Divergent Effects of the Malignant Hyperthermia-Susceptible $Arg^{615} \rightarrow Cys$ Mutation on the Ca^{2+} and Mg^{2+} Dependence of the RyR1

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ABSTRACT The sarcoplasmic reticulum (SR) Ca^{2+} release channel (RyR1) from malignant hyperthermia-susceptible (MHS) porcine skeletal muscle has a decreased sensitivity to inhibition by Mg^{2+}. This diminished Mg^{2+} inhibition has been attributed to a lower Mg^{2+} affinity of the inhibition (I) site. To determine whether alterations in the Ca^{2+} and Mg^{2+} affinity of the activation (A) site contribute to the altered Mg^{2+} inhibition, we estimated the Ca^{2+} and Mg^{2+} affinities of the A- and I-sites of normal and MHS RyR1. Compared with normal SR, MHS SR required less Ca^{2+} to half-maximally activate [^{3}H]ryanodine binding ($K_{A,Ca}$: MHS = 0.17 ± 0.01 μM; normal = 0.29 ± 0.02 μM) and more Ca^{2+} to half-maximally inhibit ryanodine binding ($K_{I,Ca}$: MHS = 519.3 ± 48.7 μM; normal = 293.3 ± 24.2 μM). The apparent Mg^{2+} affinity constants of the MHS RyR1 A- and I-sites were approximately twice those of the A- and I-sites of the normal RyR1 ($K_{A,Mg}$: MHS = 44.36 ± 4.54 μM; normal = 21.59 ± 1.66 μM; $K_{I,Mg}$: MHS = 660.8 ± 53.0 μM; normal = 299.2 ± 24.5 μM). Thus, the reduced Mg^{2+} inhibition of the MHS RyR1 compared with the normal RyR1 is due to both an enhanced selectivity of the MHS RyR1 A-site for Ca^{2+} over Mg^{2+} and a reduced Mg^{2+} affinity of the I-site.

INTRODUCTION

Depolarization of the skeletal muscle plasma membrane results in the spread of the action potential over the surface and transverse-tubule membranes. Transverse-tubule depolarization effects a structural change in the dihydropyridine receptor/L-type Ca^{2+} channel that results in the release of Ca^{2+} from the sarcoplasmic reticulum (SR) via the high-conductance Ca^{2+} release channel/ryanodine receptor protein (RyR1). RyR1 is regulated in a complex fashion by numerous endogenous effectors and, in the absence of other modulators, is activated by Ca^{2+} concentrations in the nano- to micromolar range and inhibited by Ca^{2+} concentrations in the micro- to millimolar range. Thus, it has been concluded that the RyR1 has a high-affinity divalent binding site, which when occupied by Ca^{2+} will activate the channel (A-site), and a low-affinity divalent binding site, which when occupied by Ca^{2+} will inhibit the channel (I-site) (Meissner, 1994).

RyR1 channel opening is inhibited by physiological concentrations of Mg^{2+} (Endo, 1977; Meissner, 1994). Mg^{2+} could potentially be a competitive antagonist at the A-site on the RyR1, be an agonist at the I-site, or inhibit RyR1 channel opening via an independent inhibitory site. Laver et al. (1997a) and Meissner et al. (1997) have suggested that Mg^{2+} inhibits RyR1 channel opening via its interaction with both Ca^{2+} binding sites and that the extent of the interaction of Mg^{2+} with the two regulatory sites depends on the Ca^{2+} concentration. Although the regulation of the RyR1 by Ca^{2+} and Mg^{2+} has been extensively studied, and the Ca^{2+} affinity of the A- and I-sites estimated (Zucchi and Ronca-Testoni, 1997; Meissner, 1994), the Mg^{2+} affinity of these sites has been reported only for frog RyRs (Murayama et al., 2000). In addition, the apparent affinity of each of these sites is dependent on the conditions under which the measurements are made (Fruen et al., 1996; Meissner et al., 1997). Thus, until the affinities of the two binding sites for Ca^{2+} and Mg^{2+} are determined under identical conditions, the potential physiological role of Mg^{2+} at each site cannot be concluded.

Malignant hyperthermia (MH) is a pharmacogenetic disorder originating primarily from mutations in the RyR1. Although in the human population there are 24 known RyR1 MH mutations (McCarthy et al., 2000; Jurkatt-Rott et al., 2000), the primary defect in porcine MH is a single point mutation ($Arg^{615} \rightarrow Cys$) in the RyR1 (Fujii et al., 1991). RyR1 from MH-susceptible (MHS) individuals exhibits a decreased sensitivity to inhibition by high concentrations of Ca^{2+} (Mickelson et al., 1988, 1990; Shomer et al., 1993; Richter et al., 1997). A greater sensitivity to Ca^{2+} activation has also been reported (Shomer et al., 1993; Herrmann-Frank et al., 1996; Richter et al., 1997). Although the Mg^{2+} regulation of these channels also appears to be altered, most studies have focused on the interaction of Mg^{2+} with the low-affinity I-site (Mickelson et al., 1990; Laver et al., 1997a; Owen et al., 1997). In their comparison of RyR1 from normal and MHS pigs, Laver et al. (1997b) reported that in the presence of 1 μM Ca^{2+}, a Ca^{2+} concentration where they had previously found Mg^{2+} inhibition via the A- and I-sites to be equally important (Laver et al., 1997a), a higher Mg^{2+} concentration was required to half-maximally inhibit MHS channels compared with normal channels. Although the reported decrease in the Mg^{2+} sensitivity of the I-site contributed to the diminished Mg^{2+} inhibition, the possible involvement of the A-site, via an increased Ca^{2+} or decreased Mg^{2+} affinity, cannot be excluded.

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The application of caffeine to skeletal muscle can trigger SR Ca^{2+} release and muscle contraction (Herrmann-Frank et al., 1999). MHS skeletal muscle is more sensitive to caffeine-induced contracture than skeletal muscle from normal individuals, and this enhanced caffeine sensitivity is integral to the clinical diagnosis of MH (Jurkatt-Rott et al., 2000). However, whether the MHS RyR1 itself is more sensitive to caffeine remains controversial (Shomer et al., 1994; Herrmann-Frank et al., 1996). Shomer et al. (1994) reported that the MHS RyR1 is no more sensitive to caffeine than the normal RyR1 and suggested that the increased caffeine sensitivity of MHS muscle may be secondary to an elevated resting myoplasmic Ca^{2+} concentration or altered Ca^{2+} regulation of the RyR1. Although it has been reported recently that the enhanced caffeine sensitivity of MHS muscle is mediated by an increase in the resting myoplasmic Ca^{2+} concentration (Lopez et al., 2000), the effect of caffeine on the Ca^{2+} and Mg^{2+} affinities of the A- and I-sites of the MHS and normal RyR1 have not been rigorously examined.

