Reduced Inhibitory Effect of Mg^{2+} on Ryanodine Receptor-Ca^{2+} Release Channels in Malignant Hyperthermia

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ABSTRACT Malignant hyperthermia (MH) is a potentially fatal, inherited skeletal muscle disorder in humans and pigs that is caused by abnormal regulation of Ca^{2+} release from the sarcoplasmic reticulum (SR). MH in pigs is associated with a single mutation ($Arg^{615}Cys$) in the SR ryanodine receptor (RyR) Ca^{2+} release channel. The way in which this mutation leads to excessive Ca^{2+} release is not known and is examined here. Single RyR channels from normal and MH-susceptible (MHS) pigs were examined in artificial lipid bilayers. High cytoplasmic (*cis*) concentrations of either Ca^{2+} or Mg^{2+} (>100 μM) inhibited channel opening less in MHS RyRs than in normal RyRs. This difference was more prominent at lower ionic strength (100 mM versus 250 mM). In 100 mM *cis* Cs^{+}, half-maximum inhibition of activity occurred at ~100 μM Mg^{2+} in normal RyRs and at ~300 μM Mg^{2+} in MHS RyRs, with an average Hill coefficient of ~2 in both cases. The level of Mg^{2+} inhibition was not appreciably different in the presence of either 1 or 50 μM activating Ca^{2+}, showing that it was not substantially influenced by competition between Mg^{2+} and Ca^{2+} for the Ca^{2+} activation site. Even though the absolute inhibitory levels varied widely between channels and conditions, the inhibitory effects of Ca^{2+} and Mg^{2+} were virtually identical for the same conditions in any given channel, indicating that the two cations act at the same low-affinity inhibitory site. It seems likely that at the cytoplasmic [Mg^{2+}] in vivo (~1 mM), this Ca^{2+}/Mg^{2+}-inhibitory site will be close to fully saturated with Mg^{2+} in normal RyRs, but less fully saturated in MHS RyRs. Therefore MHS RyRs should be more sensitive to any activating stimulus, which would readily account for the development of an MH episode.

INTRODUCTION

Malignant hyperthermia (MH) is an inherited skeletal muscle disorder of humans and pigs that can be triggered in susceptible individuals by anesthetics, such as halothane, by certain other agents, and even by stress (Mickelson and Louis, 1996). The disorder is due to abnormal regulation of intracellular [Ca^{2+}] in the muscle cells. If an MH episode is initiated, it results in muscle rigidity, severe metabolic changes, and excessive heat production, often leading to death if untreated. MH susceptibility in pigs is apparently caused by a single mutation ($Arg^{615} \rightarrow Cys^{615}$) in the ryanodine receptor (RyR)-Ca^{2+} release channel in the sarcoplasmic reticulum (SR), and a small percentage of human cases are attributable to a homologous mutation (MacLennan and Phillips, 1992; Mickelson and Louis, 1996). However, it is unresolved at present how this mutation in the RyR results in MH susceptibility. It has been reported previously that the peak rate of Ca^{2+}-activated Ca^{2+} release from the SR is abnormally high in muscle from MH-susceptible pigs (Ohta et al., 1989; Carrier et al., 1991). Furthermore, studies on both ryanodine binding (Mickelson et al., 1990; Shomer et al., 1993) and RyR channel activity in artificial bilayers (Fill et al., 1990; Shomer et al., 1993) show that the ability of high myoplasmic [Ca^{2+}] (e.g., $\geq$0.1 mM) to inhibit channel opening is considerably reduced (about threefold or more) in muscle from MH-susceptible pigs, with little if any change in the activating effect of low concentrations (μM) of myoplasmic Ca^{2+} (Mickelson and Louis, 1996).

It has been proposed previously that this abnormality in the inhibitory effect of high myoplasmic [Ca^{2+}] on the RyR-Ca^{2+} release channel of MH-susceptible pigs should really be viewed as an abnormality in Mg^{2+} inhibition of the channel (Lamb, 1993), because 1) studies on rabbit SR vesicles indicate that Mg^{2+} and Ca^{2+} bind with similar affinity to the same inhibitory site (half-inhibition at ~0.1 mM at physiological ionic strength; Meissner et al., 1986, 1997), and 2) in a muscle fiber this site should be effectively saturated with Mg^{2+}, which is present at ~1 mM in the myoplasm (see Lamb and Stephenson, 1992). This proposal has recently received further support from a study on isolated RyR channels, which shows at the single-channel level, in both rabbit skeletal muscle and sheep cardiac muscle, that Ca^{2+} and Mg^{2+} act with similar efficacy at the low-affinity inhibitory site (Laver et al., 1997). If Ca^{2+} and Mg^{2+} also act on a common inhibitory site in porcine muscle, it would readily explain why, in both normal and MH-susceptible muscle, the relative and absolute concentrations of Mg^{2+} required to half-maximally inhibit SR Ca^{2+} release (O'Brien, 1986; Owen et al., 1997) and ryanodine binding (Mickelson et al., 1990) are very similar to the corresponding values for Ca^{2+}. In contrast to this proposal, a recent brief report has suggested that Mg^{2+} inhibition of the RyR channels is not altered in porcine MH, and that Ca^{2+} and Mg^{2+} are therefore acting at different inhibitory sites (Shomer et al., 1995). Here we show that Mg^{2+}

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inhibition of single RyR channels from MH muscle is indeed reduced in the same way as Ca^{2+} inhibition, thus strongly supporting the proposal that the inhibitory site distinguishes poorly between the divalent cations, and readily explaining the physiological basis of the hyperresponsiveness of muscle in MH-susceptible pigs.

MATERIALS AND METHODS

Animals

Tissue and blood were obtained from three Landrace pigs and three Belgian Landrace pigs, all ~4 months old. Each animal was genetically tested for the presence of the normal and MH RyR alleles (corresponding to Arg^{615} and Cys^{615}, respectively) according to the method of Otsu et al. (1992), as described previously (Owen et al., 1997). This analysis unequivocally classified each animal as homozygous for either the normal allele (Arg/Arg) (one Belgian Landrace and two Landrace pigs) or the MH allele (Cys/Cys) (one Landrace and two Belgian Landrace pigs).

Muscle dissection

Each animal was given a premedication intramusclular injection of azaperone (Stresnil, 1.5–2 mg/kg). Once sedated, the pig was anesthetized by injection of thiopental sodium (10 mg/kg) via a cannula in the ear. Under anesthesia, three strips (4 × 1 × 0.5 cm) of the gracilis muscle were held in muscle clamps, freed of fat and fascia, and excised. The clamped strips were then bathed in oxygenated saline solution (mM: 121 NaCl: 5.4 KCl; 2.5 $CaCl_2$; 1.2 $MgSO_4$; 1.2 NaH_2PO_4; 15 $NaHCO_3$; 11.5 glucose; pH 7.4 when bubbled with carbogen), until subsequent use in the caffeine-halothane contracture test. Pigs were then killed by anesthetic overdose, and back and leg muscles (including vastus lateralis, vastus medialis, rectus fermoris, and gluteus maximus) were immediately excised for preparation of SR vesicles.

Caffeine-halothane contracture test

The caffeine-halothane contracture (CHC) test was carried out with the same techniques and criteria used in previous studies (e.g., Foster et al., 1991; Owen et al., 1997). Each muscle bundle (2.5 cm long and 3 mm in diameter) was suspended vertically under 1.5 g of resting tension in an organ bath chamber containing the above-mentioned saline solution at 37°C, and the force was recorded when the bundle was challenged with either halothane (ICI, Australia; administered with carbogen at 3% v/v through a Dragewick vaporizer) or with a range of caffeine concentrations (2 mM, 4 mM, and 8 mM) produced by cumulative addition to the bathing medium. Each treatment was performed on at least three muscle bundles from each animal.

Isolation of SR vesicles

Muscle tissue was washed free of blood in ice-cold phosphate-buffered saline containing 2 mM EGTA (pH 7.0). Crude SR vesicles were prepared by a method based on that of Meissner (1984) and modified by Ma et al. (1995). Freshly dissected tissue, trimmed of fat and connective tissue, was minced and homogenized in (in mM) 5 Tris-maleate, 100 NaCl, 2 EDTA, 0.1 EGTA, pH 6.8 (5 ml/g of tissue), in a Waring blender with four 15-s high-speed bursts and ~15-s intervening rest periods. The homogenate was centrifuged at 2600 × *g* for 30 min, and the supernatant was filtered through cotton gauze and centrifuged at 10,000 × *g* for 30 min. The pellet (P2) was collected, and the supernatant was centrifuged again at 35,000 × *g* (P3). Pellets P2 and P3 were resuspended in (mM) 5 Tris-2-(*N*-morpholino)ethanesulfonic acid (Tris-MES), 300 sucrose, 100 KCl, 2 dithiothreitol (pH 6.8); aliquoted; snap-frozen in liquid nitrogen; and stored at −70°C. All buffers contained the protease inhibitors phenylmethylsulfonyl fluoride (0.7 mM), leupeptin (1 μg/ml), pepstatin A (1 μM), and benzamidine (1 mM). SR vesicles were also prepared from muscle tissue that had been cubed, snap-frozen in liquid nitrogen, and stored at −70°C. No differences were noted in the RyR channel properties from SR vesicles isolated from fresh or frozen tissue.

