Elevated resting $[\text{Ca}^{2+}]_i$ in myotubes expressing malignant hyperthermia RyR1 cDNAs is partially restored by modulation of passive calcium leak from the SR

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Yang T, Esteve E, Pessah IN, Molinski TF, Allen PD, López JR. Elevated resting $[\text{Ca}^{2+}]_i$ in myotubes expressing malignant hyperthermia RyR1 cDNAs is partially restored by modulation of passive calcium leak from the SR. Am J Physiol Cell Physiol 292: C1591–C1598, 2007. First published January 17, 2006; doi:10.1152/ajpcell.00133.2006.—Malignant hyperthermia (MH) is a pharmacogenetic disorder of skeletal muscle triggered in susceptible individuals by inhalation anesthetics and depolarizing skeletal muscle relaxants. This syndrome has been linked to a missense mutation in the type I ryanodine receptor (RyR1) in more than 50% of cases studied to date. Using double-barreled $\text{Ca}^{2+}$ microelectrodes in myotubes expressing wild-type RyR1 ($\text{WT}_{\text{RyR1}}$) or RyR1 with one of four common MH mutations ($\text{MH}_{\text{RyR1}}$), we measured resting intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$). Changes in resting $[\text{Ca}^{2+}]_i$ produced by several drugs known to modulate the RyR1 channel complex were investigated. We found that myotubes expressing any of the $\text{MH}_{\text{RyR1}}$ had a 2.0- to 3.7-fold higher resting $[\text{Ca}^{2+}]_i$ than those expressing $\text{WT}_{\text{RyR1}}$. Exposure of myotubes expressing $\text{MH}_{\text{RyR1}}$ to ryanodine (500 μM) or (2,6-dichloro-4-aminophenyl)isopropylamine (FLA 365; 20 μM) had no effects on their resting $[\text{Ca}^{2+}]_i$. However, when myotubes were exposed to bastadin 5 alone or to a combination of ryanodine and bastadin 5, the resting $[\text{Ca}^{2+}]_i$ was significantly reduced ($P < 0.01$). Interestingly, the percent decrease in resting $[\text{Ca}^{2+}]_i$ in myotubes expressing $\text{MH}_{\text{RyR1}}$ was significantly greater than that for $\text{WT}_{\text{RyR1}}$. From these data, we propose that the high resting myoplasmic $[\text{Ca}^{2+}]_i$ in $\text{MH}_{\text{RyR1}}$ expressing myotubes is due in part to a related structural conformation of $\text{MH}_{\text{RyR1}}$ that favors “passive” calcium leak from the sarcoplasmic reticulum.

Ryanodine; FLA 365; bastadin 5; resting intracellular calcium concentration; sarcoplasmic reticulum

MALIGNANT HYPERThERMIA (MH) is a potentially fatal pharmacogenetic syndrome caused by exposure to halogenated volatile anesthetics and/or depolarizing muscle relaxants. The pathophysiology of this syndrome is not yet fully understood. However, it is generally accepted that MH susceptibility (MHS) is caused by abnormal intracellular $\text{Ca}^{2+}$ homeostasis in skeletal muscle cells (33). The ryanodine receptor gene on chromosome 19q13.1 is the primary molecular locus for MHS in humans. More than 60 mutations of type 1 ryanodine receptor (RyR1) located in three hot spots (hot spot I, 35–615 aa; hot spot II, 2163–2458 aa; and hot spot III, 4643–4898 aa) have been linked to >50% of all MHS families (36). Numerous studies have confirmed that MHS muscles exhibit increased sensitivity to direct RyR1 agonists such as caffeine, halothane, and 4-chloro-m-cresol (4-CmC), and it is thought that this increased sensitivity is likely to be the primary underlying mechanism for triggering the clinical MH syndrome (33).