We have now estimated the Ca^{2+} and Mg^{2+} affinities of the normal and MHS RyR1 A- and I-sites under identical conditions in the presence and absence of caffeine. Compared with the normal RyR1, the MHS RyR1 I-site has a lower apparent affinity for both Ca^{2+} and Mg^{2+}. In contrast, compared with the normal RyR1, the MHS RyR1 A-site has a higher apparent affinity for Ca^{2+} but a lower apparent affinity for Mg^{2+}. In addition, caffeine increased the Ca^{2+} affinity of the MHS and normal RyR1 A-sites to a similar extent. However, caffeine increased the Mg^{2+} affinity of the normal RyR1 A-site but not of the MHS RyR1 A-site. Thus, the MH mutation has opposite effects on the Ca^{2+} and Mg^{2+} affinities of the RyR1 A-site that would greatly enhance the sensitivity of the MHS RyR1 to Ca^{2+} activation in intact muscle.

MATERIALS AND METHODS

[^{3}H]Ryanodine binding

Isolation of SR vesicles

Skeletal muscle SR vesicles were prepared from porcine longissimus dorsi muscle as described previously (Mickelson et al., 1990). Briefly, muscle was homogenized in 0.1 M NaCl, 5 mM Tris maleate buffer (pH 6.8), and the membranes collected between 2,600 and 10,000 × *g* were extracted in 0.6 M KCl, 20 mM Tris (pH 6.8), centrifuged at 100,000 × *g*, and then resuspended in 0.3 M sucrose, 0.1 M KCl, 5 mM Tris (pH 6.8) buffer; all buffers contained a protease inhibitor mixture. SR vesicles were flash-frozen in liquid nitrogen and stored at −70°C.

[^{3}H]Ryanodine binding

SR vesicles (0.2 mg/ml) were incubated at 36°C in media containing 100 mM KCl, 10 mM HEPES, pH 7.4, 100 nM [^{3}H]ryanodine, and a Ca-EGTA buffer set to give the desired free Ca^{2+} concentration (Brooks and Storey, 1992). In some experiments, the binding media also included 5 mM caffeine. After 90 min, SR vesicles were collected on Whatman GF/B filters and washed with 8 ml of ice-cold 100 mM KCl buffer. Estimates of maximal [^{3}H]ryanodine binding capacity of each SR vesicle preparation were determined in media that in addition contained 500 mM KCl, 6 mM ATP, and 10 μM Ca^{2+}. Nonspecific binding was measured in the presence of 20 μM nonradioactive ryanodine. Binding experiments were performed in duplicate using seven normal and nine MHS SR preparations.

Single-channel studies

The RyR1 was purified from SR membrane vesicles as described previously (Shomer et al., 1993). Muller-Rudin planar lipid bilayers were formed by painting a lipid mixture (phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in a 5:3:2 ratio by weight, 50 mg/ml dissolved in *n*-decane) across a 250-μm aperture in a Delrin cup. The *cis* chamber was connected to the headstage input of an Axoclamp 200B patch clamp amplifier (Axon Instruments, Foster City, CA). The *trans* chamber was held at virtual ground. Data was filtered at 2 kHz with an eight-pole Bessel filter, recorded at 4.5 kHz, and stored on a Jazz disk drive (Iomega, Roy, UT). Recording solution consisted of symmetric 100 mM KCl, 10 mM HEPES, pH 7.4, 1 mM EGTA. The Mg^{2+} and Ca^{2+} concentrations were adjusted by adding small aliquots of concentrated EGTA, $CaCl_2$, and $MgCl_2$ (Brooks and Storey, 1992). Single-channel data were collected using a pulsing protocol in which the potential was held at 0 mV for 4 s between steps of 2-s duration to +70 mV (CLAMPEX program, pClamp software, Axon Instruments, Foster City, CA). Only those channels that had a conductance of at least 700 pS were used (Shomer et al., 1994). Single-channel open probability (P_o) was calculated from at least 50 2-s sweeps using FETCHAN and PSTAT analysis programs (pClamp software, Axon Instruments). When two channels were present in the bilayer, indicated by current amplitudes of twice the expected magnitude, P_o was estimated as the average P_o of the two channels, calculated as $[P_{o,level\ 1} + (P_{o,level\ 2} \times 2)]/2$. Bilayers in which three channels had incorporated were dealt with similarly; recordings were not made from bilayers containing more than three channels.

Analysis

The Ca^{2+} and Mg^{2+} affinities of the A- and I-sites of RyR1 were estimated according to the model of Murayama et al. (2000). The fraction of A-sites bound with Ca^{2+} (f_A) and the fraction of I-sites not bound with Ca^{2+} or Mg^{2+} ($1 - f_I$) were expressed as

$$f_A = [Ca^{2+}]^{n_{A,Ca}}/\{[Ca^{2+}]^{n_{A,Ca}} + K_{A,Ca}^{n_{A,Ca}} \times (1 + [Mg^{2+}]^{n_{A,Mg}}/K_{A,Mg}^{n_{A,Mg}})\} \quad (1)$$

$$1 - f_I = 1/(1 + [Ca^{2+}]^{n_{I,Ca}}/K_{I,Ca}^{n_{I,Ca}} + [Mg^{2+}]^{n_{I,Mg}}/K_{I,Mg}^{n_{A,Mg}}), \quad (2)$$

where $K_{A,Ca}$, $K_{A,Mg}$, $K_{I,Ca}$, and $K_{I,Mg}$ are the apparent affinity constants for Ca^{2+} and Mg^{2+} of the A- and I-sites, respectively. $n_{A,Ca}$, $n_{A,Mg}$, $n_{I,Ca}$, and $n_{I,Mg}$ are the Hill coefficients for Ca^{2+} and Mg^{2+} of the A- and I-sites, respectively.

The above parameters were determined in a three-step procedure. First, $K_{A,Ca}$, $n_{A,Ca}$, $K_{I,Ca}$, and $n_{I,Ca}$ were determined from the Ca^{2+} dependence of SR vesicle [^{3}H]ryanodine binding (B) in the absence of Mg^{2+} according to Eq. 3:

$$B = B_{peak} f_A (1 - f_I) = B_{peak}\{[Ca^{2+}]^{n_{A,Ca}}/([Ca^{2+}]^{n_{A,Ca}} + K_{A,Ca}^{n_{A,Ca}} \times \{1 - [Ca^{2+}]^{n_{I,Ca}}/([Ca^{2+}]^{n_{I,Ca}} + K_{I,Ca}^{n_{I,Ca}})\}. \quad (3)$$

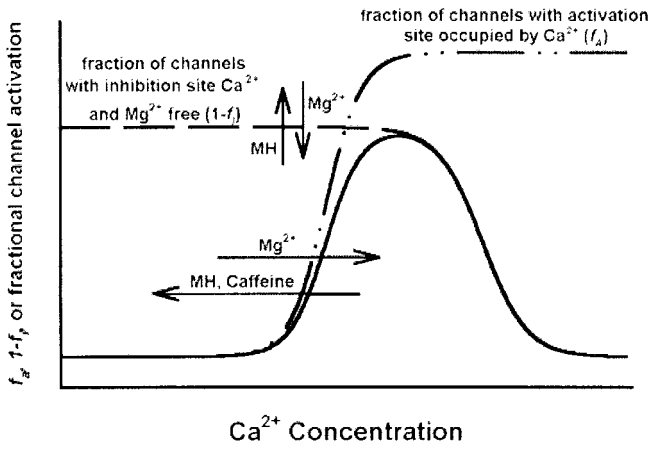

FIGURE 1 Diagrammatic representation of the model described in Materials and Methods used to derive the Ca^{2+} and Mg^{2+} affinities of the RyR1 A- and I-sites. The Ca^{2+} dependence of channel activation (——) is the product of the fraction of channels with Ca^{2+} bound to the A-site (f_A, —··—) and the fraction of channels with the I-site free of both Ca^{2+} and Mg^{2+} ($1\text{-}f_I$, — — —). Because Mg^{2+} is a competitive antagonist with Ca^{2+} at the A-site, Mg^{2+} will effectively increase the $K_{A,Ca}$ thereby shifting the Ca^{2+} dependence of channel activation to higher Ca^{2+} concentrations. As an agonist at the I-site, Mg^{2+} will decrease the fraction of channels with the I-site free of Ca^{2+} and Mg^{2+} and thus decrease the number of channels available for activation. Note that the parameters illustrated were derived in the presence of 1 mM Mg^{2+}.