Single-channel measurements

Full details of the apparatus and recording techniques are given elsewhere (Laver et al., 1995, 1997). Bilayers separating two aqueous baths (*cis* and *trans*) were formed from a mixture of phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (5:3:2, by weight) in *n*-decane, by using the film drainage technique of Mueller et al. (1962). Incorporation of ion channels with the bilayers was achieved by fusion with SR vesicles, as described by Miller and Racker (1976). SR vesicles were added to the *cis* bath, and the cytoplasmic side of the SR membrane faced the *cis* solution when fused with the bilayer.

The lumenal (*trans*) bath contained either (in mM) 50 CsCl and 0.1 $CaCl_2$ or 230 Cs-methane sulfonate ($CsCH_3SO_3$), 20 CsCl, and 0.1 $CaCl_2$. Before channel recording, the cytoplasmic (*cis*) bath contained (in mM) 500 mannitol and either 250 CsCl or 230 $CsCH_3SO_3$, 20 CsCl, and either 0.1 or 1 mM Ca^{2+}. The osmotic gradient established by mannitol alone was sufficient for vesicle fusion. All bathing solutions were buffered to pH 7.4 with 10 mM *N*-tris-(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) and CsOH. Each channel was subjected to several measurements in a range of *cis* $[Ca^{2+}]$ and $[Mg^{2+}]$. For the duration of each measurement (lasting for 30 s to 2 min), the solution at the *cis* face of the bilayer was exchanged with any one of 16 available recording solutions by flowing solutions from a vinyl tube directly onto the bilayer surface. This local perfusion technique is similar to that used by Laver and Curtis (1996). Solution flow rates of only 1–2 μl/s, which could be sustained for extended periods without rupturing the bilayer, produced complete solution exchanges within 5 s. The gating of RyRs in response to solution changes appeared to reach steady state within 5–10 s from the onset of solution exchange. The recording solutions contained (in mM) 250 CsCl or 20 CsCl and either 80 or 230 $CsCH_3SO_3$, and 10 TES (pH 7.4) and were buffered at various $[Ca^{2+}]$, by using $CaCl_2$ and either 2 1,2-bis(2-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid (BAPTA) (used for free $[Ca^{2+}] \leq 1$ μM) or 2 dibromo-BAPTA (used for free $[Ca^{2+}]$ between 3 and 10 μM). Ca^{2+} was not buffered at concentrations of ≥50 μM. Micromolar free $[Ca^{2+}]$ was measured using an ion meter (Radiometer ION83). The flow of solutions over the bilayer could be inspected visually because of the different refractive indices of the bath solution (containing mannitol) and the flow solutions (without mannitol).

Recording and analysis of single-channel data

Electrical potentials are given with respect to the *trans* chamber as ground and positive current is directed from the *cis* to the *trans* bath. Channel recordings were made at bilayer potentials of either +40 or −40 mV. During the experiments, the bilayer current and potential were recorded at a bandwidth of 5 kHz on videotape, using pulse code modulation. The current signal was replayed through a 1-kHz, low-pass, 8-pole Bessel filter and sampled at 2 or 5 kHz. Unitary current was determined from inspection of the current records.

Open probability was calculated from continuous records (10–60-s duration) using either of two methods: 1) the time-averaged current divided by the unitary current and the number of channels (up to three RyRs were sometimes present during P_o measurements); 2) the fraction of the time that the current of a single channel exceeded a threshold, which was usually set at 20% of the maximum current. The two methods gave similar results. The number of RyRs in each bilayer was determined from the number of current levels observed under conditions where the RyRs had relatively high open probability. Open channel current and time-averaged currents were measured with an in-house program (Channel2, developed by Prof.

P. W. Gage and Mr. M. Smith). The scatter in the data is given ±1 standard error of the mean (SEM), except where specifically stated otherwise.

RESULTS

Caffeine-halothane contracture test

The CHC test is used to determine whether the muscle bundles from a particular animal are hyperresponsive to in vitro challenge with caffeine or halothane, and hence to indicate whether the animal is MH-susceptible (MHS), i.e., likely to develop MH under halothane anesthesia or when severely stressed. The muscle biopsies from all three pigs homozygous for the normal RyR tested negative to the CHC test, giving no contracture response at all to either 2 mM caffeine or 3% halothane. In contrast, the muscle bundles from all three pigs homozygous for the MHS RyR gave clear positive responses to the CHC test (mean responses: 0.28 ± 0.06 g to 2 mM caffeine and 1.44 ± 0.49 g to 3% halothane), indicating that the regulation of Ca^{2+} release was abnormal in each case.

Ca^{2+} and Mg^{2+} regulation of normal and MHS RyRs

SR vesicles containing RyRs were isolated from the three MHS pigs and three normal pigs. RyRs from all pigs were activated by cytoplasmic (*cis*) [Ca^{2+}] over the range 0.1–10 μM and were inhibited by Ca^{2+} and Mg^{2+} exceeding 50 μM (e.g., Fig. 1). Such inhibition was mediated by a decrease in channel open probability, P_o. In addition to causing RyR inhibition, Ca^{2+} and Mg^{2+} both decreased the maximum current (i.e., channel conductance) with similar potency, as shown in Fig. 2. Tinker and Williams (1992) have explained this effect by competition between monovalent and divalent ions for the conduction pathway. It is also clear that divalent ions had a similar effect on the conductance of RyRs from normal and MHS muscle, which is consistent with the finding of Shomer et al. (1994b) that the MH mutation does not significantly affect the ion permeation properties of RyRs.

The inhibition of RyRs by divalent cations (either Mg^{2+} or Ca^{2+}) was characterized by fitting a Hill equation to the relationship between P_o and the total divalent cation concentration, [X^{2+}]:

$$\frac{P_o([X^{2+}])}{P_o(0)} = \frac{1}{1 + ([X^{2+}]/K_i)^H} \quad (1)$$

where $P_o(0)$ is the open probability of the fully activated channel at [Ca^{2+}] ≈ 10 μM, K_i is the [X^{2+}] that produces 50% reduction of P_o, and H is the Hill coefficient, which represents the number of ions that bind cooperatively to produce channel inhibition. The reason for analyzing inhibition in terms of the total divalent cation concentration ([X^{2+}]), rather than the concentration of each ion species, is that several studies on RyRs from rabbit skeletal muscle (Meissner et al., 1986, 1997; Soler et al., 1992; Laver et al., 1997) and sheep cardiac muscle (Laver et al., 1997) have already shown that in the presence of high concentrations of activating Ca^{2+} (e.g., ≥50 μM), the reduction in P_o asso-

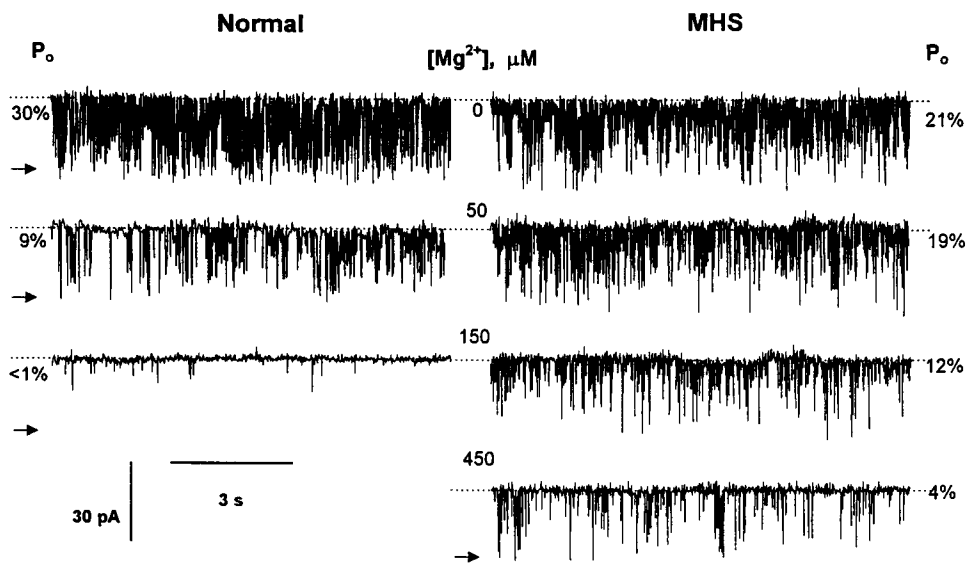

FIGURE 1 Lipid bilayer recordings of a normal RyR and a RyR from malignant hyperthermia-susceptible (MHS) muscle, which are representative of our observations. The *trans* solution contained 250 mM Cs^+, and the *cis* bath contained 100 mM Cs^+, 50 μM Ca^{2+} plus various Mg^{2+} concentrations (listed between each pair of traces). The bilayer potential was −40 mV, and the channel openings are shown by downward current steps. The current baseline for each trace is shown by the dashed lines, and the arrows point to the maximum level in each record. The maximum level does not appear in the bottom left trace because strongly inhibited channels rarely opened to their maximum conductance. The records shown in each panel are from the same channel. The addition of either Mg^{2+} or Ca^{2+} (not shown) at submillimolar concentrations inhibited RyR currents primarily by reducing the channel open probability (P_o, listed with each trace). RyRs from MHS muscle were significantly less inhibited by cytosolic Mg^{2+} than RyRs from normal muscle.