Intracellular $\text{Ca}^{2+}$ measurements obtained using $\text{Ca}^{2+}$-selective microelectrodes and fluorescent indicators have demonstrated that there is a significantly elevated resting intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) in MH adult muscle fibers possessing unidentified human MH mutations in swine expressing the Arg614Cys MH mutation (24, 25, 27) and in cultured cells expressing MH mutations (13, 51, 52). The chronically increased resting $[\text{Ca}^{2+}]_i$ in MHS muscle cells has been suggested to be associated with the increased sensitivity of these cells to caffeine and 4-CmC stimulation (26, 28). Thus these changes in resting $[\text{Ca}^{2+}]_i$ in MHS muscle cells and their possible contribution to the pathophysiology of MH represent another interesting aspect. On the other hand, other studies using the same fluorescent indicators in adult swine (14, 15) and dyspedic myotubes or human embryonic kidney (HEK) cells transfected with RyR1 cDNAs possessing some MH mutations (12, 48) have failed to measure such an elevation. Furthermore, the mechanistic relationship between $\text{MH}_{\text{RyR1}}$ mutations and the possible dysfunction of intracellular $\text{Ca}^{2+}$ homeostasis seen in MH-susceptible individuals with these mutations remain to be established.

In the current investigation, we have used double-barreled $\text{Ca}^{2+}$ microelectrodes to measure the resting myoplasmic $[\text{Ca}^{2+}]_i$ in myotubes that were transduced with herpes simplex virus (HSV) virions to express wild-type RyR1 ($\text{WT}_{\text{RyR1}}$) or one of four common MH mutations ($\text{MH}_{\text{RyR1}}$), including at least one from each of the three mutation hot spots. The new findings are as follows: 1) myotubes expressing any of the four MH mutations had a higher resting $[\text{Ca}^{2+}]_i$ than those expressing $\text{WT}_{\text{RyR1}}$; 2) blocking of $\text{MH}_{\text{RyR1}}$ with ryanodine or FLA 365 had little or no influence on resting $[\text{Ca}^{2+}]_i$ of myotubes; and 3) bastadin 5 alone or ryanodine and bastadin 5 together significantly lowered the resting $[\text{Ca}^{2+}]_i$ of myotubes expressing $\text{MH}_{\text{RyR1}}$, and this effect was more pro-

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nounced in myotubes expressing \( \textit{MH RyR1} \) than in myotubes expressing \( \textit{wR RyR1} \). On the basis of these data, we propose that the mechanism causing increased resting myoplasmic \([\text{Ca}^{2+}]_i\) in \( \textit{MH RyR1} \)-expressing myotubes is not the result of a height-ened gating activity of \( \textit{MH RyR1} \) release channels. Rather, the chronic increase in resting \([\text{Ca}^{2+}]_i\) results from a structural conformation of \( \textit{MH RyR1} \) that favors a constant “passive” \([\text{Ca}^{2+}]_i\) release from the SR that can be rescued by allosteric modula-tors of the RyR1 complex.

**MATERIALS AND METHODS**

**Construction and expression of \( \textit{MH RyR1} \) cDNAs.** A detailed explana-tion of construction and expression of the \( \textit{MH RyR1} \)s used in the current study has been previously described (53). With the use of a helper virus-free packaging system, all mutated and \( \textit{wR RyR1} \) cDNAs were packaged into HSV virions, which were then used to transduce 1B5 myotubes (49) at a multiplicity of infection (MOI) of 0.5 for 2 h and then cultured for 48 h before measurement of \([\text{Ca}^{2+}]_i\), at room temperature. As described previously (53), 1B5 cells (RyR1, RyR2, and RyR3 null) were cultured in growth medium (15% FBS in low-glucose DMEM; Gibco no. 11885-084) at \( 37^\circ\text{C} \) to pass-sages 5–10 and then plated on Matrigel (BD Bioscience, San Jose, CA)-coated plates (96-well plates; Opticel Costar 3614). Differentiation was started when cell density reached 20–30% confluence by changing growth medium to differentiation medium (0.2% horse serum in low-glucose DMEM). Typical multinuclear myotubes as shown in Fig. 1 were obtained by continued incubation of the cells in differentiation medium at \( 37^\circ\text{C} \) for 5–7 days.

**Preparation of bastadin 5.** The bromotyrosine derivative bastadin 5 was extracted and purified from the marine sponge \textit{Ianthella basta} as previously described (30, 40).

