expressing RyR1 WT and mutants R164C, Y523S, R2163H, E2347-del, G2434R, R2435L, R2435H, R2454H, Y4796C, T4826I, L4838V, A4940T, G4943V, and P4973L were generated using the Flp-In T-REx Core Kit from Invitrogen [17,18]. Briefly, Flp-In T-REx HEK293 cells were co-transfected with the inducible expression vector pcDNA5/FRT/TO containing the RyR1 mutant cDNA and the pOG44 vector encoding the Flp recombinase in 1 : 5 ratios using the Ca^{2+} phosphate precipitation method. The transfected cells were washed with PBS (phosphate-buffered saline, 137 mM NaCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , and 2.7 mM KCl, pH 7.4) 24 h after transfection followed by a change into fresh medium for 24 h. The cells were then washed again with PBS, harvested, and plated onto new dishes. After the cells had attached

(~4 h), the growth medium was replaced with a selection medium containing 200 µg/ml hygromycin (Invitrogen). The selection medium was changed every 3–4 days until the desired number of cells was grown. The hygromycin-resistant cells were pooled, aliquoted (1 ml), and stored at –80°C. These positive cells are believed to be isogenic, because the integration of the RyR1 cDNA is mediated by the Flp recombinase at a single FRT site. HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 0.1 mM minimum Eagle's medium nonessential amino acids, 4 mM L-glutamine, 100 units of penicillin/ml, 100 mg of streptomycin/ml, 4.5 g of glucose/l, and 10% fetal calf serum at 37°C under 5% CO₂.

Single-cell Ca²⁺ imaging (luminal Ca²⁺ measurements)

Endoplasmic reticulum (ER) luminal Ca²⁺ levels in HEK293 cells expressing RyR1 WT or mutants were measured using single-cell Ca²⁺ imaging and the FRET (fluorescence resonance energy transfer)-based ER luminal Ca²⁺-sensitive cameleon protein D1ER as described previously [20,37]. The cells were grown to 95% confluence in a 75 cm² flask, dissociated with PBS, and plated in 100-mm-diameter tissue culture dishes at ~10% confluence 18–20 h before transfection with the D1ER cDNA using the Ca²⁺ phosphate precipitation method. After transfection for 24 h, the growth medium was then changed to an induction medium containing 1 µg/ml tetracycline. After induction for ~22 h, the cells were perfused continuously with KRH buffer [125 mM NaCl, 5 mM KCl, 6 mM glucose, 1.2 mM MgCl₂, and 25 mM HEPES (pH 7.4)] containing 2 mM caffeine and increasing concentrations of CaCl₂ (0, 0.2, 0.5, 1.0, 2.0, and 5.0 mM) or 5.0 mM CaCl₂ plus tetracaine (1 mM) for estimating the store capacity or 5.0 mM CaCl₂ plus caffeine (20 mM) for estimating the minimum store level by depleting the ER Ca²⁺ stores at room temperature (23°C). Images were captured with the Compix Simple PCI 6 software every 2 s using an inverted microscope (Nikon TE2000-S) equipped with an S-Fluor 20×/0.75 objective. The filters used for D1ER imaging were $\lambda_{\text{ex}} = 436 \pm 20$ nm for CFP and $\lambda_{\text{ex}} = 500 \pm 20$ nm for YFP, and $\lambda_{\text{em}} = 465 \pm 30$ nm for CFP and $\lambda_{\text{em}} = 535 \pm 30$ nm for YFP with a dichroic mirror (500 nm). The amount of FRET was determined from the ratio of the light emission at 535 and 465 nm.

Single-cell Ca²⁺ imaging (cytosolic Ca²⁺ measurements)