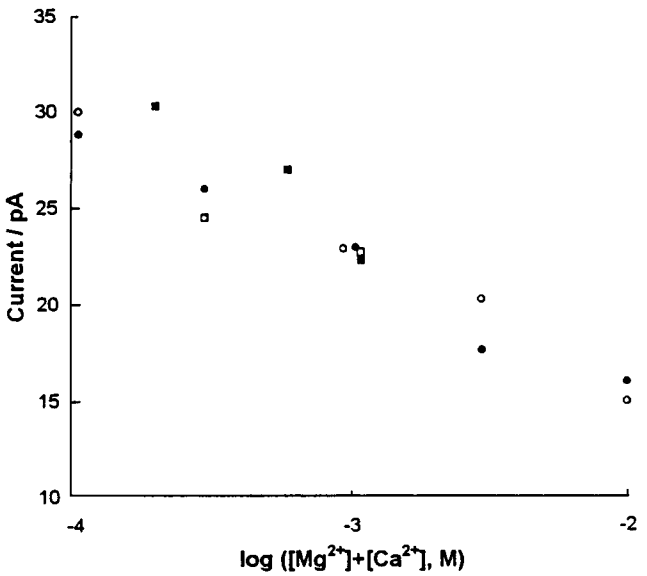

FIGURE 2 The maximum current through normal and MHS RyRs, at +40 mV, plotted against total cytoplasmic divalent ion concentration (either Ca^{2+} or Mg^{2+} + 100 μM Ca^{2+}). The *cis* compartment also contained 250 mM CsCl, and the *trans* compartment contained 50 mM CsCl and 100 μM $CaCl_2$. Data points show the mean of several experiments. ■, Effect of Mg^{2+} on normal RyRs (n = 8). □, Effect of Mg^{2+} on MHS RyRs (n = 4). ●, Effect of Ca^{2+} on normal RyRs (n = 11). ○, Effect of Ca^{2+} on MHS RyRs (n = 9).

ciated with inhibition depends only on the total divalent cation concentration. Laver et al. (1997) found that the effects of Ca^{2+} and Mg^{2+} on the detailed gating kinetics associated with RyR inhibition are indistinguishable, provided *cis* [Ca^{2+}] exceeded 10 μM for sheep cardiac RyR and 1 μM for rabbit skeletal RyRs.

RyRs from MHS muscle were generally less sensitive to inhibition by Ca^{2+} and Mg^{2+} than those from normal muscle (e.g., Fig. 1). RyRs in membrane pellets P2 and P3 obtained from different pigs of the same genotype gave similar responses. We also found that the concentration for half-inhibition of RyRs by Ca^{2+} and Mg^{2+} measured in CsCl solutions at +40 mV was not distinguishable from that measured in $CsCH_3SO_3$ solutions at −40 mV. There was also no noticeable difference between the P_o data obtained from bilayers containing multiple RyRs and bilayers with single RyRs.

Ca^{2+} and Mg^{2+} inhibition at lower ionic strength

In one series of experiments, we examined separately the Ca^{2+} inhibition and the Mg^{2+} inhibition of RyR activity in the presence of 100 mM *cis* $CsCH_3SO_3$ (and pH and Ca^{2+} buffers), as this is close to the ionic strength in vivo. In such conditions, RyRs were maximally activated by cytoplasmic [Ca^{2+}] between 1 and 50 μM (average maximum P_o values at 1 and 50 μM Ca^{2+}, respectively: 0.17 ± 0.03 (n = 10) and 0.23 ± 0.03 (n = 20) for normal RyRs; 0.23 ± 0.06 (n = 5) and 0.23 ± 0.04 (n = 19) for MHS RyRs). In these experiments, Mg^{2+} inhibition was examined in the presence of a high concentration of activating Ca^{2+} (50 μM), to eliminate any additional inhibitory effects due to competition of Mg^{2+} with the activating Ca^{2+} (see earlier and Discussion). In both the Ca^{2+} inhibition and Mg^{2+} inhibition experiments, Hill curves were fitted to the data from all individual bilayer recordings in which the P_o of the RyRs was measured over a sufficiently broad range of divalent ion concentrations to get a reliable fit. These K_i values are shown as frequency distributions in Fig. 3, *A* and *B*, respec-

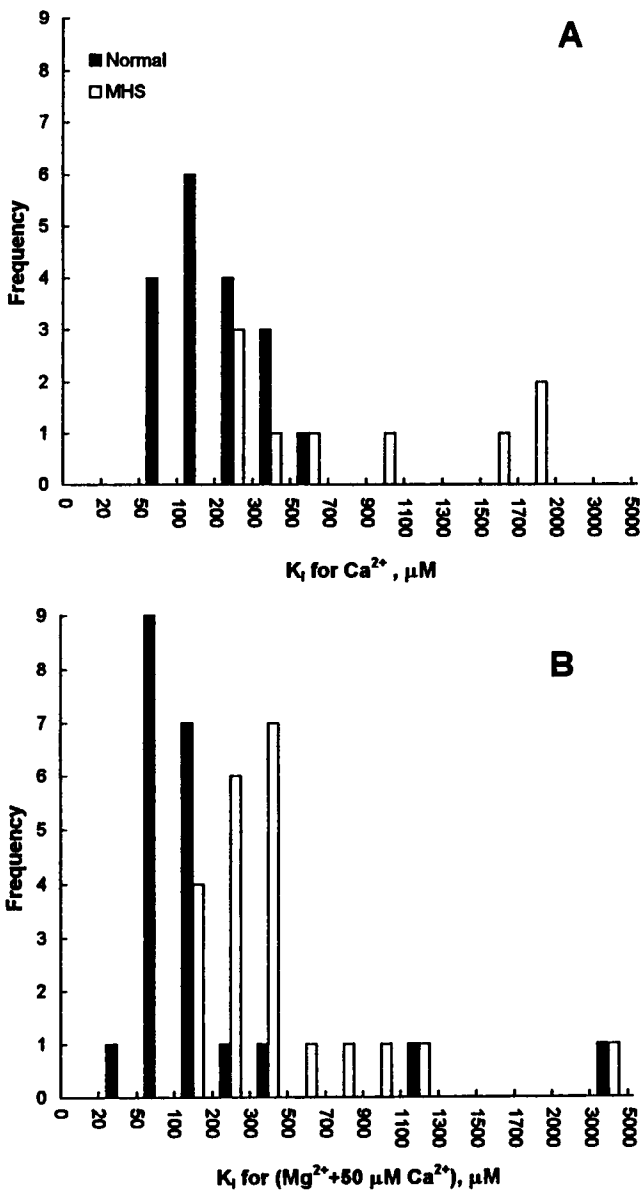

FIGURE 3 Frequency distributions of the concentration (K_i) for half-maximum inhibition of individual RyRs from normal and MHS muscle by Ca^{2+} (*A*) or Mg^{2+} (*B*), in the presence of 100 mM *cis* Cs^+; values were obtained by fitting the data for each bilayer experiment separately. Mg^{2+} inhibition was measured in the presence of 50 μM Ca^{2+}, and K_i was derived from plots of P_o versus ([Ca^{2+}] + [Mg^{2+}]), as discussed in the text.

tively. Individual K_i values for both Ca^{2+} and Mg^{2+} inhibition showed considerable variation within the same genotype, but the individual Hill coefficients were ~2 in all cases where they could be estimated reliably (2.1 ± 0.3 (n = 9) and 1.7 ± 0.15 (n = 4) for Mg^{2+} and Ca^{2+} inhibition, respectively, in normal RyRs and 1.9 ± 0.2 (n = 9) for Mg^{2+} inhibition in MHS RyRs). The mean K_i (± SE) for Ca^{2+} inhibition was 204 ± 32 μM (n = 18) in normal RyRs and 957 ± 251 μM (n = 9) in MHS RyRs (see Fig. 3 *A*). Although a Student's t-test indicates that such values are significantly different, it is not clear that the underlying assumption that the values are normally distributed is valid in the case of the MHS data. Such divergence from a normal distribution is even more apparent in the data for Mg^{2+} inhibition of the normal RyRs shown in Fig. 3 *B*, where it is clear that two of the RyRs were very much less sensitive to Mg^{2+} inhibition (i.e., 7- to 20-fold) than the rest of the population, perhaps reflecting differences existing in vivo or caused during vesicle isolation or experimentation (see Discussion, as well as Laver et al., 1997). Owing to these two extreme values (1.3 and 5 mM), the mean of the K_i values for Mg^{2+} inhibition of the normal RyRs (412 ± 237 μM, n = 21) was very much larger than the median value for the population (~110 μM). The corresponding mean K_i for the MHS RyRs was ~637 ± 215 μM (n = 22), whereas the median value was ~400 μM; this difference was due primarily to the presence of one extreme value (5 mM). Such distributions clearly made it inappropriate to compare the different populations with a t-test. Statistical analysis of the values of K_i by the Wilcoxon-Mann-Whitney rank test, which does not make any assumption about the population distributions, confirmed that the MHS RyRs were indeed significantly less inhibited than the normal RyRs by both Mg^{2+} and Ca^{2+} (probability $p < 0.001$ in both cases), irrespective of whether the extreme values were included in the analysis.