**Immunostaining.** Immunostaining for RyR1 expression was performed using monoclonal antibody 34C (Airey JA and Sutko JL, ISHB, University of Iowa, Iowa City, IA) that recognizes both RyR1 and RyR3, and Cy3-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA). To help identify the cells in which resting \([\text{Ca}^{2+}]_i\) was measured, pictures were taken with grid labels attached to the bottom of the plate before each experiment and then matched to pictures taken with the same grid after immunostaining.

\( \text{Ca}^{2+} \)-selective microelectrodes. Double-barreled \( \text{Ca}^{2+} \)-selective microelectrodes were prepared as described previously (28). They were backfilled with the neutral carrier ETI 129 (Fluka, Ronkonkoma, NY) and then with pCa 7. Each \( \text{Ca}^{2+} \)-selective micro-electrode was individually tested as described previously (23), and only those with a linear relationship between pCa at 24°C and a response >16–20 mV between pCa 7 and pCa 8 were used experimentally. All elec-trodes were then recalibrated after taking measurements of resting \([\text{Ca}^{2+}]_i\), and if the two calibration curves did not agree within 2.5 mV in the relevant range of the calibration curve (pCa 6–8), the data from that microelectrode was discarded. The calcium sensitivity of the \( \text{Ca}^{2+} \) microelectrodes was not affected by any of the drugs used in the present study, as determined by direct calibration.

**Intracellular \( \text{Ca}^{2+} \) measurements.** Single myotube impalements were observed through an inverted microscope (Zeiss Axiovert 10) fitted with a \( \times10 \) eyepiece and a \( \times40 \) dry objective. The potentials from the 3 M KCl barrel \( (V_{\text{m}}) \) and the \( \text{Ca}^{2+} \) barrel \( (V_{\text{Cae}}) \) were recorded via a high-impedance amplifier at \( >10^{11} \text{M} \Omega \) (model FD-223; WPI, Sarasota, FL). The potential of the voltage microelectrode \( (V_{\text{m}}) \) was subtracted electronically from the potential of the \( \text{Ca}^{2+} \) electrode \( (V_{\text{Cae}}) \) to give the differential signal \( (V_{\text{D}}) \) that represents the resting myoplasmic \([\text{Ca}^{2+}]_i\) concentration. \( V_{\text{m}} \) and \( V_{\text{Cae}} \) potentials were filtered using a low-pass filter (LPF-30; WPI) at 10–30 kHz, acquired

with a frequency of 1,000 Hz with A xoGraph (version 4.8; Axon Instruments, Foster City, CA), and stored for further analysis.

Three criteria were used as key elements to accept or reject individual measurements of resting \([\text{Ca}^{2+}]_i\): 1) the calibrations before and after the intracellular \([\text{Ca}^{2+}]_i\) determination had to agree with one another within 2.5 mV; 2) an instantaneous change in potential \( (V_{\text{m}} \) and \( V_{\text{Cae}}) \) had to occur during the cell impalement, followed by a stable recording \( (\pm 2–3 \text{ mV}) \) for at least 40 s; and 3) the membrane potential had to be more negative than \(-60 \text{ mV}\). In experiments where resting \([\text{Ca}^{2+}]_i\) determinations were carried out twice in the same cell, before and after drug treatment, cells were identified with grid labels attached to the bottom of the plate to allow the identification of an individual cell.

**Statistics.** All values are expressed as means \( \pm \) SE. Prism software (version 4.0b) was used for statistical analysis (GraphPad Software, San Diego, CA; www.graphpad.com). One-way ANOVA and Tukey’s mul-tiple comparison tests were used to compare the resting \([\text{Ca}^{2+}]_i\), values among cells expressing different RyR1 constructs; paired t-tests were used to compare the resting \([\text{Ca}^{2+}]_i\), values in a single cell before and after drug treatment.