Cytosolic Ca²⁺ levels in stable, inducible HEK293 cells expressing RyR1 WT or mutants (R164C and Y523S) were monitored using single-cell Ca²⁺ imaging and the fluorescent Ca²⁺ indicator dye Fura-2 AM as described previously [17,18]. Briefly, cells grown on glass coverslips for 18 h after induction by 1 µg/ml tetracycline were loaded with 5 µM Fura-2, AM in KRH buffer plus 0.02% pluronic F-127, and 0.1 mg/ml BSA for 20 min at room temperature (23°C). The coverslips were then mounted in a perfusion chamber (Warner Instruments) on an inverted microscope (Nikon TE2000-S). The cells were perfused continuously with KRH buffer containing 2 mM caffeine and increasing concentrations of CaCl₂ (0, 0.2, 0.5, 1.0, 2.0, and 5.0 mM). Caffeine (10 mM) was applied at the end of each experiment to confirm the expression of active RyR2 channels. To determine the effect of dantrolene or carvedilol on SOICR, RyR1 mutant cells were perfused continuously with KRH buffer containing 2 mM caffeine and 5 mM Ca²⁺, followed by the addition of dantrolene (100 nM) or carvedilol (30 µM). The dantrolene- or carvedilol-treated cells were

then perfused with caffeine (20 mM). Time-lapse images (0.25 frame/s) were captured and analyzed with the Compix Simple PCI 6 software. Fluorescence intensities were measured from regions of interest centered on individual cells. Only cells that responded to caffeine were analyzed. The filters used for Fura-2 imaging were $\lambda_{\text{ex}} = 340 \pm 26$ and 387 ± 11 nm and $\lambda_{\text{em}} = 510 \pm 84$ nm with a dichroic mirror (410 nm).

Statistical analysis

All values shown are mean $\pm$ SEM unless indicated otherwise. To test for differences between groups, we used Student's *t*-test (two-tailed) or one-way ANOVA with a *post hoc* test. A value of $P < 0.05$ was considered to be statistically significant.

Results

MH/CCD RyR1 mutations located in different regions of the channel reduce the threshold for SOICR

We previously reported that the N-terminal MH RyR1 mutation R615C markedly reduced the threshold for SOICR [29]. To determine whether the reduced SOICR threshold is a common defect of MH/CCD RyR1 mutations located in other regions of the channel, we generated stable, inducible HEK293 cell lines expressing RyR1 mutations Del-E2347, R2163H, G2434R, R2435L, R2435H, and R2454H located in the central region, and Y4796C, T4826I, L4838V, A4940T, G4943V, and P4973L located in the C-terminal region of the channel (Figure 1). Each of these MH/CCD RyR1 mutations is located in close proximity to one or more CPVT RyR2 mutations in the three-dimensional (3D) structure of RyR (Figure 1). To assess the impact of these RyR1 mutations on SOICR, we determined the luminal Ca^{2+} threshold at which SOICR occurs using an FRET-based ER Ca^{2+} sensor protein, D1ER [20,37]. SOICR in HEK293 cells expressing RyR1 WT or mutants was induced by elevating $[\text{Ca}^{2+}]_o$ in the presence of 2 mM caffeine to sensitize the RyR1 channel to SOICR [29]. The ER luminal Ca^{2+} dynamics during store Ca^{2+} overload were continuously monitored using single-cell FRET imaging. As shown in Figure 2, the ER luminal Ca^{2+} level in HEK293 cells expressing RyR1 WT gradually increased upon the elevation of $[\text{Ca}^{2+}]_o$ (Figure 2A). When the luminal Ca^{2+} reached a threshold level (F_{SOICR}), SOICR occurred, leading to Ca^{2+} release and a sudden decrease in ER luminal Ca^{2+} level as reflected by the downward deflections in the FRET signal (Figure 2A). To estimate the maximum attainable luminal Ca^{2+} level (F_{max}) in these cells, SOICR was inhibited by the addition of 1.0 mM tetracaine, an inhibitor of RyR. Caffeine (20 mM) was then used to empty the RyR-gated Ca^{2+} stores to estimate the minimum luminal Ca^{2+} level (F_{min}). The SOICR threshold was then determined by calculating the % of maximum luminal Ca^{2+} store capacity at which SOICR occurs based on the formula $[(F_{\text{SOICR}} - F_{\text{min}})/(F_{\text{max}} - F_{\text{min}})] \times 100\%$. Using this method, we found that HEK293 cells expressing RyR1 WT displayed an SOICR threshold of $95 \pm 1.5\%$ ($n = 4$). The luminal Ca^{2+} dynamics during store Ca^{2+} overload in cells expressing the central region RyR1 mutations, Del-E2347, R2163H, G2434R, R2435L, R2435H, and R2454H, are shown in Figure 2B–G. The luminal Ca^{2+} level at which SOICR occurred (the SOICR threshold) in each of these mutant cells was significantly reduced compared with that in WT cells (Figure 1H; $P < 0.05$). Figure 3 shows the luminal Ca^{2+} dynamics during store Ca^{2+} overload in cells expressing the C-terminal