B_{peak} in these equations indicates the maximal SR vesicle [^{3}H]ryanodine binding under the conditions of the experiment. Then, the concentration dependence of Mg^{2+} inhibition of SR vesicle [^{3}H]ryanodine binding was measured at a Ca^{2+} concentration much greater than $K_{A,Ca}$. At this Ca^{2+} concentration, Ca^{2+} is bound to the A-site and the inhibition by Mg^{2+} occurs via the I-site. $K_{I,Mg}$ and $n_{I,Mg}$ were determined by fitting the data from these experiments with Eq. 4 and including the values previously obtained for $K_{A,Ca}$,$n_{A,Ca}$,$K_{I,Ca}$,and$n_{I,Ca}$:

$$B = B_{peak}(1 - f_I) = B_{peak}\{1/1 + ([Ca^{2+}]^{n_{I,Ca}}/K_{I,Ca}^{n_{I,Ca}} + [Mg^{2+}]^{n_{I,Mg}}/K_{I,Mg}^{n_{I,Mg}})\}. \quad (4)$$

Finally, the concentration dependence of Mg^{2+} inhibition of SR vesicle [^{3}H]ryanodine binding was measured at a Ca^{2+} concentration near $K_{A,Ca}$. At this Ca^{2+} concentration, competitive inhibition by Mg^{2+} at the A-site predominates. $K_{A,Mg}$ and $n_{A,Mg}$ were determined by fitting the data from these experiments with Eq. 5, using values previously obtained for $K_{A,Ca}$, $n_{A,Ca}$, $K_{I,Ca}$, $n_{I,Ca}$, $K_{I,Mg}$, and $n_{I,Mg}$:

$$B = B_{peak} f_A (1 - f_I) = B_{peak}\left\{\frac{[Ca^{2+}]^{n_{A,Ca}}}{[Ca^{2+}]^{n_{A,Ca}} + K_{A,Ca}^{n_{A,Ca}}(1 + [Mg^{2+}]^{n_{A,Mg}}/K_{A,Mg}^{n_{A,Mg}})}\right\} \times \left\{1\Big/\left(1 + \frac{[Ca^{2+}]^{n_{I,Ca}}}{K_{I,Ca}^{n_{I,Ca}}} + \frac{[Mg^{2+}]^{n_{I,Mg}}}{K_{I,Mg}^{n_{I,Mg}}}\right)\right\}. \quad (5)$$

The model is depicted diagrammatically in Fig. 1 using parameters derived in the presence of 1 mM Mg^{2+}. The Ca^{2+} dependence of the fraction of channels activated, i.e., SR vesicle [^{3}H]ryanodine binding (solid line), is the product of the fraction of channels with the A-site bound with Ca^{2+} (f_A, dashed and dotted line) and the fraction of channels with the I-site free of both Ca^{2+} and Mg^{2+} ($1 - f_I$, dashed line). As a competitive antagonist of Ca^{2+} for binding to the A-site, Mg^{2+} effectively increases $K_{A,Ca}$, shifts the Ca^{2+} dependence of f_A to higher Ca^{2+} concentrations, and increases the Ca^{2+} required for channel activation. Because Mg^{2+} is an agonist at the I-site, Mg^{2+} will decrease the fraction of channels with the I-site free of both Ca^{2+} and Mg^{2+}, resulting in a reduction in the number of channels available for activation. Thus, it is clear that the diagram is drawn as would occur in the presence of a Mg^{2+} concentration somewhat below the $K_{I,Mg}$ (at very low Ca^{2+} concentrations, $1 - f_I > 0.5$). It should be pointed out that in the presence of Mg^{2+}, differences in the Ca^{2+} dependence of MHS and normal RyR1 channel activation could potentially occur via an increase in the Ca^{2+} affinity of the A-site, by a decrease in the Mg^{2+} affinity of the A-site, or both.

In an initial experiment, to determine whether MHS and normal RyR1s differ in their sensitivities to inhibition by Mg^{2+}, the Mg^{2+} concentration dependence of MHS and normal SR [^{3}H]ryanodine binding was compared in the presence of 10 μM Ca^{2+}. In this experiment the inhibitory effect of Mg^{2+} could not be attributed to its action at a single site. Therefore, the half-inhibitory (IC_{50}) concentrations were determined using the Hill equation. Curve fitting was performed using SigmaPlot 5.0 (SPSS, Richmond, CA) software. All data are expressed as mean ± SEM. Comparisons between muscle types or treatments performed were made via two-sample *t*-tests with the level of significance set at $p < 0.05$.

RESULTS

The model used to estimate the Ca^{2+} and Mg^{2+} affinities of the RyR1 A- and I-sites (Murayama et al., 2000) is dependent on the assumption that Mg^{2+} is a competitive inhibitor with Ca^{2+} at the A-site and is an agonist at the I-site. This assumption was confirmed in single-channel studies (Fig. 2). Thus, if channels were activated by low concentrations of Ca^{2+} (near $K_{A,Ca}$), the subsequent addition of a low concentration of Mg^{2+} should compete with Ca^{2+} for the A-site and decrease the mean single-channel percent open time. Under these conditions, increasing concentrations of Ca^{2+} would effectively compete with Mg^{2+} for the A-site and increase the single-channel percent open time. As shown in Fig. 2 *A*, a normal RyR1 channel activated by 300 nM *cis* Ca^{2+} had a mean single-channel percent open time of 4.19. The addition of 50 μM Mg^{2+} to the *cis* chamber decreased the percent open time to 0.92. Increasing the Ca^{2+} concentration in the *cis* chamber to 3 μM increased the single-channel percent open time to 5.06. Similar results were obtained in all six experiments, although the sensitivity of the channels to Ca^{2+} and Mg^{2+} varied (Fig. 2 *C*). Thus, these single-channel experiments are consistent with the hypothesis that Mg^{2+} can act as a competitive antagonist with Ca^{2+} at the A-site.