**RESULTS**

**Measurements of resting \([\text{Ca}^{2+}]_i\).** Differentiated 1B5 myo-tubes were infected with HSV virion particles containing \( \textit{wR RyR1} \) or \( \textit{MH RyR1} \) cDNAs at an MOI of 0.5 for 2 h. Forty-eight hours after incubation with the virions, \([\text{Ca}^{2+}]_i\), measurements were performed on 10–15 myotubes in each well, using double-barreled \( \text{Ca}^{2+} \)-selective microelectrodes. Representative data traces of \( V_{\text{Cae}} \) and \( V_{\text{m}} \) measurements in (untransduced) 1B5 myotubes and those expressing \( \textit{wR RyR1} \) or \( \textit{T4825 RyR1} \) are shown in Fig. 1A. Phase-contrast images of the same cells are pictured in Fig. 1B. To make certain that measured cells were transduced and expressed no ryanodine receptor, \( \textit{wR RyR1} \), or \( \textit{MH RyR1} \), we performed immunostain-ning immediately after obtaining the measurements of resting \([\text{Ca}^{2+}]_i\), (Fig. 1C). Grid labels attached to the bottom of the culture plate allowed identification of each cell before and after immunostaining (see MATERIALS AND METHODS) and appeared as the bar-shaped shadows in the background of panel B pictures.

Expression of \( \textit{MH RyR1} \)s is associated with higher resting \([\text{Ca}^{2+}]_i\), than expression of \( \textit{wR RyR1} \). Although our previous studies clearly showed that human and porcine MH muscle fibers had an elevated myoplasmic resting \([\text{Ca}^{2+}]_i\), compared with nonsusceptible individuals, the mechanism for this in-crease has never been systematically tested in situ in myotubes expressing \( \textit{MH RyR1} \). In the present study, \( \textit{wR RyR1} \) and four \( \textit{MH RyR1} \)s, R164C, R615C, R2163C, and T4825I, were packed into HSV virions and used to transduce differentiated 1B5 myotubes(35). Measurements of membrane potential and resting \([\text{Ca}^{2+}]_i\) in \( \textit{MH RyR1} \)-expressing myotubes showed that expression of \( \textit{MH RyR1} \)s and \( \textit{wR RyR1} \)s caused no significant change in resting membrane potential compared with untrans-dived 1B5 myotubes. However, cells expressing any of the four MH mutations had a significantly \((P < 0.001)\) increased resting \([\text{Ca}^{2+}]_i\); compared with cells expressing \( \textit{wR RyR1} \) (Fig. 2). The degree of elevation of resting \([\text{Ca}^{2+}]_i\) varied significantly among mutations, with expression of T4825I causing the largest increase \((418 \pm 10.89 \text{ nM}, n = 23) \) and R164C producing the smallest increase \((242.3 \pm 11.45 \text{ nM}, n = 18) \) compared with the \( \textit{wR RyR1} \) \((112 \pm 1.9 \text{ nM}, n = 11) \). The resting \([\text{Ca}^{2+}]_i\) in T4825I-expressing myotubes was also significantly higher \((P < 0.001)\) than that of all other con-
resting myoplasmic $[Ca^{2+}]_i$ in myotubes expressing $\text{wtRyR1}$ or one of the four $\text{MH-RyR1}$s (R164C, R615C, R2163C, and T4825I) before and after incubation with 500 $\mu$M ryanodine for 45 min or before and after incubation with 20 $\mu$M FLA 365 for 5 min. As shown in Fig. 3, treatment with ryanodine or FLA 365 did not have any significant effect on resting $[Ca^{2+}]_i$ in myotubes expressing any RyR1 construct.