In Fig. 4 we show the result of pooling all of the Ca^{2+} inhibition data (Fig. 4 *A*) and Mg^{2+} inhibition data (Fig. 4 *B*) for the experiments with 100 mM Cs^+; at each divalent cation concentration, the P_o measurements from all of the RyRs of one genotype were averaged together, and Hill curves were fitted to the resulting mean values. It is common to pool single-channel data in this manner, but we note below that several major problems arise with this type of analysis. The best-fit K_i for the pooled Ca^{2+} inhibition data was 204 μM for the normal RyRs and 1160 μM for the MHS RyRs (Fig. 4 *A*), which are both close to the mean of individual K_i values for the respective data (see above). In the case of Mg^{2+} inhibition (Fig. 4 *B*), the pooled data do not include values from the two normal RyRs and one MHS RyR that had K_i values far greater than the remainder of the respective populations (i.e., >10 times the median value). The K_i of the best fits to these pooled Mg^{2+} inhibition data were 93 μM and 292 μM for the normal and MHS RyRs, respectively. If the values from all RyRs are included, irrespective of their extreme nature, the mean P_o data show a small though prominent "tail" at high values of

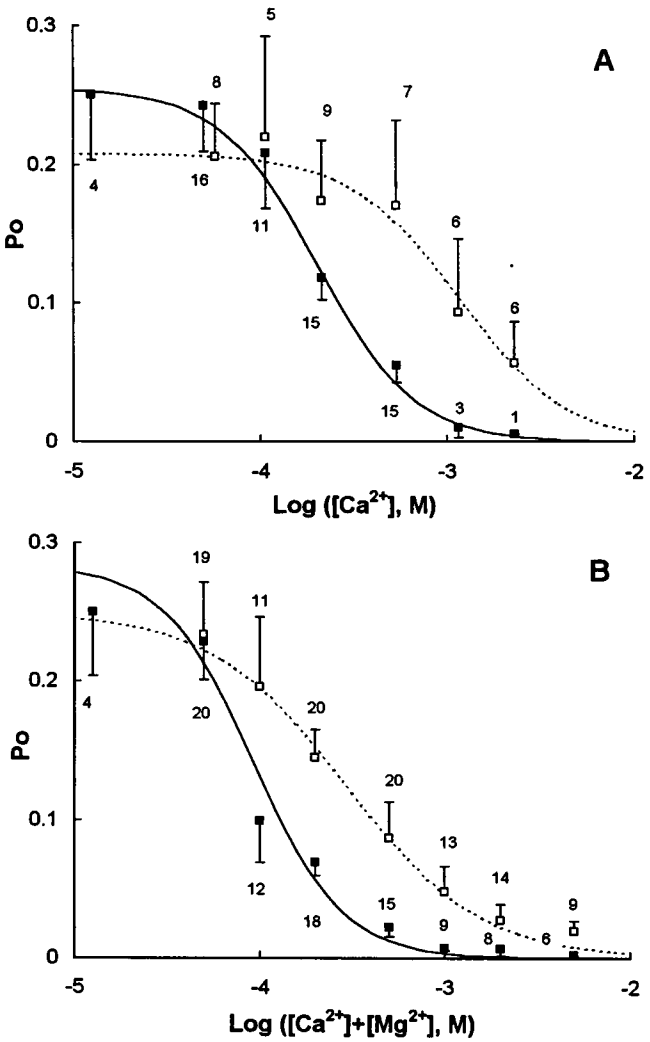

FIGURE 4 Pooled data of open probability, P_o, of normal RyRs (■) and MHS RyRs (□) in the presence of 100 mM *cis* Cs^+, showing the inhibition of these channels by cytoplasmic $[Ca^{2+}]$ (*A*) or by cytoplasmic $[Mg^{2+}]$ in the presence of 50 μM Ca^{2+} (*B*). The data points and error bars show the mean and standard errors, and the labels show the number of experiments included in each average. Data from two normal RyRs and one MHS RyR that showed far less Mg^{2+} inhibition than the remainder of the respective populations (see Fig. 3 *B*) were not included in the averages (see text). The curves show fits of the Hill equation (Eq. 1) to the normal (——) and MHS (·····) data. The total number of experiments, n (and the number with one or two RyRs in the bilayer) were 18 (11, 7) for normal RyRs with Ca^{2+} and 9 (4, 5) for MHS RyRs with Ca^{2+}, 21 (14, 7) for normal RyRs with Mg^{2+} and 22 (12, 10) for MHS RyRs with Mg^{2+}. Parameters for the best fits (by weighted least squares): (*A*) ——, P_{max} = 0.25, H = 1.7, K_i = 204 μM; ·····, P_{max} = 0.21, H = 1.5, K_i = 1160 μM. (*B*) ——, P_{max} = 0.28, H = 1.8, K_i = 93 μM; ·····, P_{max} = 0.25, H = 1.2, K_i = 292 μM.

($[Mg^{2+}]+[Ca^{2+}]$), which is not fit well by a simple Hill curve, but the best-fit K_i is little affected (~78 μM and ~215 μM, respectively, with H_I reduced to ~1). Thus, as could be expected, the K_i for the fit to the pooled P_o data is less sensitive to the presence of a small proportion of poorly inhibited RyRs than is the mean of the individual K_i values (see above), and as such perhaps gives a better indication of

the half-inhibitory divalent cation concentration when a single representative value is required.

Although pooling of the data can give an overview of the inhibitory effect of the divalent cations on the total activity of the two RyR genotypes and may give a good indication of the divalent ion concentration required for half-inhibition of each population, we emphasise that it does not accurately convey the effects on individual RyRs. In particular, unless the half-inhibitory concentration (K_i) is identical in all members of a population (and this was clearly not the case here; see Fig. 3), pooling the data "smears out" the decrease in activity occurring with increasing cation concentration, giving a lower apparent Hill coefficient (only ~1.5; see Fig. 4) than the true value for the individual RyRs (~2; see above). Furthermore, appropriate statistical comparisons of two populations based only on such pooled data (e.g., normal versus MHS RyRs in Fig. 4 *A*) is extremely difficult, because the standard error measure associated with each mean is a complex function of both the steepness of the inhibitory effect in each channel and the spread of the K_i values between different channels within each population. However, in the case of the data for 100 mM Cs^+, where the difference in the mean K_i values between the two populations is large compared to the spread of the individual K_i values (Fig. 3), the pooled data (i.e., Fig. 4) still clearly indicate that the divalent cation inhibition is markedly reduced in MHS RyRs compared to normal RyRs.

Inhibitory effect of Mg^{2+} at a lower activating [Ca^{2+}]

We also examined the inhibitory behavior of Mg^{2+} in the presence of only 1 μM Ca^{2+} to gain more information on the mechanism of Mg^{2+} inhibition and to verify that the inhibitory effect of Mg^{2+} detailed in the preceding section was not dependent on using a particular concentration of activating Ca^{2+} (50 μM). The K_i was determined for each RyR as described above. In both normal and MHS RyRs, there was a considerable spread in the K_i values for Mg^{2+} inhibition in 100 mM Cs^+ with 1 μM activating Ca^{2+}, and the values were very similar to those obtained in the same genotype with 50 μM activating Ca^{2+}; the mean values at 1 μM Ca^{2+} were 182 ± 24 μM (n = 10) and 397 ± 118 μM (n = 5), respectively (compare Fig. 3). The corresponding values for H_I were 3.2 ± 0.8 (n = 4) and 3.6 ± 0.2 (n = 4). Thus the level of Mg^{2+} inhibition found here was not critically dependent on the activating [Ca^{2+}].