RyR1 mutations, Y4796C, T4826I, L4838V, A4940T, G4943V, and P4973L. Similarly, we found that the SOICR threshold in HEK293 cells expressing each of these C-terminal RyR1 mutations was significantly reduced compared with that in cells expressing RyR1-WT (Figure 3G; $P < 0.05$). Therefore, these data demonstrate that a reduced SOICR threshold is a common defect of MH/CCD RyR1 mutations located in the central and C-terminal regions of the channel.

Dantrolene and carvedilol suppress SOICR in HEK293 cells expressing the central and C-terminal RyR1 mutations

We previously found that dantrolene effectively suppresses SOICR in HEK293 cells expressing the N-terminal MH RyR1 mutation R615C [29]. To determine whether dantrolene also suppresses SOICR in cells expressing the central and C-terminal RyR1 mutations, we assessed the impact of dantrolene on SOICR in HEK293 cells expressing the RyR1 mutations R2163H and R2435H located in the central region, and Y4796C located in the C-terminal region. As shown in Figure 4, HEK293 cells expressing these RyR1 mutations exhibited Ca^{2+} oscillations in the presence of 5 mM $[\text{Ca}^{2+}]_o$ and 2.0 mM caffeine (Figure 4A–D). Importantly, dantrolene (100 nM) markedly suppressed these Ca^{2+} oscillations, significantly reducing the fraction of HEK293 cells that displayed Ca^{2+} oscillations (Figure 4E). These data demonstrate that dantrolene is a potent suppressor of SOICR mediated by MH/CCD RyR1 mutants located in the central and C-terminal regions of the channel.

We recently reported that carvedilol, a nonselective beta-blocker, effectively inhibits SOICR in HEK293 cells and cardiac cells expressing the disease-causing RyR2 R4496C mutation, and suppresses stress-induced, SOICR-evoked ventricular tachyarrhythmias in RyR2 R4496C knock-in mice [31]. We also demonstrated that carvedilol suppresses RyR2-mediated SOICR by directly interacting with the RyR2 channel and reducing the open duration of RyR2 independent of carvedilol's β - and α -blocking and antioxidant activities [31]. To test if carvedilol is also effective in suppressing RyR1-mediated SOICR, we determined the impact of carvedilol on enhanced SOICR in HEK293 cells expressing MH/CCD-associated RyR1 mutants (R2163H and R2435H located in the central region, and Y4796C located in the C-terminal region). As shown in Figure 5, carvedilol (30 μM) substantially inhibited SOICR in these mutant cells ($P < 0.05$). Taken together, these observations indicate that carvedilol is capable of inhibiting both RyR1- and RyR2-mediated SOICR, and suggest that carvedilol, an effective treatment for heart failure and cardiac arrhythmias, may represent a potential new treatment for MH/CCD.

Dantrolene and carvedilol inhibit SOICR in HEK293 cells expressing leaky N-terminal RyR1 mutations

The N-terminal RyR1 mutations R164C and Y523S have been shown to cause severe SR Ca^{2+} leak, leading to significant Ca^{2+} store depletion, and elevated resting cytosolic Ca^{2+} levels [35,38–41]. To determine whether dantrolene or carvedilol suppresses enhanced RyR1 Ca^{2+} leak caused by these mutations, we generated stable, inducible HEK293 cell lines expressing the R164C and Y523S mutants. As shown previously, increasing $[\text{Ca}^{2+}]_o$ triggered Ca^{2+} oscillations in HEK293 cells expressing RyR1 WT (Figure 6A). However,

unlike the Ca^{2+} oscillations in RyR1 WT-expressing cells, elevating $[\text{Ca}^{2+}]_o$ led to a stepwise, sustained increase in cytosolic Ca^{2+} in R164C- and Y523S-expressing cells (Figure 6B,C). The sustained increase in cytosolic Ca^{2+} upon elevation of extracellular Ca^{2+} in these RyR1 mutant cells probably resulted from excessive RyR1 Ca^{2+} leak, leading to store Ca^{2+} depletion and activation of store-operated Ca^{2+} entry that exceeds the capacity of Ca^{2+} removal. Thus, these N-terminal RyR1 mutations greatly enhance spontaneous Ca^{2+} release that leads to store depletion, consistent with that reported previously in muscle cells [35,38–41]. Importantly, both dantrolene (100 nM) and carvedilol (30 μM) suppressed spontaneous Ca^{2+} release in R164C- and Y523S-expressing cells (Figure 7). Thus, dantrolene and carvedilol are also effective in suppressing spontaneous Ca^{2+} leak resulting from MH/CCD RyR1 mutant channels.