Probing the mechanism of the $\text{MH-RyR1}$-induced increase in myoplasmic $[Ca^{2+}]_i$: Bastadin 5 alone or ryanodine and bastadin 5 in combination partially restore myoplasmic $[Ca^{2+}]_i$. Investigations using bastadins in isolated junctional SR and BC3H1 cells have suggested a pharmacological link between ryanodine-sensitive actively gating $Ca^{2+}$ channels and ryanodine-insensitive leak pathways (30, 40). In combination with 500 $\mu$M ryanodine, bastadin 5 nearly eliminates the $Ca^{2+}$ leak unmasked by thapsigargin without causing any $Ca^{2+}$ store depletion, whereas ryanodine alone does not exhibit such an effect (40). To test whether these ryanodine-insensitive leak pathways contribute to the increased resting myoplasmic $[Ca^{2+}]_i$, in myotubes expressing $\text{MH-RyR1}$s, we measured the resting $[Ca^{2+}]_i$ in myotubes expressing $\text{wtRyR1}$ or $\text{MH-RyR1}$s after incubation with 10 $\mu$M bastadin 5 for 5 min. Interestingly, bastadin 5 alone significantly decreased the resting $[Ca^{2+}]_i$ in both types of myotubes (Fig. 4). To test the hypothesis that the effect of bastadin 5 allosterically changes the conformation of RyR1 from a ryanodine-insensitive “RyR1 leak conformation” into RyR1s in an actively gating conformation that is sensitive to ryanodine block (see DISCUSSION for detailed description of these proposed RyR1 conformations), we then measured myoplasmic resting $[Ca^{2+}]_i$ in myotubes before and after incubation with 500 $\mu$M ryanodine for 45 min followed by incubation with 10 $\mu$M bastadin 5 for 5 min. As shown previously in this study (see Fig. 3), incubation with ryanodine alone did not change the resting $[Ca^{2+}]_i$ in myotubes expressing either $\text{wtRyR1}$ or any of the $\text{MH-RyR1}$s tested. However, incubation of ryanodine-blocked myotubes in 10 $\mu$M bastadin 5 for 5 min significantly lowered resting myoplasmic $[Ca^{2+}]_i$ in all myotubes expressing $\text{wtRyR1}$ or one of the four $\text{MH-RyR1}$s (R164C, R615C, R2163C, and T4825I).
No significant difference was found between [Ca\(^{2+}\)] in cells expressing 

cells was confirmed by immunostaining. Data are presented as means 
greater for all measured immediately before and after the incubation, using the same micro-
tubes studied (Fig. 5A). Furthermore, the degree of reduction in 
resting [Ca\(^{2+}\)], induced by the two drugs in combination was 
greater for all MT1RyR1s than for w7RyR1 (P < 0.05) in both absolute and percent values (Fig. 5B). These data are consistent with a mechanism by which bastadin 5 converts RyR1s that are in a ryanodine-insensitive RyR1 leak conformation into the actively gating conformation that is ryanodine sensi-
tive. The ryanodine-sensitive conformation is largely accepted to represent the high-conductance channel that is typically reconstituted in bilayer lipid membranes. Under resting conditions, the population of ryanodine-insensitive MT1RyR1s in the RyR1 leak conformation is larger than the population of w7RyR1 in the RyR1 leak conformation.

**DISCUSSION**

Mutations of RyR1 (52) have been implicated in the patho-
physiology of the MH syndrome in more than 50% of susceptible 
patients. In the present study we measured intracellular resting [Ca\(^{2+}\)]; in myotubes expressing either w7RyR1 or one of four common missense MT1RyR1s found in humans, using double-barreled Ca\(^{2+}\) microelectrodes. The new findings in the present study are as follows: 1) myotubes expressing any of the four MT1RyR1s (at least 1 from all 3 mutation hot spots) had a higher resting [Ca\(^{2+}\)], than those expressing w7RyR1; 2) the elevated resting [Ca\(^{2+}\)], observed in myotubes expressing the four MT1RyR1s varied among the individual mutations; 3) treatment of myotubes expressing w7MT1RyR1s with ryanodine or FLA 365 had no effect on resting [Ca\(^{2+}\)]; 4) incubation of myotubes with bastadin 5 or ryanodine and bastadin 5 in combination significantly lowered resting [Ca\(^{2+}\)], in myotubes expressing either w7RyR1 and MT1RyR1s; and 5) the percent decrease in resting [Ca\(^{2+}\)], after treatment with bastadin 5 or the combination of ryanodine and bastadin 5 in myotubes expressing MT1RyR1s was significantly greater than in myotubes expressing w7RyR1. Each of these new findings is discussed below.