Single-channel studies also confirmed the assumption that at Ca^{2+} concentrations sufficient to saturate the A-site, Mg^{2+} interacts with the I-site. Thus, the single normal channel in Fig. 2 *B*, activated by 300 μM Ca^{2+} had a mean single-channel percent open time of 1.23. The addition of 50 μM Mg^{2+} to the *cis* chamber lowered the percent open time to 1.05. However, in contrast to experiments in 300 nM Ca^{2+}, this channel could not be reactivated by the subse-

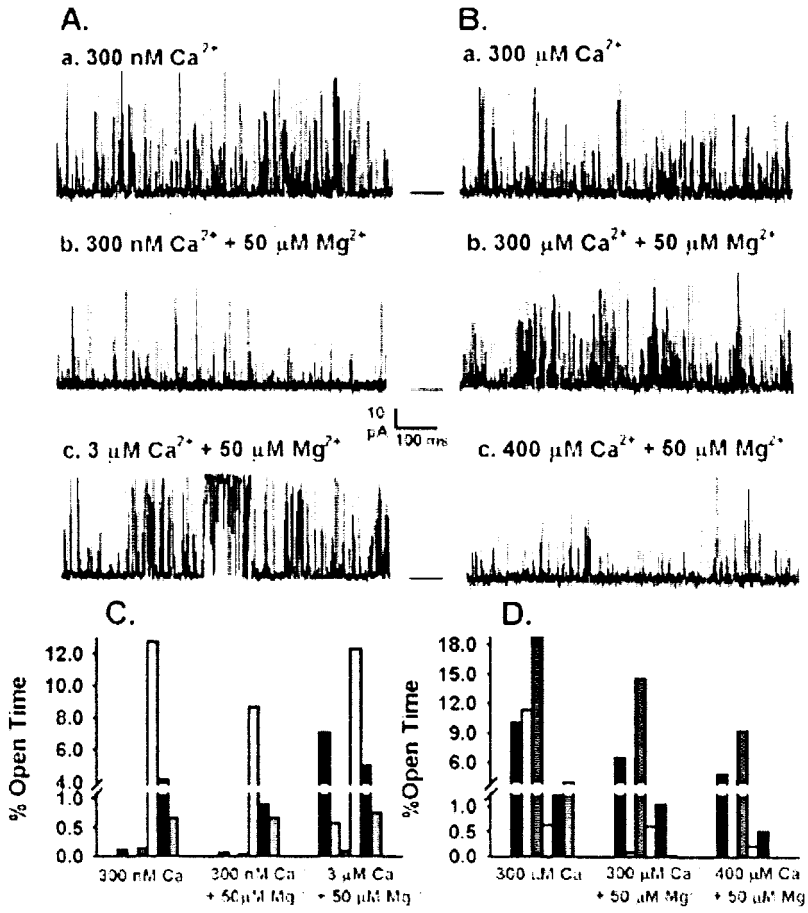

FIGURE 2 Effects of Ca^{2+} and Mg^{2+} in combination on the single-channel activity of the RyR1. Single-channel activity of normal channels was recorded as described in Materials and Methods. Recording solution contained 100 mM KCl, 10 mM HEPES (pH 7.4), 2 mM EGTA, and $CaCl_2$ and $MgCl_2$ to obtain the indicated ionized Ca^{2+} and Mg^{2+} concentrations. Single-channel currents were obtained with voltage steps from 0 mV to +70 mV. The solid line indicates the closed state of the channels; openings are upward. (*A*) (*a*) Single channel activated by 300 nM *cis* Ca^{2+}. Percent open time (%OT) = 4.19, mean open time (OT) = 1.0 ms, and mean closed time (CT) = 8.4 ms; (*b*) Addition of 50 μM *cis* Mg^{2+} decreased the %OT to 0.92 (OT = 0.9 ms, CT = 21.4 ms); (*c*) Increasing *cis* Ca^{2+} to 3 μM reactivated the channel (%OT = 5.06, OT = 1.2 ms, CT = 8.8 ms). (*B*) (*a*) Single channel activated by 300 μM *cis* Ca^{2+} (%OT = 1.23, OT = 1.0 ms, CT = 16.6 ms); (*b*) Addition of 50 μM *cis* Mg^{2+} decreased %OT to 1.05 (OT = 0.9 ms, CT = 17.2 ms); (*c*) Increasing *cis* Ca^{2+} to 400 μM further decreased the %OT to 0.51 (OT = 1.0 ms, CT = 32.5 ms). Each color bar in *C* and *D* represents a different experiment. In all experiments, the addition of 50 μM Mg^{2+} to the *cis* chamber reduced the mean single-channel percent open time when the free Ca^{2+} concentration was either 300 nM, $n = 6$ (*C*) or 300 μM, $n = 6$ (*D*). (*C*) When the Ca^{2+} concentration was increased from 300 nM to 3 μM in the presence of Mg^{2+}, the %OT increased. (*D*) When the Ca^{2+} concentration was increased from 300 μM to 400 μM in the presence of Mg^{2+}, the %OT decreased.

quent addition of Ca^{2+}. Indeed, the additional 100 μM Ca^{2+} added to the *cis* chamber further reduced the percent open time to 0.51. Similar results were obtained with all six experiments (Fig. 2 *D*). Although the extent of inhibition was variable, in no case did increasing *cis* Ca^{2+} increase the percent open time of Mg^{2+}-inhibited channels. Thus, when the Ca^{2+} concentration is greater than that required to maximally activate the RyR1 (i.e., when the A-site is in the Ca^{2+} bound state), Mg^{2+} is an agonist at the low-affinity I-site.

The maximal [^{3}H]ryanodine binding (i.e., in 500 mM KCl, 10 μM Ca^{2+}, and 6 mM ATP) for 9 normal (11.6 $\pm$ 1.1 pmol/mg protein) and 11 MHS (9.5 $\pm$ 0.8 pmol/mg protein) SR preparations were not significantly different. Both MHS and normal SR exhibited the characteristic bell-shaped Ca^{2+} dependence of [^{3}H]ryanodine binding. However, compared with normal SR, MHS SR [^{3}H]ryanodine binding was more sensitive to Ca^{2+} activation and less sensitive to inhibition by Ca^{2+} (Fig. 3). The $K_{A,Ca}$, $n_{A,Ca}$, $K_{I,Ca}$, and $n_{I,Ca}$ of the RyR1 determined for both muscle types according to Eq. 3 are presented in Table 1. The MHS $K_{A,Ca}$ was significantly smaller than the normal $K_{A,Ca}$; in contrast, the MHS $K_{I,Ca}$ was more than 1.7-fold greater than the normal $K_{I,Ca}$. Thus, compared with the normal RyR1, the MHS RyR1 A-site had a higher apparent affinity for Ca^{2+} whereas the I-site had a lower apparent affinity for Ca^{2+}.