Ca^{2+} and Mg^{2+} inhibition at higher ionic strength

The ryanodine binding studies of Shomer et al. (1993) indicate that the potency of Ca^{2+} inhibition decreases with increasing ionic strength of the bathing medium, and that the relative difference in Ca^{2+} inhibition between normal and MHS RyRs is progressively lost (see Discussion). We examined whether this effect could be directly observed in single-channel activity by recording in the presence of solutions with 250 mM *cis* Cs^+. We also examined whether the inhibitory effect of Mg^{2+} was similarly affected by raised ionic strength, as this would provide further evidence of the common inhibitory action of Ca^{2+} and Mg^{2+} at high concentrations. In *cis* solutions containing 250 mM Cs^+, RyRs were maximally activated by cytoplasmic [Ca^{2+}] between 1 and 100 μM, with average P_o values of 0.35 (± 0.16 SD, n = 32) for normal RyRs and 0.32 (± 0.15, n = 5) for MHS RyRs. As above, the K_i values for Ca^{2+} and Mg^{2+} inhibition were determined for each RyR. Fig. 5

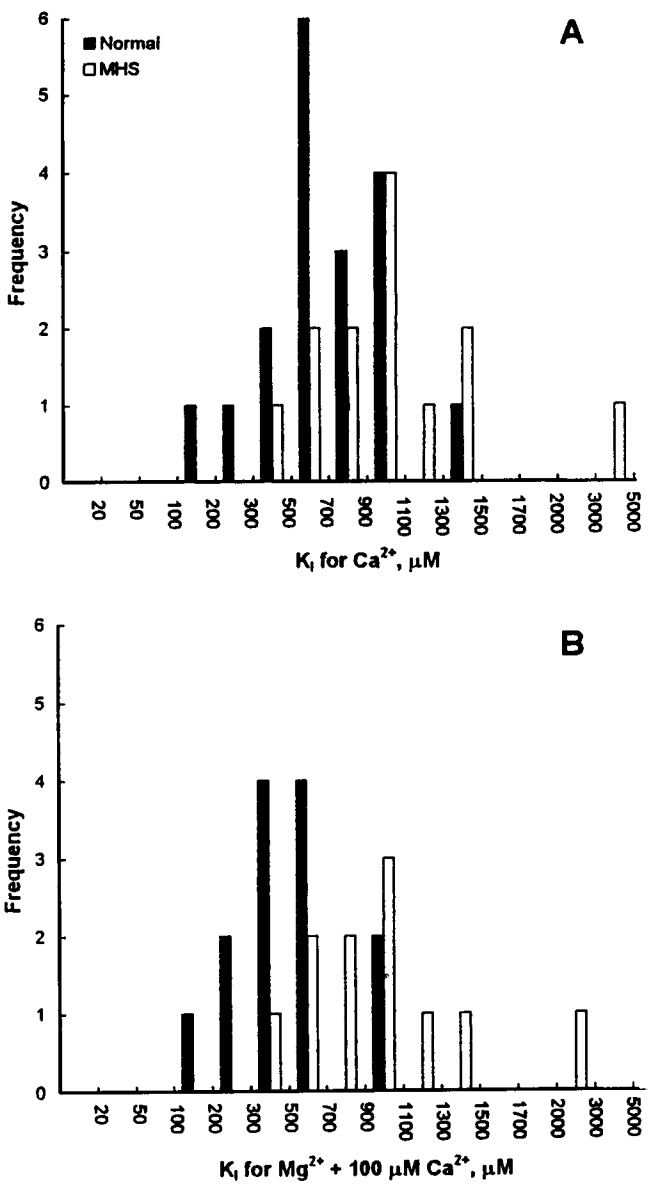

FIGURE 5 Frequency distributions of the concentration (K_i) for half-maximum inhibition of individual RyRs from normal and MHS muscle by Ca^{2+} (*A*) or Mg^{2+} (*B*), in the presence of 250 mM *cis* Cs^+; values obtained by fitting the data for each bilayer experiment separately. Mg^{2+} inhibition was measured in the presence of 100 μM Ca^{2+}, and K_i was derived from plots of P_o versus ([Ca^{2+}] + [Mg^{2+}]).

shows the frequency distributions of the K_i values for Ca^{2+} and Mg^{2+} inhibition for the two RyR genotypes; the Hill coefficients were ~2 in cases where they could be reliably determined. (Hill coefficients of inhibition of MHS RyRs were 2.1 ± 0.2 (n = 10) for Mg^{2+} and 2.2 ± 0.3 (n = 7) for Ca^{2+}, and those in normal RyRs were 2.2 ± 0.2 (n = 8) for Mg^{2+} and 1.9 ± 0.2 (n = 8) for Ca^{2+}). Normal and MHS RyRs were inhibited by Ca^{2+}, with mean K_i values of 710 ± 80 μM (n = 18) and 1160 ± 220 μM (n = 13), respectively. The corresponding K_i values for inhibition by Mg^{2+} + Ca^{2+} (i.e., Mg^{2+} in the presence of 100 μM activating Ca^{2+}) were 488 ± 67 μM (n = 13) and 1030 ± 160 μM (n = 11), respectively. If it is assumed that the K_i values are normally distributed in each case (see Fig. 5), a one-tailed Student's t-test indicates that the MHS RyRs are significantly less sensitive than normal RyRs to inhibition by both Ca^{2+} ($p < 0.05$) and Mg^{2+} ($p < 0.01$). Statistical analysis of the data by the Wilcoxon-Mann-Whitney rank test, which makes no assumption that the data are normally distributed, also establishes that MHS RyRs are significantly less sensitive than normal RyRs to inhibition by both Ca^{2+} ($p < 0.02$) and Mg^{2+} ($p < 0.005$). Furthermore, comparison of Figs. 3 and 5 clearly indicates that both Ca^{2+} and Mg^{2+} inhibited RyR activity with lower affinity in the 250 mM Cs^+ than in the 100 mM Cs^+ solutions (see also Table 1).

The P_o measurements obtained in 250 mM Cs^+ from all RyRs of the same genotype were also pooled together, and best-fit Hill plots were calculated for the mean data, as shown in Fig. 6. The K_i values of the best fits to the Ca^{2+} inhibition data were 614 μM and 1090 μM in normal and MHS RyRs, respectively, and the corresponding values for Mg^{2+} inhibition were 517 μM and 838 μM, respectively. These values for the fits to the pooled data are very similar to the respective mean K_i values for the fits to individual RyR data (see above) and again indicate that, in the presence of 250 mM Cs^+, the MHS RyRs were ~50% less sensitive to divalent cation inhibition than the normal RyRs. As pointed out above for the 100 mM Cs^+ data, such pooling of the data 1) "smears out" the data, giving an erroneous indication of the Hill coefficient of the individual RyRs (~1.5 for pooled data (see Fig. 6) versus ~2 for individual RyRs (see above)), and 2) does not allow any simple statistical analysis of the relative inhibitory effect in the two genotypes. We show the pooled data here for illustrative purposes only, and reiterate that in cases such as this, where the difference in the mean inhibitory effect between normal and MHS RyRs is comparatively small (because of the increase in ionic strength), tests of statistical difference must be based on the data for the individual RyRs, as detailed above.

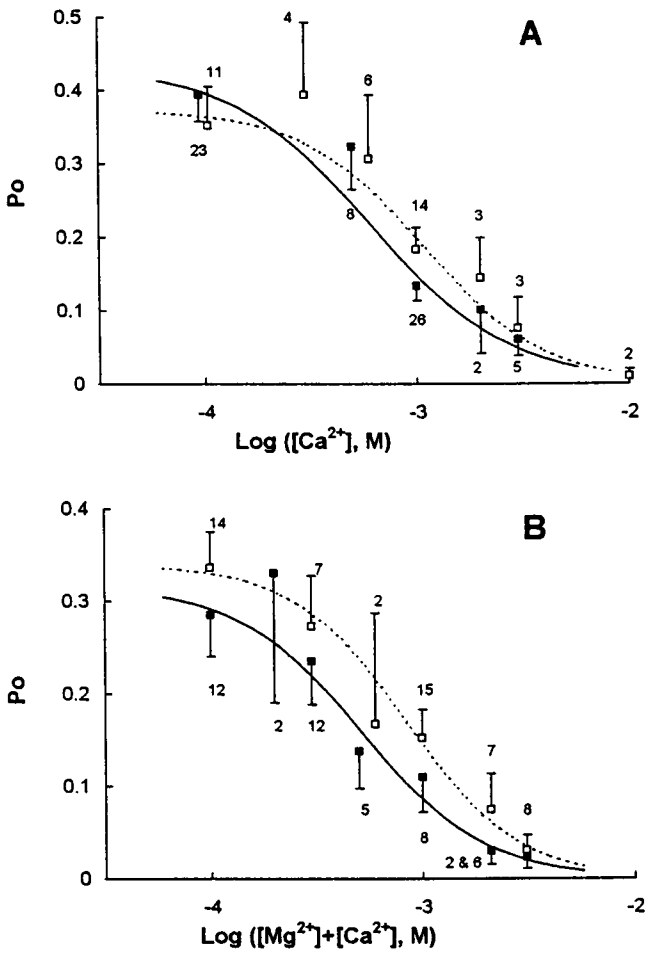

FIGURE 6 Pooled data of open probability, P_o, for all normal RyRs (■) and MHS RyRs (□), showing the inhibition of these channels by cytoplasmic [Ca^{2+}] (A) or by cytoplasmic [Mg^{2+}] in the presence of 100 μM Ca^{2+} (B). Solutions also contained 250 mM Cs^+ in the *cis* bath and 50 mM Cs^+ in the *trans* bath. The average P_o (± SE) from different experiments at each [Ca^{2+}] or ([Ca^{2+}] + [Mg^{2+}]) was fitted (by weighted least squares) with the Hill equation (Eq. 1) to the normal (——) and MHS data (·····). The data labels show the number of experiments included in each average. The total number of experiments, n (and the number with one, two, or three RyRs in the bilayer) were 13 (8, 3, 2) for normal RyRs with Mg^{2+}, 15 (7, 8, 0) for MHS RyRs with Mg^{2+}, 26 (20, 4, 2) for normal RyRs with Ca^{2+}, and 15 (9, 6, 0) for MHS RyRs with Ca^{2+}. Parameters for the best fits: (A) ——, P_{max} = 0.43, H = 1.3, K_i = 614 μM; ·····, P_{max} = 0.37, H = 1.5, K_i = 1090 μM; (B) ——, P_{max} = 0.32, H = 1.5, K_i = 517 μM; ·····, P_{max} = 0.34, H = 1.65, K_i = 838 μM.