Discussion

Uncontrolled skeletal muscle contraction is the most salient feature during an MH episode. It has long been proposed that this uncontrolled muscle contraction is caused by spontaneous Ca^{2+} release from the SR [24–28]. However, the mechanism by which MH/CCD-associated RyR1 mutations enhance the propensity for spontaneous SR Ca^{2+} release is not well understood. An important clue to the understanding of the impact of MH/CCD RyR1 mutations comes from studies of the mechanism of CPVT-associated RyR2 mutations. Like MH, CPVT is also caused by spontaneous SR Ca^{2+} release. Functional studies demonstrated that CPVT-associated RyR2 mutations enhance the propensity for spontaneous SR Ca^{2+} release during SR Ca^{2+} overload (or SOICR) by reducing the threshold for SOICR. The resultant increase in SOICR activity can in turn cause DAD, triggered activity, ventricular tachyarrhythmias, and sudden death [9]. Interestingly, MH/CCD-associated RyR1 mutations and CPVT-associated RyR2 mutations are largely located in the same domains in the corresponding 3D structure of RyR (Figure 1). The similarity of their 3D locations suggests that MH/CCD-associated RyR1 and CPVT-associated RyR2 mutations may alter the same functional properties of the RyR channel [6,9,11]. Indeed, in the present study, we found that like CPVT-associated RyR2 mutations, MH/CCD-associated RyR1 mutations located in different regions of the channel reduce the threshold for SOICR. Thus, a reduced threshold for SOICR is a common defect of both MH/CCD-associated RyR1 and CPVT-associated RyR2 mutations [6,9,11]. A reduced SOICR threshold would expect to lead to enhanced spontaneous Ca^{2+} release, increased cytosolic Ca^{2+} , or decreased store Ca^{2+} level. Indeed, some human MH/CCD RyR1 mutations have been shown to increase spontaneous Ca^{2+} release, elevate resting cytosolic Ca^{2+} concentrations, or decrease resting Ca^{2+} store content [42].

An important question then is how a reduced SOICR threshold predisposes individuals with the RyR1 mutation to MH. MH is a pharmacogenetic disorder triggered by volatile anesthetics, depolarizing muscle relaxants, or stress [6,11]. It has been shown that volatile anesthetics such as halothane and RyR activators such as caffeine potentiate spontaneous SR Ca^{2+} release by reducing the threshold for SOICR [29]. Since MH/CCD-associated RyR1 mutations also reduce the threshold for SOICR, individuals harboring MH/CCD-associated RyR1 mutations would be more sensitive to volatile anesthetics- and caffeine-induced SOICR compared with normal individuals. Therefore, our findings support a model in which

volatile anesthetics trigger spontaneous SR Ca^{2+} release, uncontrolled muscle contraction, and MH in individuals with RyR1 mutations by further lowering an already-reduced SOICR threshold.

Another important question is how dantrolene suppresses MH. It has long been shown that dantrolene markedly suppresses halothane- or caffeine-induced muscle contraction [43,44]. We previously showed that dantrolene (100 nM) effectively suppresses SOICR in HEK293 cells expressing the porcine MH mutation R615C [29]. Here, we found that dantrolene (100 nM) also substantially suppresses SOICR in HEK293 cells expressing MH/CCD RyR1 mutations located in the central and C-terminal regions. Consistent with these observations, azumolene, a dantrolene analog, decreases the frequency of spontaneous Ca^{2+} sparks in muscle fibers [45]. Dantrolene at nanomolar concentrations has also been shown to directly inhibit the RyR1 channel, and this dantrolene inhibition of RyR1 channel activity requires calmoduline [46–48]. Dantrolene (up to 50 μM) also partially suppressed voltage- or Ca^{2+} -induced SR Ca^{2+} release [46,49–51]. Since MH is caused by spontaneous SR Ca^{2+} release, the inhibitory action of dantrolene on spontaneous SR Ca^{2+} release or SOICR probably accounts for its remarkable suppression of MH. The effect of dantrolene on voltage-induced SR Ca^{2+} release may also contribute to its therapeutic benefits.