To determine whether the normal and MHS RyR1 also differ in their sensitivity to Mg^{2+} inhibition, the Mg^{2+}

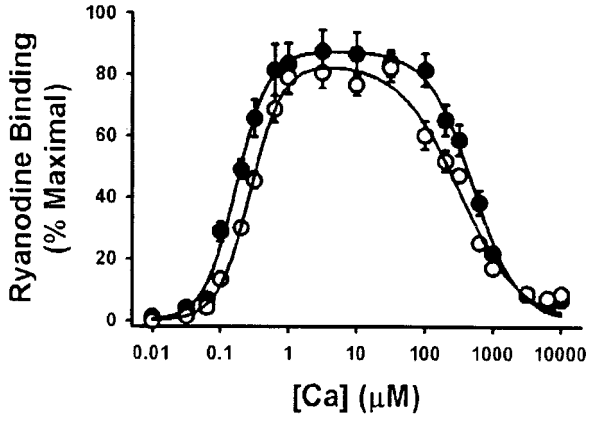

FIGURE 3 Comparison of the Ca^{2+} dependence of [^{3}H]ryanodine binding to normal and MHS SR vesicles. [^{3}H]Ryanodine binding to normal (○) and MHS (●) skeletal muscle SR vesicles was determined as described in Materials and Methods. Media contained 100 mM KCl, 10 mM HEPES (pH 7.4), and Ca-EGTA buffer set to provide the indicated free Ca^{2+} concentrations. Data are expressed as percentages of the maximal [^{3}H]ryanodine binding capacity of the SR preparations. Solid lines are based on fits to Eq. 3 (see Materials and Methods). Means ± SEM are of seven independent experiments performed in duplicate (seven different SR vesicle preparations).

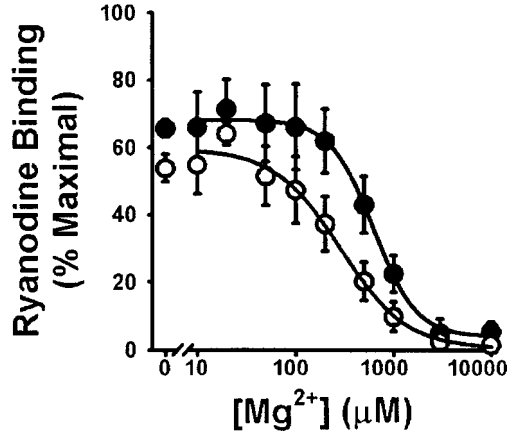

FIGURE 4 Inhibition of [^{3}H]ryanodine binding to normal and MHS SR by Mg^{2+}. [^{3}H]Ryanodine binding to normal (○) and MHS (●) skeletal muscle SR vesicles was determined as described in Materials and Methods. Media contained 100 mM KCl, 10 mM HEPES (pH 7.4), and 2 mM EGTA. Ca^{2+} and Mg^{2+} concentrations were adjusted to maintain an ionized Ca^{2+} concentration of 10 μM and various ionized Mg^{2+} concentrations as indicated. Data are expressed as percentages of the maximal [^{3}H]ryanodine binding capacity of the SR vesicle preparations; solid lines are based on fits to the Hill equation. Means ± SEM are of five independent experiments preformed in duplicate (five different SR vesicle preparations).

dependence of SR vesicle [^{3}H]ryanodine binding was determined in the presence of 10 μM Ca^{2+}, a Ca^{2+} concentration that is more than 30 times the $K_{A,Ca}$. As shown in Fig. 4, in the presence of 10 μM Ca^{2+} the concentration dependence of Mg^{2+} inhibition of [^{3}H]ryanodine binding to MHS SR was shifted to significantly higher Mg^{2+} concentrations compared with that of normal SR. The IC_{50} values, derived from the Hill equation, for MHS and normal SR were 652.0 ± 46.5 μM and 304.9 ± 49.6 μM Mg^{2+}, respectively. Thus, [^{3}H]ryanodine binding to MHS SR appears to be less sensitive to inhibition by Mg^{2+} than is [^{3}H]ryanodine binding to normal SR.

From the experiments described above, we conclude that compared with the normal RyR1, the MHS RyR1 A-site has a higher Ca^{2+} affinity, the I-site has a lower Ca^{2+} affinity, and the MHS channels are less sensitive to inhibition by Mg^{2+}. However, because Mg^{2+} inhibition occurs via its binding to both regulatory sites, and there are significant differences in the Ca^{2+} affinities of both sites, it is not possible, from the data presented in Fig. 4, to identify the mechanism responsible for the decreased inhibition of the MHS RyR1 by Mg^{2+}. The MHS RyR1 I-site may have a reduced Mg^{2+} affinity as suggested by Laver et al. (1997b), the MHS RYR1 A-site may have a reduced Mg^{2+} affinity, or both. Furthermore, an increased affinity of the MHS RyR1 A-site for Ca^{2+}, with no change in the Mg^{2+} affinity, could also result in a decreased competitive inhibition of the MHS RyR1 by Mg^{2+}. To distinguish between these possibilities, we estimated the affinities of the normal and MHS RyR1 A- and I-sites for Mg^{2+}.

We determined the Mg^{2+} dependence of the inhibition of [^{3}H]ryanodine binding in the presence of 300 μM Ca^{2+}, a concentration of Ca^{2+} that is 1000-fold greater than the RyR1 $K_{A,Ca}$ (Table 1). At this Ca^{2+} concentration the

TABLE 1 Ca^{2+} and Mg^{2+} parameters for the activation and inhibition sites of normal and MHS RyR1

	Ca^{2+} parameters				Mg^{2+} parameters			
	$K_{A,Ca}$ (μM)	n_A	$K_{I,Ca}$ (μM)	n_I	K_{AMg} (μM)	n_A	K_{IMg} (μM)	n_I
No caffeine								
Normal	0.29 ± 0.2	1.8 ± 0.2	293.3 ± 24.2	1.0 ± 0.1	21.59 ± 1.66	1.0 ± 0.1	299.2 ± 24.5	0.8 ± 0.1
MHS	0.17 ± .01*	1.6 ± 0.1	519.3 ± 48.7*	1.3 ± 0.1*	44.36 ± 4.54*	1.2 ± 0.1	660.8 ± 53.0*	1.2 ± 0.1
5 mM caffeine								
Normal	0.071 ± .005†	2.1 ± 0.3	376.1 ± 45.8	1.2 ± 0.1	5.59 ± 1.28†	1.2 ± 0.1	285.6 ± 51.5	0.7 ± 0.1
MHS	0.051 ± .003*†	1.3 ± 0.1*	584.1 ± 27.2*	1.3 ± 0.1	50.45 ± 1.93*	1.5 ± 0.1*†	531.0 ± 41.5*	1.1 ± 0.1*

*Significantly different from normal, $p < 0.05$.
†Significantly different from the absence of caffeine, $p < 0.05$.

A-sites should be fully occupied by Ca^{2+}, and inhibition of [^{3}H]ryanodine binding by Mg^{2+} should occur primarily via the I-site. Fitting this data with Eq. 4, using the previously determined Ca^{2+} affinities and Hill coefficients, allowed us to estimate the Mg^{2+} affinity of the I-sites of both MHS and normal RyR1 (Fig. 5; Table 1). This analysis indicated that the $K_{I,Mg}$ of the MHS RyR1 I-site was approximately twice that of the normal RyR1 I-site. However, the relative Mg^{2+}/Ca^{2+} affinities for MHS and normal RyR1 were both ~1 (Table 2), indicating the MHS mutation decreases the affinity of the I-site for both divalent ions in a similar fashion.