Correlation of Mg^{2+} and Ca^{2+} inhibition in individual RyRs

Finally, the above results show that the average inhibitory effects of Ca^{2+} on RyRs were altered in the same way as those of Mg^{2+}, both by the MH mutation and by changes in ionic strength. However, there was a considerable spread in the individual K_i values in each circumstance, making it difficult to be certain that the inhibitory effects of the two cations were indeed related. Consequently, we further examined the relationship between Ca^{2+} inhibition and Mg^{2+} inhibition by comparing them in the same channel. Fig. 7 shows that in individual RyRs, irrespective of genotype, ionic strength, or unexplained RyR variability, there is a close correlation in the abilities of Ca^{2+} and Mg^{2+} to cause inhibition.

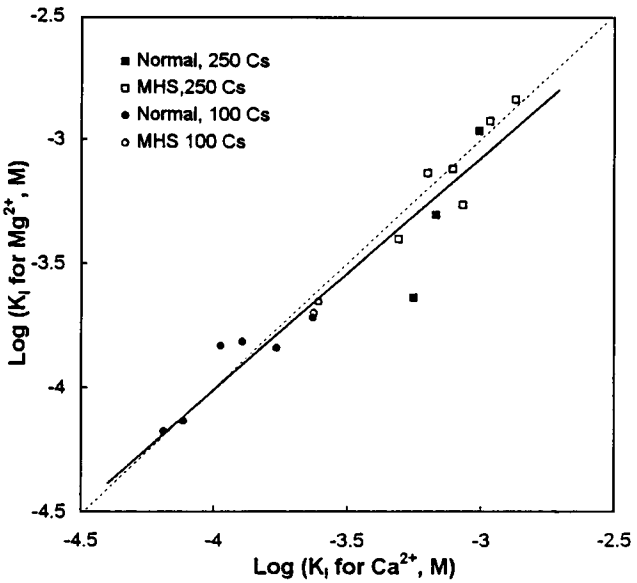

FIGURE 7 A correlation plot showing the concentration, K_i, for half-maximum inhibition by Mg^{2+} plotted against the value obtained for half-maximum inhibition by Ca^{2+} in the same experiment. Measurements were made in both normal and MHS RyRs, in the presence of *cis* 100 or 250 mM Cs^+, as indicated. This figure shows that in individual RyRs, irrespective of genotype and ionic strength, Ca^{2+} and Mg^{2+} have a virtually identical inhibitory effect at high concentration (best fit shown as *solid line*; correlation coefficient $r^2 = 0.95$). The dashed line indicates identical K_i for Ca^{2+} and Mg^{2+} inhibition.

DISCUSSION

Ca^{2+} and Mg^{2+} inhibition

In agreement with previous single-channel studies of RyRs in pigs (Fill et al., 1990; Shomer et al., 1993), we found here that high *cis* (i.e., myoplasmic) [Ca^{2+}] has a significantly smaller inhibitory effect on MHS RyRs than it does on normal RyRs (Figs. 3 *A* to 6 *A*). Most importantly, we also found that the inhibitory effect of myoplasmic Mg^{2+} on the MHS RyRs is also reduced to a similar extent compared to normal RyRs, both at lower (100 mM Cs^+; Figs. 3 *B* and 4 *B*) and higher (250 mM Cs^+, Figs. 5 *B* and 6 *B*) ionic strengths. We have shown previously (Laver et al., 1997) that, in isolated RyRs from rabbit and sheep muscle, Mg^{2+} can inhibit channel activity by two independent mechanisms: 1) by acting at a low-affinity inhibitory site that discriminates poorly between Ca^{2+} and Mg^{2+} and 2) by competitively interfering with Ca^{2+} binding at the Ca^{2+} activation site. However, because the relative affinity of the activation site for Mg^{2+} was ~1000-fold lower than that for Ca^{2+}, the latter type of inhibition was negligible, provided that there was at least 1 μM activating Ca^{2+}. The results reported here suggest that a similar situation exists in porcine skeletal RyRs. In the experiments described here, the net inhibitory effect of Mg^{2+} was very similar in the presence of either 1 μM or 50 μM activating Ca^{2+}. This indicates that, under these conditions, the inhibitory effect of Mg^{2+} due to competition at the activation site contributes relatively little to the total inhibitory effect, even with the lower activating [Ca^{2+}], and that certainly this effect must be quite negligible with the higher activating [Ca^{2+}] (i.e., for Figs. 3–6). (We note that the Hill coefficients for Mg^{2+} inhibition were considerably higher for 1 μM activating Ca^{2+} (~3.4) than for 50 μM Ca^{2+} (~2), which might indicate that the second inhibitory mechanism is starting to become appreciable when the activating [Ca^{2+}] is lowered to 1 μM; this would be best examined further by comparing the net inhibitory effect of Mg^{2+} for the two conditions in the same RyR).

Importantly, we also show that, when examined in the same RyR, the low-affinity inhibitory effect of Mg^{2+} is virtually identical to that of Ca^{2+} (Fig. 7), irrespective of both the RyR genotype and the ionic strength used. This is very strong evidence that these two divalent cations act on the same inhibitory site in RyRs from pig skeletal muscle, as concluded previously for RyRs from other species (Meissner et al., 1986, 1997; Soler et al., 1992; Laver et al., 1997). (It also shows that any apparent difference in the mean inhibitory effects of Ca^{2+} and Mg^{2+} found in different matching experiments (e.g., normal RyRs in Fig. 4 *A* versus Fig. 4 *B*) is due entirely to sampling bias). The similarity in the inhibitory actions of Ca^{2+} and Mg^{2+} in both normal and MHS porcine muscle is also apparent from the findings of many different types of studies of RyR channel activity, as can be seen from Table 1 when values at similar ionic strength are compared. Studies of ryanodine binding, SR Ca^{2+} release, and single RyR activity in normal porcine muscle all indicate that, when the ionic strength is ~100 mM (actually slightly above this because of the presence of ions used in pH buffering), half-inhibition of channel activity occurs between 75 μM and 200 μM Ca^{2+} and between 100 μM and 230 μM Mg^{2+}. Similarly, in MHS muscle the corresponding ranges are 250 μM to ~1000 μM for Ca^{2+} and 250 μM to 780 μM for Mg^{2+}. Note also that in each study the half-inhibitory concentration of the divalent cation was 1.5–5 times higher for the MHS RyR than for the normal RyR, for both Ca^{2+} and Mg^{2+} (Table 1). In summary, the results of this and other studies indicate that, in both the normal and MHS porcine RyR, Mg^{2+} and Ca^{2+} indeed bind to the same inhibitory site, and that this site is aberrant in the MHS RyR, with its affinity for the divalent cations being reduced severalfold at physiological ionic strength.

The similar affinity of the RyR inhibitory site for Mg^{2+} and Ca^{2+} should not be surprising, because previous work has indicated that this low-affinity site discriminates poorly between all divalent metal cations examined (e.g., Ca^{2+}, Mg^{2+}, Sr^{2+}, Mn^{2+}, Soler et al., 1992; Mg^{2+}, Co^{2+}, Ni^{2+}, Ba^{2+}, Nagura et al., 1988; Ca^{2+}, Sr^{2+}, Mg^{2+}, Ba^{2+}, Meissner et al., 1997). In fact, trivalent metal cations have a similar inhibitory effect, but with ~100-fold higher affinity than the divalent cations ($K_i \approx 1$ μM; Soler et al., 1992). Of course, the affinities of the inhibitory site for different divalent cations, although similar, may well still differ by a

TABLE 1 [Ca^{2+}] or [Mg^{2+}] causing half-inhibition of various measures of RyR channel activity in porcine muscle.