Given the high degree of sequence similarity between RyR1 and RyR2, one may expect that agents that suppress RyR1-mediated SOICR and SOICR-evoked MH may also be able to suppress RyR2-mediated SOICR and SOICR-evoked CPVT. Indeed, it has been shown that dantrolene can also suppress RyR2-mediated spontaneous Ca^{2+} release in cardiomyocytes from diseased or aged hearts [52–55]. Dantrolene has also been shown to suppress cardiac arrhythmias [56–60]. On the other hand, one could also expect that agents that suppress RyR2-mediated SOICR may also inhibit RyR1-mediated SOICR. We recently reported that carvedilol, a β -blocker of choice for the treatment of heart failure and cardiac arrhythmias, effectively suppresses RyR2-mediated SOICR and SOICR-evoked CPVT [31]. We also showed that carvedilol suppresses RyR2-mediated SOICR by reducing the mean open time of single RyR2 channels independent of carvedilol's β - and α -blocking and antioxidant activities [31]. In the present study, we found that carvedilol also suppresses SOICR in HEK293 cells expressing MH/CCD-associated RyR1 mutations located in different regions (N-terminal, central, and C-terminal regions) of the channel.

The molecular mechanisms by which dantrolene and carvedilol suppress SOICR are unknown. We have previously shown that residue E4872 located in the helix bundle-crossing region of the RyR2 channel is critical for luminal Ca^{2+} activation and the occurrence of SOICR [61]. Comparison of the 3D structures of RyR2 in the closed and open states reveals that residue E4872 is involved in the formation of salt bridges with R4874 in the closed state. However, in the open state, R4874 moves away from E4872, and E4872 and E4878 may be involved in the formation of a putative luminal Ca^{2+} -binding pocket [62]. In other words, it appears that the luminal Ca^{2+} activation site is only formed in the open state. This open state-dependent formation of a putative luminal Ca^{2+} -binding pocket suggests that luminal Ca^{2+} would enhance the RyR channel activity by stabilizing the open state. Accordingly, disease-causing RyR mutations that stabilize the open state would enhance luminal Ca^{2+} activation of the channel and SOICR. On the other hand, agents such as

dantrolene or carvedilol that stabilize the closed state (or destabilize the open state) would suppress luminal Ca^{2+} activation and SOICR. Hence, we propose that dantrolene or carvedilol suppresses the open state-dependent luminal Ca^{2+} activation and SOICR by stabilizing the closed state of the channel.

Currently, dantrolene is the only acute treatment for MH. Considering its toxicity to liver and kidney function [63], dantrolene is not commonly used as a long-term treatment for MH/CCD. On the other hand, carvedilol is commonly used as a long-term treatment for heart failure and cardiac arrhythmias. Our novel finding that, like dantrolene, carvedilol also suppresses the enhanced RyR1-mediated SOICR suggests that carvedilol may represent a potential new therapeutic agent for long-term treatment of MH/CCD and other RyR1-associated disorders, assuming that it exhibits similar action in adult skeletal muscle.

HEK293 cells have been widely used to study the impact of disease-associated RyR1 and RyR2 mutations on the intrinsic properties of the RyR channel. These studies have revealed important insights into the molecular defects of RyR mutations. However, HEK293 cells lack many muscle-specific proteins, such as the L-type Ca^{2+} channels, calsequestrin, triadin, and junctin. Thus, whether the intrinsic defects in RyR function of all the MH/CCD mutations observed in HEK293 cells will be manifested in skeletal muscle cells has yet to be determined. Similarly, although carvedilol at 30 μM is capable of diminishing SOICR in HEK293 cells expressing RyR1 MH/CCD mutants, whether carvedilol at clinically relevant concentrations is capable of suppressing SOICR in skeletal muscle cells and whether carvedilol is effective in suppressing MH in animal models or MH/CCD patients have yet to be investigated. Future studies using patient-specific, induced pluripotent stem cell-derived skeletal muscle cells or mouse models harboring some of these RyR1 mutations will be needed to directly address these questions. Encouragingly, consistent with our findings observed in HEK293 cells that MH/CCD RyR1 mutations reduce the threshold for SOICR and thus the propensity for spontaneous Ca^{2+} release, increased incidence of spontaneous Ca^{2+} release has also been observed in dyspedic myotubes expressing MH/CCD-RyR1 mutants [42]. Therefore, the HEK293 cell expression system represents an appropriate and valuable experimental approach to investigating the intrinsic defects of disease-associated RyR mutations.