Next, we examined the concentration dependence of the inhibition of SR [^{3}H]ryanodine binding by Mg^{2+} in the presence of 300 nM Ca^{2+}, a Ca^{2+} concentration near the $K_{A,Ca}$. At this Ca^{2+} concentration, Mg^{2+} will inhibit RyR1 channel opening primarily via its action at the RyR1 A-site. Fitting these data in Fig. 5 with Eq. 5 provided values for $K_{A,Mg}$. As shown in Table 1, the MHS $K_{A,Mg}$ was approximately twice the value for the normal RyR1. Consequently, in contrast to its effect on the I-site, the MHS mutation altered the apparent affinity of the A-site for Ca^{2+} and Mg^{2+} in opposite ways, increasing the Ca^{2+} affinity and decreasing the Mg^{2+} affinity. As a result, the selectivity of the MHS RyR1 A-site for Ca^{2+} over Mg^{2+} was ~3.5-fold greater than the normal RyR1 A-site (Table 2).

If the model described in the methods and derived parameters in Table 1 are valid, it should be possible to predict the Ca^{2+} dependence of [^{3}H]ryanodine binding to SR vesicles in the presence of various concentrations of Mg^{2+}. Therefore, we determined the Ca^{2+} dependence of ryanodine binding to normal and MHS SR vesicles in the presence of 100 μM and 500 μM Mg^{2+} and fit the data according to Eq. 5 using the parameters given in Table 1 (Fig. 6). If the conditions are established such that Mg^{2+} inhibition occurs primarily as a result of its binding to the A-site, a shift in the activation side of the Ca^{2+} dependence curve to higher Ca^{2+} concentrations, with no change in the inactivation side of the curve would be expected. Thus, 100 μM Mg^{2+}, a Mg^{2+} concentration near the $K_{A,Mg}$ increased the Ca^{2+} concentration required to activate normal and MHS SR vesicle [^{3}H]ryanodine binding compared with experiments performed in the absence of Mg^{2+}. Half-activating Ca^{2+} concentrations (EC_{50}) of 0.50 ± 0.03 μM and 0.34 ± 0.03 μM were derived for normal and MHS SR, respectively (compare with $K_{A,Ca}$ in Table 1). In contrast, the Ca^{2+} dependence of RyR1 inhibition was not significantly altered in either muscle type. The IC_{50} values in the presence of 100 μM Mg^{2+} were 344.7 ± 22.6 μM for normal and 416.3 ± 36.1 μM for MHS SR (compare with $K_{I,Ca}$ in Table 1). Thus, low concentrations of Mg^{2+} (≤100 μM Mg^{2+}) affect [^{3}H]ryanodine binding primarily via competition with Ca^{2+} for the A-site on the RyR1.

The lower-affinity I-site has a similar affinity for Ca^{2+} and Mg^{2+} (Table 1). Therefore, a Mg^{2+} concentration near the $K_{I,Ca}$ should inhibit ryanodine binding by acting at both the A- and I-sites. Accordingly, 500 μM Mg^{2+} shifted both the Ca^{2+} dependence of activation and decreased the maximal extent of Ca^{2+} activation (Fig. 6). This concentration

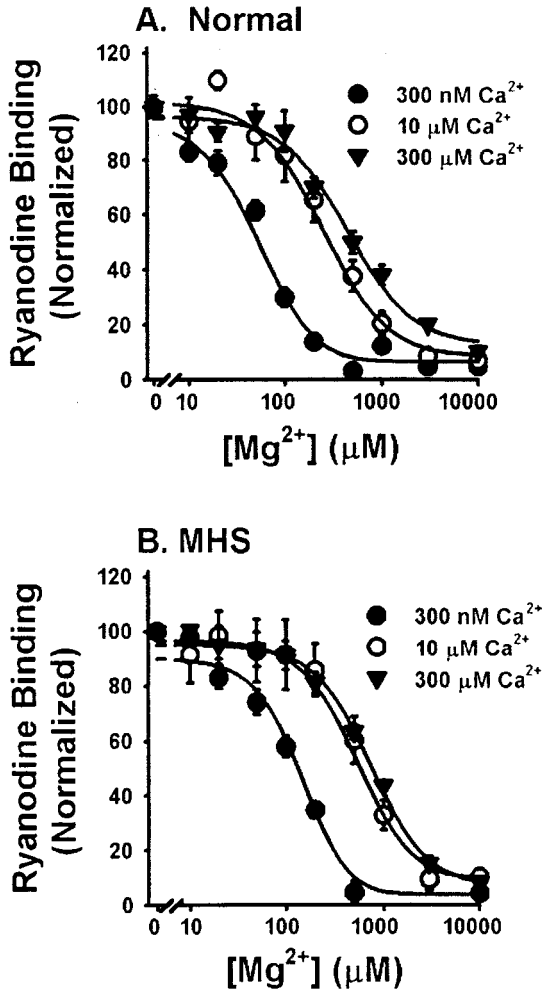

FIGURE 5 Mg^{2+} dependence of [^{3}H]ryanodine binding to normal and MHS SR in the presence of various concentrations of Ca^{2+}. [^{3}H]Ryanodine binding to normal (*A*) and MHS (*B*) skeletal muscle SR was determined as described in Materials and Methods. Media contained 100 mM KCl, 10 mM HEPES (pH 7.4), and 2 mM EGTA. The concentrations of $MgCl_2$ and $CaCl_2$ were adjusted to maintain the ionized Ca^{2+} concentration of 300 nM (●), 10 μM (○), or 300 μM (▼) and Mg^{2+} as indicated in the figure. Data for 10 μM Ca^{2+} (○) are replotted from Fig. 3 for comparison with 300 nM and 300 μM Ca^{2+}. Data are expressed as percentages of the [^{3}H]ryanodine binding in the absence of Mg^{2+}; solid lines are based on fits to Eq. 5 (see Materials and Methods). Means ± SEM are of five independent experiments preformed in duplicate (five different SR vesicle preparations).

TABLE 2 Relative Mg^{2+} and Ca^{2+} affinities of the activation and inhibition sites of normal and MHS RyR1

	No caffeine		5 mM caffeine	
	$K_{A,Mg}/K_{A,Ca}$	$K_{I,Mg}/K_{Ica}$	$K_{A,Mg}/K_{A,Ca}$	$K_{I,Mg}/K_{Ica}$
Normal	74.5	1.0	78.7	0.8
MHS	260.9	1.3	989.2	0.9