	Normal RyR (μM)	MHS RyR (μM)	Reference
Lower ionic strength			
Ca^{2+}			
Ryanodine binding (100 mM NaCl)	100	300	Shomer et al. (1993)
Single channel (100 mM CsCl)	75	250	Shomer et al. (1993)
Single channel (100 mM CsCl)	200	~1000	This study
Mg^{2+}			
Ryanodine binding (100 mM KCl)	210	340	Mickelson et al. (1990)
Ca^{2+} release (100 mM KCl)	~170	~250	Carrier et al. (1991)
Ca^{2+} release (100 mM KCl)	230	780	O'Brien (1986)
Ca^{2+} release (50 mM K_2 HDTA)	100	≥200	Owen et al. (1997)
Single channel (100 mM CsCl)	~100	~300	This study
Higher ionic strength			
Ca^{2+}			
Single channel (250 mM CsCl)	~200	~2500	Fill et al. (1990)
Single channel (250 mM CsCl)	610	1090	This study
Mg^{2+}			
Single channel (250 mM CsCl)	520	840	This study

Carrier et al. (1991) data were estimated from figure 5 in that paper.
Owen et al. (1997) data show [Mg^{2+}] threshold for self-reinforcing Ca^{2+} release at 125 mM K/50 mM HDTA
Values given for this study are indicative both of the K_i for the best fit to the pooled data for all RyRs and the median K_i of all the individual RyRs
Values for half-inhibition by Ca^{2+} very similar to those above in normal RyRs from pig were also obtained in rabbit muscle: 100 μM Ca^{2+} for Ca^{2+} release in 100 mM KCl; 49 μM Ca^{2+} for ryanodine binding in 100 mM KCl; and 523 μM, 290 μM, and 255 μM Ca^{2+} for ryanodine binding in 250 mM NaCl, CsCl, and KCl, respectively (Meissner et al., 1986, 1997).

factor of 2 or more, and this factor may vary according the exact conditions (e.g., ionic strength, etc.). Nevertheless, the near-identity of the K_i values found for Mg^{2+} and Ca^{2+} when examined in the same channel (Fig. 7) indicates that any such difference between the affinities for Ca^{2+} and Mg^{2+} is small.

The location of the low-affinity inhibitory binding site on the RyR molecule has not yet been specifically identified. A likely candidate is the very distinctive sequence of 30 consecutive negative amino acids (with only one intervening positive residue) at positions 1873–1903 in the skeletal muscle RyR, which might be expected to act as a low-affinity cation-binding site that shows little specificity for ions of similar charge. Significantly, the homologous sequence in the cardiac RyR, where the divalent cation affinity is 10-fold lower (Laver et al., 1997, and references therein), has a far lower net charge. In further support of this proposal, Bhat et al. (1997) have recently reported that a mutated RyR in which amino acids 1641–2437 were absent, formed a functional, Ca^{2+}-activated channel, but required a 10-fold higher [Ca^{2+}] for "inactivation" than the native RyR. If this region is the low-affinity inhibitory site, the findings of this study on the MHS RyR with an $Arg^{615}Cys$ mutation, would indicate that the aberration of channel inhibition in MHS RyRs involves interactions between widely separated parts along the channel protein, presumably reflecting critical aspects of the protein's tertiary structure.

Reduction in Ca^{2+}/Mg^{2+} inhibition at high ionic strength

Shomer et al. (1993) found that the concentration of Ca^{2+} needed to half-maximally inhibit ryanodine binding in SR vesicles from normal and MHS pigs became progressively higher (up to ~50–100-fold) as the concentration of NaCl in the solution was increased from 100 mM to 1 M, indicating that the affinity of the inhibitory site on the RyR for Ca^{2+} was reduced by increased ionic strength. (In fact, this effect may depend principally on the anion concentration rather than the ionic strength per se; see Meissner et al., 1997.) Interestingly, the 3-fold difference in the half-maximum inhibitory [Ca^{2+}] between normal and MHS RyR that was apparent in 100 mM NaCl was progressively lost with increasing ionic strength, with both RyR types requiring a nearly identical, high [Ca^{2+}] for inhibition (~15 mM) in the presence of 1 M NaCl. The results presented here, found by direct observation of RyR channel activity, show a similar effect of ionic strength on Ca^{2+} inhibition, with the K_i of normal RyRs increasing from ~200 μM at 100 mM Cs^+ to 610 μM at 250 mM Cs^+, and with the already elevated K_i in MHS RyR showing little if any change (~1000–1090 μM). Thus the relative difference in the K_i values between normal and MHS RyRs decreased from about 5-fold at 100 mM Cs^+ to only 1.8-fold at 250 mM Cs^+. Importantly, we also found a similar effect of ionic strength on the K_i for Mg^{2+} (a 3-fold difference at 100 mM Cs^+, decreasing to a

1.6-fold difference at 250 mM Cs^+; see Table 1), further supporting the conclusion that Mg^{2+} and Ca^{2+} act on the same inhibitory site. It is also significant that this inhibitory effect of Ca^{2+} and Mg^{2+} is most marked in normal RyRs at close to physiological ionic strength (e.g., 100 mM NaCl or CsCl plus ions added for pH buffering) and that the MHS RyR shows proportionately greater difference from the normal RyR under such conditions. This is particularly important when considering the magnitude and role of this inhibitory effect in a functional muscle fiber (see below).

Heterogeneity in Ca^{2+}/Mg^{2+} inhibition

It is clear from the data in Figs. 3–6 that there was considerable variation between different RyRs in the affinity of the inhibitory site for Ca^{2+} and Mg^{2+}, although we emphasize that the inhibitory effects of the two cations were very similar when examined in the same RyR (Fig. 7). In most cases (e.g., normal RyRs in Fig. 5), it could perhaps have been assumed that the individual K_i values were normally distributed and arose from a homogeneous population of RyRs. However, at least in the case of the K_i values for Mg^{2+} inhibition in normal RyRs in 100 mM CsCl (i.e., *filled columns* in Fig. 3 *B*), the great disparity in values for two RyRs (1.3 mM and 5 mM) compared to the remaining 19 channels (40–303 μM, with a mean of 122 μM), suggests that there were at least two distinct populations of RyRs present. This heterogeneity highlights the need to obtain measurements from a substantial number of channels for each condition if one wishes to determine whether there is a significant difference in the properties of the RyRs from normal and MHS animals. It also shows that any statistical test for determining whether the inhibitory effect of Mg^{2+} or Ca^{2+} is different between normal and MHS RyRs must be based on measurements from the individual channels (e.g., Fig. 3 *B*) rather than on a Hill fit to the average data (e.g., Fig. 4 *B*), which 1) can be "stretched out" compared to the fits to the individual RyR (i.e., have a lower Hill coefficient) owing to the variation in individual K_i values (see discussion in Laver et al., 1995), and 2) is inappropriate for a bimodal distribution of K_i values.

The basis of the heterogeneity in the K_i values is not clear. It might reflect differences in the state of the RyRs in vivo, due to phosphorylation (Hain et al., 1994) or the presence or absence of associated proteins such as calmodulin (Tripathy et al., 1995) or FK506-binding protein (FKBP) (Timerman et al., 1993). In fact, removal of FKBP has been shown to cause a ~10-fold decrease in the inhibitory effect of Ca^{2+} (Ahern et al., 1997). The heterogeneity might also reflect different responses of the four splice variants of the RyR (Futatsugi et al., 1995). Alternatively, the heterogeneity might have arisen because of alterations occurring during the isolation and recording procedure. If the latter, it might then be proposed that the difference in the inhibitory effects of Ca^{2+} and Mg^{2+} between normal and MHS RyRs is merely the result of a greater sensitivity of the MHS RyRs to such treatment, rather than of some difference in the RyRs in vivo. Such a criticism would also have to be made of all other studies on isolated SR and single RyRs, particularly given that they are consistent with an aberration in the Ca^{2+}/Mg^{2+} inhibition in MHS RyRs (e.g., Table 1). However, it seems unlikely that differential sensitivity of the MHS RyRs to experimentation-induced alteration could account for observed differences in K_i, because reduced Mg^{2+} inhibition of Ca^{2+} release has also been observed in mechanically skinned muscle fibers from MHS pigs, where the RyRs remain in situ, and normal excitation-contraction coupling is preserved (Owen et al., 1997).