In summary, we demonstrate that MH/CCD-associated RyR1 mutations located in different regions of the channel reduce the threshold for spontaneous Ca^{2+} release during store Ca^{2+} overload (SOICR). The enhanced SOICR activity in MH/CCD RyR1 mutant-expressing cells can be suppressed by both dantrolene, an acute treatment for MH, and carvedilol, a commonly used β -blocker. Thus, carvedilol may represent a potential new therapeutics for the long-term treatment of MH/CCD and RyR1-associated disease.

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Abbreviations

3D	three-dimensional
CCD	central core disease
CFP/YFP	cyan/yellow fluorescent protein
CPVT	catecholaminergic polymorphic ventricular tachycardia
DADs	delayed afterdepolarizations
ER	endoplasmic reticulum
FRET	fluorescence resonance energy transfer
FRT	flippase recognition target
KRH	Kreb-Ringer-HEPES
MH	malignant hyperthermia
PBS	phosphate-buffered saline
RyR1	skeletal muscle ryanodine receptor
RyR2	cardiac ryanodine receptor
RyRs	Ryanodine receptors
SOICR	store overload-induced Ca^{2+} release
SR	sarcoplasmic reticulum
WT	wild type

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Figure 1. Co-localization of disease-associated RyR1 and RyR2 mutations in the 3D structure of RyR

The 3D structure of two RyR monomers [62,64,65] is shown. RyR1 mutations associated with MH and CCD are largely clustered in three hotspot regions: MH/CCD mutation hotspot I (highlighted in yellow), MH/CCD mutation hotspot II (in green), and MH/CCD mutation hotspot III (in cyan). MH/CCD-associated RyR1 mutations tested in the present study are highlighted in red/magenta. RyR2 mutations associated with CPVT are also clustered in these three hotspot regions. In addition to these three mutation hotspots, RyR2 also harbors another disease mutation hotspot between residues 3778–4201 (highlighted in violet) [6,9]. CPVT-associated RyR2 mutations that are co-localized with the MH/CCD RyR1 mutations tested in the present study are highlighted in black. CPVT RyR2 mutations that coincide with the MH/CCD RyR1 mutations (R164C, R2435L/H, R2454H, and P4973L) are highlighted in magenta.

Figure 2. MH/CCD-associated RyR1 mutations located in the central region of the channel reduce the threshold for SOICR

Stable, inducible HEK293 cell lines expressing RyR1 WT (A), E2347-del (B), R2163H (C), G2434R (D), R2435L (E), R2435H (F), and R2454H (G) mutants were transfected with the FRET-based ER luminal Ca^{2+} -sensing protein D1ER for 48 h. The expression of RyR1 WT and mutants was induced by tetracycline 24 h before imaging. The cells were perfused with KRH buffer containing caffeine (2 mM) and increasing levels of extracellular Ca^{2+} (0–5 mM) to induce SOICR. This was followed by the addition of 1 mM tetracaine to inhibit SOICR and then 20 mM caffeine to deplete the ER Ca^{2+} store. FRET recordings from representative RyR1 WT (A) and mutant (B–G) cells (from a total of 15–91 cells) are shown. To minimize the influence by CFP/YFP cross-talk, we used relative FRET measurements for calculating the SOICR threshold (H), which was determined by the equation $((F_{\text{SOICR}} - F_{\text{min}})/(F_{\text{max}} - F_{\text{min}})) \times 100\%$. F_{SOICR} indicates the FRET level at which SOICR occurs. The maximum FRET signal F_{max} is defined as the FRET level after tetracaine treatment. The minimum FRET signal F_{min} is defined as the FRET level after caffeine treatment. Data shown are mean $\pm$ SEM from three to five separate experiments (* $P < 0.05$ vs. WT).