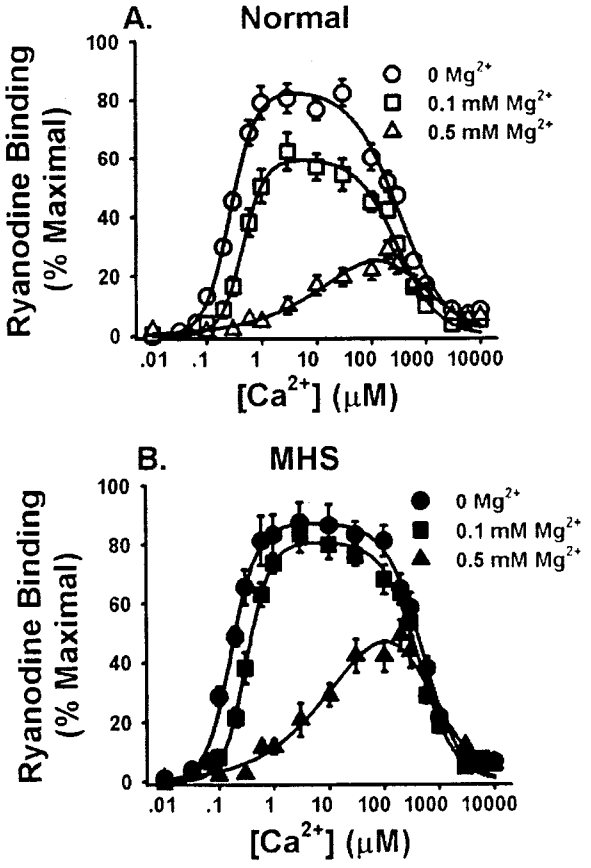

FIGURE 6 Ca^{2+} dependence of [^{3}H]ryanodine binding to normal and MHS SR in the presence or absence of various concentrations of Mg^{2+}. [^{3}H]Ryanodine binding to normal (*A*) and MHS (*B*) skeletal muscle SR was determined as described in Materials and Methods. Media contained 100 mM KCl, 10 mM HEPES (pH 7.4), and 2 mM EGTA. The concentrations of $MgCl_2$ and $CaCl_2$ were adjusted to maintain an ionized Mg^{2+} concentration of either 0 mM (○, ●), 0.1 mM (□, ■) or 0.5 mM (△, ▲) and Ca^{2+} as indicated in the figure. Data are expressed as percentages of the maximal [^{3}H]ryanodine binding capacity of the SR vesicle preparations; solid lines are based on fits to Eq. 5 (see Materials and Methods) and the parameters presented in Table 1. Data for 0 Mg^{2+} (○, ●) are replotted from Fig. 1 for comparison with 0.1 and 0.5 mM Mg^{2+}. Means ± SEM are of five to seven independent experiments performed in duplicate (five to seven different SR vesicle preparations).

of Mg^{2+} increased the Ca^{2+} EC_{50} for both types of SR (MHS: 5.86 ± 1.06 μM; normal: 6.36 ± 1.32 μM). Likewise, the Ca^{2+} IC_{50} was also increased for both MHS (740 ± 8 μM) and normal (630 ± 13 μM) SR.

The fitted lines in Fig. 6 derived from Eq. 5 and using the parameters presented in Table 1 appear to fit the data well and adequately describe the combined effects of Ca^{2+} and Mg^{2+} on ryanodine binding to both MHS and normal SR. Thus, the data presented in Fig. 6 support the validity of both the model and the derived parameters.

Although MHS skeletal muscle fibers are more sensitive to caffeine-induced contraction, Shomer et al. (1994) suggested that an increased Ca^{2+} sensitivity of the MHS RyR1 channel, rather than increased caffeine affinity, underlies the greater responsiveness of MHS muscle to caffeine. Therefore, we examined the effects of 5 mM caffeine on the Ca^{2+} and Mg^{2+} affinities of the normal and MHS RyR1 A-and I-sites (Table 1). Caffeine did not significantly alter the Ca^{2+} or Mg^{2+} affinity of the I-site of either the MHS or normal RyR1. Caffeine had a similar effect on the MHS and normal A-site Ca^{2+} affinity, increasing the apparent affinity approximately 3–4-fold. In contrast, caffeine had divergent effects on the Mg^{2+} affinity of the MHS and normal A-site. The caffeine-induced increase in the Mg^{2+} affinity (3.9-fold) of the normal RyR1 A-site was similar to the increase in the Ca^{2+} affinity (4.1-fold). In contrast, caffeine did not significantly increase the affinity of the MHS RyR1 A-site for Mg^{2+}. Thus, 5 mM caffeine increased the Ca^{2+} affinity of the A-site of both channel types to a similar extent. However, caffeine increased the selectivity of the MHS but not the normal A-site for Ca^{2+} over Mg^{2+} (Table 2).

DISCUSSION

In agreement with previous reports (Mickelson et al., 1988; Shomer et al., 1993; Herrmann-Frank et al., 1996), [^{3}H]ryanodine binding to SR from pigs with the Arg^{615}→Cys RyR1 MHS mutation was more sensitive to Ca^{2+} activation and less sensitive to Ca^{2+} inhibition than SR from normal individuals (Fig. 3; Table 1). An increased sensitivity of Ca^{2+} activation has also been reported for SR isolated from individuals with the RyR1 Gly^{2434}→Arg human MH mutation (Richter et al., 1997) as well as individuals with positive MH contracture tests but unknown and probably variable genotypes (Valdivia et al., 1991). Therefore, we conclude that the porcine Arg^{615}→Cys mutation, its human homolog, and likely other MH mutations as well, increase the sensitivity of the RyR1 to activation by Ca^{2+} and decrease the sensitivity of the RyR1 to inhibition by Ca^{2+}.

Consistent with Mickelson et al. (1990), we found that Mg^{2+} is a less effective inhibitor of [^{3}H]ryanodine binding to MHS than to normal SR (Fig. 4). Mg^{2+} has also been shown to be less effective at inhibiting MHS RyR1 channel opening (Laver et al., 1997b) and Ca^{2+} release in mechanically peeled MHS muscle fibers (Owen et al., 1997).

Because Mg^{2+} competes with Ca^{2+} for binding to the two divalent cation regulatory sites on RyR1, and the extent of Mg^{2+} inhibition at each site is dependent on the Ca^{2+} concentration (Figs. 5 and 6), the relative magnitude of the Mg^{2+} effect at each site has been difficult to assess. This is of particular importance as it relates to the molecular basis of MH, as it is now clear that RyR1 channels with the Arg^{615}→Cys mutation are more sensitive to activation by Ca^{2+} and less sensitive to inhibition by both Ca^{2+} and Mg^{2+}. Although the decreased sensitivity of the MHS RyR1 to inhibition by Mg^{2+} has been extensively studied, it has not been clear whether the decreased inhibition of the MHS RyR1 by Mg^{2+} is due solely to a decreased affinity of

the MHS RyR1 I-site for Mg^{2+} or whether alterations in the affinity of the MHS RyR1 A-site for divalent cations also plays a role. Either an increase in the affinity of the A-site for Ca^{2+}, or a decreased affinity of the A-site for Mg^{2+} would enhance the ability of Ca^{2+} to compete with Mg^{2+} for the A-site and activate the RyR1. We show here that in addition to a decreased affinity of the MHS RyR1 I-site for Mg^{2+}, alterations in the MHS RyR1 A-site contribute to the decreased Mg^{2+} inhibition; i.e., both an increased Ca^{2+} affinity and a decreased Mg^{2+} affinity of the MHS RyR1 A-site contribute to the decreased Mg^{2+} inhibition of the MHS RyR1.