The presence of a single MT1RyR1 missense mutation from 
any and all of the three MH hot spots produces a chronically elevated resting myoplasmic [Ca\(^{2+}\)], when compared with myotubes expressing w7RyR1. These results strongly suggest that an increased resting [Ca\(^{2+}\)], is a consistent phenotype of myotubes expressing any MT1RyR1. In addition, this finding is in agreement with all of our previous studies showing increased resting [Ca\(^{2+}\)], in muscle cells in both MH patients and swine (25–28) and with some recent studies using fluorescent Ca\(^{2+}\) indicators in human and mouse myotubes expressing other MH mutations [T2206M (51), V2168M (13), R2163H (2), or R1086H in the dihydropyridine receptor (DHPR) (52)].
However, some other studies on the [Ca\textsuperscript{2+}], in MH muscle cells have yielded conflicting results as to whether or not the resting [Ca\textsuperscript{2+}], is higher in resting MHS skeletal muscle, and studies using fluorescent indicators frequently have not been able to discern a difference between WT and MH cells (12, 24, 29, 48, 51). At present we cannot explain the basis for why different studies on the same expressed mutation yield different results other than to suggest it is due to the difference in experimental methods. It is important to note that there is a broad variation in the reported absolute resting myoplasmic [Ca\textsuperscript{2+}], for WT RyR1-expressing myotubes and fibers between different studies using the same or different fluorescent Ca\textsuperscript{2+} indicators (~110 nM (48); ~50 nM (2); and 35–40 nM (13)), and there is an even larger variation in the absolute values from cells studied in the same laboratory using this technique. We propose that whatever is responsible for this broad variation may be the underlying reason for the inconsistency among studies. In general, the absolute resting [Ca\textsuperscript{2+}], in muscle fibers and myotubes measured using fluorescent Ca\textsuperscript{2+} indicators is always lower than that measured using electrodes. The most probable explanation for this is the result of buffering of intracellular Ca\textsuperscript{2+} by these fluorescent dyes, all of which are derivatives of the Ca\textsuperscript{2+} chelator BAPTA, coupled with the uncertainty about the accuracy of their calibration (K\textsubscript{eq}, temperature, viscosity, intracellular pH, ionic strength) (47).

One alternative suggestion that could be made is that the resting membrane potential of our cells was low, and that the increased Ca\textsuperscript{2+} was the result of an abnormal response to partial depolarization. Although it is true that the membrane potential observed in this study was less than resting membrane potential recorded in myotubes and adult cells at 37°C (~80 mV), the value for the resting membrane potential found in all types of myotubes studied are in accordance with the membrane potential of myotubes and adult cells recorded at temperatures ranging between 20 and 25°C by several other groups (3, 16, 22, 32, 44). Furthermore, previous studies performed at 37°C in intact humans and pigs also had an higher than normal resting [Ca\textsuperscript{2+}], suggesting that the increased resting [Ca\textsuperscript{2+}], is not simply an artifact caused by partial depolarization.

We cannot explain why some MH RyR1 mutations cause a higher resting [Ca\textsuperscript{2+}], than others, as shown in Fig. 3. However, the increased resting [Ca\textsuperscript{2+}], that we found in the present study seems supportive of our previous results from \textsuperscript{[3]}H ryanodine binding analysis, which demonstrated similar differences in sensitivity to caffeine activation and to Ca\textsuperscript{2+} and Mg\textsuperscript{2+} inhibition common among MH mutations (53). The high resting [Ca\textsuperscript{2+}], associated with T4825I might suggest that like COOH-terminal mutations of RyR1 associated with central core disease, MH mutations in this region may be more “leaky,” as previous studies have suggested (48), and related to more significant functional changes than the mutations in the other two hot spots.