Figure 3. MH/CCD RyR1 mutations located in the C-terminal region reduce the SOICR threshold

Stable, inducible HEK293 cell lines expressing RyR1 Y4796C (A), T4826I (B), L4838V (C), A4940T (D), G4943V (E), and P4973L (F) mutants were transfected with the FRET-based ER luminal Ca²⁺-sensing protein D1ER for 48 h. The expression of RyR1 mutants was induced by tetracycline 24 h before imaging. The cells were perfused with KRH buffer containing caffeine (2 mM) and increasing levels of extracellular Ca²⁺ (0–5 mM) to induce SOICR. This was followed by the addition of 1 mM tetracaine to inhibit SOICR and then 20 mM caffeine to deplete the ER Ca²⁺ store. FRET recordings from representative RyR1 mutant (A–F) cells (from a total of 31–138 cells) are shown. (G) The SOICR threshold was determined by the equation $((F_{\text{SOICR}} - F_{\text{min}})/(F_{\text{max}} - F_{\text{min}})) \times 100\%$. F_{SOICR} indicates the FRET level at which SOICR occurs. The maximum FRET signal F_{max} is defined as the FRET level after tetracaine treatment. The minimum FRET signal F_{min} is defined as the FRET level after caffeine treatment. Data shown are mean $\pm$ SEM from three to five separate experiments (* $P < 0.05$ vs. WT).

Figure 4. Dantrolene suppresses SOICR in HEK293 cells expressing the central and C-terminal region MH/CCD RyR1 mutations

Stable, inducible HEK293 cells expressing the RyR1 R2136H (A), R2435H (B), or Y4796C (C) mutant were loaded with Fura-2, AM and perfused with KRH buffer containing 5 mM $[Ca^{2+}]_o$ plus 2 mM caffeine in the absence or the presence of 100 nM dantrolene. (D) The fraction of R2136H, R2435H, or Y4796C mutant cells that displayed Ca^{2+} oscillations before (control) and after the addition of 100 nM dantrolene (from a total of 39–87 cells). Data shown are mean $\pm$ SEM from three separate experiments (* $P < 0.05$ vs. control).

Figure 5. Carvedilol suppresses RyR1-mediated SOICR in HEK293 cells

Stable, inducible HEK293 cells expressing the RyR1 R2136H (A), R2435H (B), or Y4796C (C) mutant were loaded with Fura-2, AM and perfused with KRH buffer containing 5 mM [Ca²⁺]_o plus 2 mM caffeine in the absence or the presence of 30 μM carvedilol. (D) The fraction of R2136H, R2435H, or Y4796C mutant cells that displayed Ca²⁺ oscillations before (control) and after the addition of 30 μM carvedilol (from a total of 51–94 cells). Data shown are mean ± SEM from three separate experiments (**P* < 0.05 vs. control).

Figure 6. Sustained cytosolic Ca^{2+} elevation in HEK293 cells expressing leaky MH/CCD RyR1 mutations R164C and Y523S

Stable, inducible HEK293 cells expressing RyR1 WT (A) and R164C (B), and Y523S (C) mutants were loaded with 5 μM Fura-2, AM in KRH buffer. The cells were then perfused continuously with KRH buffer containing increasing levels of extracellular Ca^{2+} (0–5 mM) plus 2 mM caffeine to induce SOICR. Fura-2 ratios were recorded using epifluorescence single-cell Ca^{2+} imaging. Traces of Fura-2 ratios of representative RyR1 WT, R164C, and Y523S cells are shown.

Figure 7. Dantrolene and carvedilol inhibit R164C- and Y523S-mediated spontaneous Ca^{2+} leak Stable, inducible HEK293 cells expressing the RyR1 R164C (A and C) or Y523S (B and D) mutant were loaded with Fura-2, AM and perfused with KRH buffer containing 5 mM $[Ca^{2+}]_o$ plus 2 mM caffeine in the absence or the presence of 100 nM dantrolene (A and B) or 30 μ M carvedilol (C and D). The fraction of R164C or Y523S mutant cells that displayed Ca^{2+} leak before (control) and after the addition of 100 nM dantrolene (E) or 30 μ M carvedilol (F) (from a total of 314–417 cells). Data shown are mean $\pm$ SEM from three separate experiments (* P < 0.05 vs. control).