Although the increased sensitivity of MHS skeletal muscle to caffeine-induced contracture is integral to the clinical diagnosis of MH (Jurkatt-Rott et al., 2000), the mechanistic basis for the differential response of normal and MHS muscle to caffeine has been unclear (Shomer et al., 1994; Herrmann-Frank et al., 1996). Shomer et al. (1994) reported no difference in the apparent affinity of the MHS and normal RyR1 for caffeine and suggested the increased caffeine sensitivity of MHS muscle may be due to an increased resting myoplasmic Ca^{2+} concentration and/or alterations in the Ca^{2+} affinity of the RyR1. Recent measurements of intracellular Ca^{2+} in MHS skeletal muscle fibers are consistent with the former hypothesis (Lopez et al., 2000). However, the effects of caffeine on the Ca^{2+} and Mg^{2+} affinity of normal and MHS RyR1 have not been thoroughly examined. The data presented suggest that the increased Ca^{2+} affinity of the MHS RyR1 A-site does indeed contribute to the increased caffeine sensitivity of MHS skeletal muscle. Because caffeine increased the Ca^{2+} affinity of the A-site of both the normal and MHS RyR1 to a similar extent (3–4-fold) the Ca^{2+} affinity of the MHS RyR1 A-site remained significantly higher than the normal RyR1 A-site (Table 1). This alone could increase the sensitivity of MHS skeletal muscle to caffeine-induced contraction. However, because the extent of the caffeine-induced increase in the affinity of the normal RyR1 A-site for Ca^{2+} and Mg^{2+} were similar, the selectivity of the A-site for Ca^{2+} over Mg^{2+} did not change. In contrast, caffeine did not alter the affinity of the MHS A-site for Mg^{2+}; thus, caffeine further increased the selectivity of the MHS A-site for Ca^{2+} over Mg^{2+} (Table 2). Thus, both an increased Ca^{2+} affinity of the MHS RyR1 A-site and a greater selectivity of the MHS A-site for Ca^{2+} over Mg^{2+} contribute to the enhanced caffeine sensitivity of MHS skeletal muscle.

The extent of RyR1 channel activation reflects the product of the fraction of channels with Ca^{2+} bound to the A-site (f_A) and the fraction of channels with the I-site free of both Ca^{2+} and Mg^{2+} ($1 - f_I$). Mg^{2+} shifts the Ca^{2+} dependence of f_A to higher Ca^{2+} concentrations, and decreases $1 - f_I$ at all Ca^{2+} concentrations (Murayama et al., 2000). Consequently, Mg^{2+} decreases the maximal Ca^{2+} activation of the RyR1 and shifts the Ca^{2+} dependence of activation to higher Ca^{2+} concentrations (see Fig. 8. of Murayama et al., 2000). The Arg^{615}→Cys RyR1 mutation opposes the effects of Mg^{2+} on RyR1. Thus, in a manner similar to caffeine, in the presence of Mg^{2+}, the MH mutation shifted the Ca^{2+} dependence of f_A to lower Ca^{2+} concentrations (Fig. 1). However, in contrast to caffeine, at low Ca^{2+} concentrations, the MH mutation also increased $1 - f_I$ (Fig. 1). As a result, maximal Ca^{2+} activation of the MHS RyR1 is increased and the Ca^{2+} dependence of activation is shifted to lower Ca^{2+} concentrations compared with the normal RyR1.

The concentration of free Mg^{2+} present in the myoplasm is sufficient to inhibit activation of the RyR1 by Ca^{2+} in intact muscle (Endo, 1977). Indeed, based on the parameters given in Table 1, and using Eqs. 1 and 2, at a resting Ca^{2+} concentration of 0.1 μM and 1 mM Mg^{2+} (Konishi, 1998) the I-sites of normal and MHS RyR1 are predicted to be partially occupied (~86% and 72%, respectively) by Mg^{2+}. Mg^{2+} is predicted to occupy >99% of the A-sites of both normal and MHS RyR1. Thus, at physiological levels of Mg^{2+} both MHS and normal RyR1 channels can be partially activated (~15% and 30%, respectively) by increasing the Ca^{2+} concentration. If the channels are to be maximally activated, the Mg^{2+} inhibition at the I-site must be removed. At the same time, Ca^{2+} must replace Mg^{2+} occupying the A-site. However, the resting myoplasmic Ca^{2+} concentration is not sufficient to activate RyR1 channel opening. Therefore, if maximal SR Ca^{2+} release is to occur, the Ca^{2+} sensitivity of the RyR1 A-site must be increased concurrent with the removal of the Mg^{2+}-dependent inhibition of the RyR1. The increased Ca^{2+} affinity of the A-site could depend in part on other endogenous effectors of the RyR1, such as ATP (Meissner, 1994) and calmodulin (Fruen et al., 2000), or alternatively on the interaction of the RyR1 with the dihydropyridine receptor.

The single point mutation in the porcine MHS RyR1 results in an increased sensitivity of the muscle to voltage activation (Gallant et al., 1982; Dietze et al., 2000). Although the mechanism by which this occurs is unclear, Dietze et al. (2000) suggested the MHS mutation alters the equilibrium for a voltage-independent transition of the RyR1 from the closed to the open state. The association of the increased voltage sensitivity of MHS SR Ca^{2+} release with the enhanced sensitivity of the MHS RyR1 to activation by Ca^{2+} raises the possibility that endogenous effectors may modulate the voltage-independent transition. Thus, the 3.5-fold increase in the selectivity of the MHS RyR1 A-site ($K_{A,Mg}/K_{A,Ca}$, Table 2) for Ca^{2+} over Mg^{2+} might provide the basis for the enhanced sensitivity of MHS muscle to voltage activation.

The Arg^{615}→Cys mutation likely alters the affinity of the A- and I-sites via a conformational change transmitted over a significant distance because the mutation is a substantial distance along the primary sequence from putative locations of the A- (near amino acid 3885 of RyR3) (Chen et al., 1998) and I-sites (between amino acids 3726 and 5037) of

RyR1 (Du and MacLennan, 1999). The proposal that Arg^{615} is not located in either of the RyR1 Ca^{2+}/Mg^{2+}-binding sites is supported by the observation that the human MH mutation, $Gly^{2434} \rightarrow Arg$, has a similar effect on the Ca^{2+} sensitivity of activation and inhibition of the RyR1 (Richter et al., 1997). Although it is difficult to envision how residues 615 and 2434 could form part of both the high- and low-affinity Ca^{2+}-binding sites, the mechanism by which these and other MH mutations alter RyR1 function will remain a matter of speculation until the relationship of the primary sequence to the tertiary structure of the RyR1 is resolved in detail.

In summary, we have determined the Ca^{2+} and Mg^{2+} affinities of the A- and I-sites of both the MHS and normal RyR1. Although the I-site displayed no preference for Ca^{2+} over Mg^{2+}, the affinity of the MHS RyR1 I-site for these ions was reduced nearly twofold compared with the normal RyR1. The A-site of the normal RyR1, however, had ~75-fold higher affinity for Ca^{2+} compared with Mg^{2+}, whereas the MHS A-site had more than a 250-fold greater preference for Ca^{2+} over Mg^{2+}. This significant increase in Ca^{2+} selectivity over Mg^{2+} may contribute not only to the increased voltage sensitivity of MHS skeletal muscle but also to the increased sensitivity of MHS muscle to caffeine and other pharmacological activators.

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