The opening and closing of the Ca\textsuperscript{2+} release channels present within the terminal cisternae of SR are basic events in the Ca\textsuperscript{2+} release process in skeletal muscle. Ryanodine is a neutral plant alkaloid that is frequently used as a specific ligand for characterizing the gating mechanism of ryanodine receptor (31, 42). It has been shown that at high concentrations (>200 μM), ryanodine irreversibly inhibits the ability of the RyRs to release Ca\textsuperscript{2+} without changing the passive electrical properties of the bilayer (8, 41). Likewise, FLA 365 reversibly inhibits the Ca\textsuperscript{2+} release channel at the SR in both skeletal and cardiac muscle cells (9, 11). As shown in RESULTS, treatment of myotubes expressing either WT RyR1 or MH RyR1s with ryanodine (500 μM) for 45 min or with FLA 365 (20 μM) for 5 min did not significantly modify resting [Ca\textsuperscript{2+}],. This suggests that, similar to cells expressing WT RyR1, the mechanism for the increased resting [Ca\textsuperscript{2+}], seen in cells expressing MH RyR1s is not due to an increase in the spontaneous activity of these channels as a result of the missense mutation leading to enhanced efflux of Ca\textsuperscript{2+} from the SR. In fact, the current results support previous conclusions that RyR1 in its ryanodine-sensitive actively gating conformation does not contribute to the filling state of the SR stores (40). This is not surprising with regard to the data of Ward et al. (50), who demonstrated that the frequency of Ca\textsuperscript{2+} sparks (which represent spontaneous active gating) in intact myotubes expressing WT RyR1 was very low. In addition, RyR1 gating states are in strong negative control by their association with the DHPR, further reducing the possible contribution that actively gating channels would have on resting Ca\textsuperscript{2+} levels (21, 54).
What may be the most exciting result of this study, the effect of exposure to bastadin 5 alone or the simultaneous exposure to ryanodine and bastadin 5 on the resting \([\text{Ca}^{2+}]\), is that RyRs expressing WT/MH RyR1, appears to shed some light on the possible mechanism(s) for the chronically elevated resting \([\text{Ca}^{2+}]\), in MH muscle cells. Bastadin 5 is a natural product isolated from the marine sponge *I. basta*, which selectively modulates RyR1’s conformation through an allosteric mechanism requiring an intact FKBP12/RyR1 complex. In previous studies (30, 40), our group has shown that RyRs appear to exist in two conformations. The first is an active gating conformation. In the actively gating “channel” conformation, RyR1 has a finite but variable number of rapid transitions from a closed state to one or more high-conductance open states (\(\sim 25-100\) pS for \([\text{Ca}^{2+}]\)). The “gating channel” is readily measured in bilayer lipid membrane reconstitutions, where closed and open states and substates can be clearly defined. The probability that the channel is in an open state \((P_o)\) when the \([\text{Ca}^{2+}]\) concentration is in the physiological range in the myoplasm \((60–300 \text{nM})\) is very low \((<0.01)\). When \([\text{Ca}^{2+}]\) is increased to 100 \(\mu\text{M}\), \(P_o\) of channels nominally increases to a maximum of \(0.4–0.8\), making it possible to see this high-conductance channel rapidly open and close. When a RyR1 in the channel conformation is in the open state, it can bind ryanodine and can be blocked by FLA 365 and ruthenium red. The present studies with bastardin 5 confirm previous results with isolated junctional SR showing that, in addition to the gating channel conformation, which can be completely blocked by all three compounds, an alternate RyR1 leak conformation exists that is insensitive to these drugs and is responsible for setting the filling state of the SR \([\text{Ca}^{2+}]\) store (30, 40). The activity of the RyR1 leak conformation is not easily measured in bilayer studies, because based on the rates of efflux that have been previously measured (40), it is likely to have a much smaller conductance than actively gating channels; and although never directly measured, the RyR1 leak conformation is likely to have a high \(P_o\), with few transitions to its closed state. Thus it would make only a small contribution to the large background currents typically incorporated in bilayer reconstitutions.

Thus, if incorporated into bilayers, the RyR1 leak conformation would likely make only a small contribution to the relatively large offset current typically incorporated with gating channels. RyR1s in this conformation would be ignored as baseline current. RyR1s in this RyR1 leak conformation cannot and do not bind ryanodine with high affinity. Similarly RyR1 channel blockers such as FLA 365 or ruthenium red cannot and do not act as blockers of the RyR1 leak conformation. The concept of a channel switching from a leak conformation to one that actively gates is not unique to RyR1. In fact, RyR1 is just one of several in a class of channels that can exist in both conformations. The archetypical channels in this class are the two-pore domain potassium (K2P) channels (for recent reviews, see Refs. 5, 20). More than sixteen K2P channel genes have been identified in the mammalian genome. All have properties of background or leak \(K^+\) channels and play a crucial role setting the resting membrane potential and regulating cell excitability. Some of these channels are the targets of \(G\) proteins or AKAP150 and can be converted to an actively gating state (17–19, 43). RyR1’s smaller “sister” endoplasmic reticulum calcium release channel, the inositol trisphosphate receptor, also has been shown to function as a \([\text{Ca}^{2+}]\) leak channel when the cytoplasmic control domains are uncoupled from the channel domain of the receptor; however, the mechanism for this uncoupling is as yet unknown (46).

The evidence for the existence of a RyR1 leak conformation from our previous study was that in the presence of bastardin 5, maximum binding for ryanodine binding of SR vesicles could be increased more than twofold while \(K_d\) remained constant. Likewise, \([\text{Ca}^{2+}]\) uptake in SR vesicles treated with bastardin 5 increased nearly 2.5-fold in the presence of ryanodine or ryanodine receptor (RR) (20) compared with untreated vesicles or vesicles treated with ryanodine or RR alone. Our present data represent the first direct pharmacological demonstration that RyR1s set the resting \([\text{Ca}^{2+}]\), in wild-type myotubes and contribute significantly to the chronic elevation in resting \([\text{Ca}^{2+}]\), associated with MH. The current study also establishes a relationship between resting \([\text{Ca}^{2+}]\), and changes in the filling capacity of the SR store previously reported. Both rely on the RyR1 leak conformation. It is interesting to note that with ryanodine and bastardin 5 treatment, the reduction in the elevated resting \([\text{Ca}^{2+}]\), in muscle cells expressing *m*RYR1s is not only absolutely greater but also significantly greater when measured as a percentage of the resting value before treatment. This would imply that expression of *m*RYR1s is associated with an absolute increase in the percentage of RyR1s that assume the ryanodine-insensitive RyR1 leak conformation. However, the fact that resting \([\text{Ca}^{2+}]\), did not fall to normal values in myotubes expressing *m*RYR1s after cotreatment with ryanodine and bastardin 5 suggests that this is not the only mechanism contributing to the elevated resting \([\text{Ca}^{2+}]\). The remaining elevation could come from the possibility that we did not use sufficient concentrations of bastardin 5 to convert all of the *m*RYR1s from their RyR1 leak conformation to an actively gating conformation, since these actions are dose dependent, and/or, more likely, that other mechanisms are also involved.

Other possible mechanisms that could contribute to the remaining intracellular \([\text{Ca}^{2+}]\) dysfunction observed in muscle cells expressing *m*RYR1s could include *m*RYR1-related mitochondrial dysfunction (6) that could cause a change in energetics. Such a change, in turn, could lead to an increased resting \([\text{Ca}^{2+}]\), by changing SR \([\text{Ca}^{2+}]\) pump efficiency, causing a possible dysfunction of normal sarcosomal \([\text{Ca}^{2+}]\) entry, or by causing a possible dysfunction in \([\text{Ca}^{2+}]\) extrusion mechanisms. A change in basal energetics in MH-susceptible individuals, demonstrated using \(^{31}\text{P}\) NMR spectroscopy as an increase in the P/ phosphocreatine ratio, was suggested (37–39), but later studies were unable to confirm this abnormality (4, 34). Furthermore, none of the studies of muscle metabolism that have measured skeletal muscle lactate production using microdialysis have shown any measurable change in baseline values between MH and wild-type humans or pigs (1, 7, 43). Thus the theory that a defect in metabolism could be the mechanism for the unresolved increase in resting \([\text{Ca}^{2+}]\), seems unlikely or at best is highly controversial. However, it is possible that neither of these two techniques are sensitive enough to account for the change in metabolism that would be sufficient to cause the small non-RyR-related increase in resting \([\text{Ca}^{2+}]\), that we observed. Further work is required to resolve this issue and answer the question.

Finally, it is important to note that the mechanisms discussed above and the results of this study are only relevant to MH
caused by RyR1 mutations and that the mechanisms causing MH resulting from other causes or mutations independent of RyR1 may be entirely different.

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