Contrary conclusions

It has been briefly reported that the half-inhibitory concentration of Mg^{2+} is not significantly different between normal and MHS RyRs (Shomer et al., 1995; Balog et al., 1997). The reason for these contrary results is not entirely clear. RyRs in lipid bilayers generally show considerable unexplained variations in their open probability and their response to ligands such as Ca^{2+} and Mg^{2+} (Meissner, 1994), as illustrated by the large spread in our values of K_i (Figs. 3 and 5), and the large error bars on the open probability estimates of RyRs shown by Shomer et al. (1993). Thus variability within a small sample of only five RyRs from each genotype (Shomer et al., 1995) might have masked differences in Mg^{2+} inhibition between the normal and MHS RyRs. Shomer et al. (1995) also report that the $[Mg^{2+}]$ causing half-maximum inhibition of ryanodine binding was not significantly different in SR vesicles from normal and MHS muscle (217 ± 113 μM and 292 ± 95 μM, respectively), although this is at variance with an earlier study of theirs (Mickelson et al., 1990), which found a significant difference (210 ± 10 μM and 340 ± 50 μM, respectively). Furthermore, as the inhibitory effect of Ca^{2+} on ryanodine binding is indeed reduced in MHS RyRs (Mickelson et al., 1990; Shomer et al., 1993), the lack of a similar change in Mg^{2+} inhibition cannot be reconciled with the conclusion of other studies that both divalent cations act on the same low-affinity inhibitory site (Meissner et al., 1986, 1997; Soler et al., 1992; Laver et al., 1997; this study). Finally, Shomer et al. (1995) found that the optimal $[Ca^{2+}]$ for activation of isolated RyRs was increased from ~10 μM to ~4 mM in the presence of 5 mM Mg^{2+}, from which the authors concluded that Mg^{2+} was acting solely by competing with Ca^{2+} at the activation site. However, a large shift in the optimal activating $[Ca^{2+}]$ is entirely consistent with Mg^{2+} inhibiting RyR activity, both by competing with Ca^{2+} at the activation site and by having a combined effect with Ca^{2+} on a common inhibitory site. In the presence of 5 mM Mg^{2+}, any increase in the $[Ca^{2+}]$ would be stimulatory because it would reduce the inhibitory effect of Mg^{2+} at the activation site, but would cause virtually no additional inhibition via the common inhibitory site, as the latter would already be effectively saturated by the high $[Mg^{2+}]$.

In summary, the data presented by Shomer and colleagues do not necessarily conflict substantially with the data and conclusions presented here.

Physiological consequences of the reduced Ca^{2+}/Mg^{2+} inhibition

The reduced inhibitory effect of Ca^{2+} and Mg^{2+} on the MHS RyR readily explains the hyperresponsiveness of MHS muscle. Because the intracellular [Mg^{2+}] in an intact muscle fiber is ~1 mM (see review by Lamb and Stephenson, 1992), the Ca^{2+}/Mg^{2+} inhibitory site on the RyR in normal muscle will be almost fully saturated with Mg^{2+} (half-inhibition at ~0.1–0.2 mM at physiological ionic strength; see above and Table 1), and consequently the RyR will be potently inhibited and mostly closed. This conclusion is based not only on the inhibition seen here with single RyRs in the lipid bilayer at 1 mM Mg^{2+} (i.e., Fig. 4 *B*), but also on the potent inhibitory effect of myoplasmic [Mg^{2+}] observed 1) in mechanically skinned muscle fibers under conditions of physiological [ATP] and SR Ca^{2+} loading (Lamb and Stephenson, 1991, 1994) and 2) in SR vesicles with 5 mM AMP (Meissner et al., 1997). The effective saturation of the site with Mg^{2+} in vivo underlies our previous proposal that the so-called Ca^{2+} inactivation of the RyR occurring at submillimolar [Ca^{2+}] should actually be thought of and referred to as Mg^{2+} inhibition or Ca^{2+}/Mg^{2+} inhibition (Lamb, 1993).

The results of this study (e.g., Fig. 4 *B*) make it clear that the MHS RyR in vivo will not be as fully inhibited by the intracellular [Mg^{2+}] as the normal RyR. Again, it is apparent from experiments with mechanically skinned fibers that this difference in the inhibitory effect of Mg^{2+} between normal and MHS RyRs also occurs when the channels are in their normal in vivo position, with physiological [ATP], etc. (Owen et al., 1997). Thus the MHS RyRs in vivo should have a slightly higher open probability than the normal RyRs at the same resting [Ca^{2+}]. Consequently, the leakage of Ca^{2+} out of the SR should be slightly higher in MHS muscle. Whether this causes a detectable increase in the resting [Ca^{2+}] (Lopez et al., 1986) or not (Iaizzo et al., 1988) will depend critically on 1) the ability of the SR pump to counter the increased Ca^{2+} leakage, 2) the sensitivity of the measuring technique, and 3) whether the measuring technique actually perturbs the system and helps induce further release. Importantly, the MHS RyR in vivo, being less inhibited by myoplasmic [Mg^{2+}], will also be more readily activated by any stimulus, and consequently it will be more susceptible than the normal RyR to a self-reinforcing cycle of Ca^{2+}-induced Ca^{2+} release. Thus the weaker Mg^{2+} inhibition readily explains the hyperresponsiveness of MHS muscle to caffeine, halothane, or, indeed, any other form of stimulation (Mickelson and Louis, 1996), including the increase in the sensitivity of MHS muscle to small depolarizations (Gallant and Lentz, 1992; El-Hayek et al., 1995). Nevertheless, this phenomenon is totally consistent with the reported similarity in the caffeine sensitivity of normal and MHS RyRs (Shomer et al., 1994a), as such sensitivity was studied in isolated RyRs under conditions where differences in Ca^{2+}/Mg^{2+} inhibition would not have affected the channel activity and where there was no ability for self-reinforcement of the activating stimulus. The increased responsiveness of the RyR in vivo means that MHS muscle operates close to an inherently unstable state in which released Ca^{2+} triggers further release that eventually exceeds the ability of the SR pump to maintain the myoplasmic [Ca^{2+}] at a low level, leading to the catastrophic changes involved in an MH episode.

Finally, we have shown here that the RyR mutation in MH causes reduced Ca^{2+}/Mg^{2+} inhibition. It is possible that the mutation also separately causes other functional alterations in the MHS RyR, such as that responsible for the apparent increase in the peak rate of Ca^{2+} release from the SR in MH (Ohta et al., 1989; Carrier et al., 1991; see Introduction). However, it is quite possible that this latter effect is also due to the reduced affinity of the inhibitory site on the MHS RyR, because Ca^{2+} efflux through the RyR in SR vesicles and skinned fibers could be locally modulating further release (Tripathy and Meissner, 1996), particularly when myoplasmic Mg^{2+} is absent and the activating [Ca^{2+}] is low. Irrespective of this, the reduced inhibitory effect of Mg^{2+} on the low-affinity Ca^{2+}/Mg^{2+} inhibitory site in the $Arg^{615}Cys$-mutated RyR reported here seems likely to play a crucial role in the pathogenesis of porcine MH.

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REFERENCES

Ahern, G. P., P. R. Junankar, and A. F. Dulhunty. 1997. Subconductance states in single-channel activity of skeletal muscle ryanodine receptors after removal of FKBP12. *Biophys. J.* 72:146–162.

Balog, E. M., N. H. Shomer, B. Fruen, J. R. Mickelson, and C. F. Louis. 1997. Regulation of malignant hyperthermia-susceptible (MHS) Ca^{2+} release channels by Mg^{2+}. *Biophys. J.* 72:A274.

Bhat, M. B., J. Zhao, S. Hayek, E. C. Freeman, H. Takeshima, and J. Ma. 1997. The negatively charged D3 region of skeletal muscle ryanodine receptor is involved in ion conduction and Ca-dependent inactivation of the Ca release channel. *Biophys. J.* 72:A169.

Carrier, L., M. Villaz, and Y. Dupont. 1991. Abnormal rapid Ca^{2+} release from sarcoplasmic reticulum of malignant hyperthermia susceptible pigs. *Biochem. Biophys. Acta.* 1064:175–183.

El-Hayek, R., M. Yano, B. Antoniu, J. R. Mickelson, C. F. Louis, and N. Ikemoto. 1995. Altered E-C coupling in triads isolated from malignant hyperthermia-susceptible porcine muscle. *Am. J. Physiol.* 268: 1381–1386.

Fatatsugi, A., G. Kuwajima, and K. Mikoshiba. 1995. Tissue-specific and developmentally regulated alternative splicing in mouse skeletal muscle ryanodine receptor mRNA. *Biochem. J.* 305:373–378.

Fill, M., R. Coronado, J. R. Mickelson, J. Vilven, J. Ma, B. A. Jacobson, and C. F. Louis. 1990. Abnormal ryanodine receptor channels in malignant hyperthermia. *Biophys. J.* 50:471–475.

Foster, P. S., K. C. Hopkinson, N. Payne, and M. A. Denborough. 1991. The effect of azumolene on hypercontractility and sarcoplasmic reticu-

lum Ca^{2+}-dependent ATPase activity on malignant hyperpyrexia-susceptible porcine skeletal muscle. *Clin. Exp. Pharmacol. Physiol.* 18:489–495.

Gallant, E. M., and L. R. Lentz. 1992. Excitation-contraction coupling in pigs heterozygous for malignant hyperthermia. *Am. J. Physiol.* 262: 422–426.

Hain, J., S. Nath, M. Mayrleitner, S. Fleischer, and H. Schindler. 1994. Phosphorylation modulates the function of the calcium release channel of sarcoplasmic reticulum from skeletal muscle. *Biophys. J.* 67: 1832–1833.

Iaizzo, P. A., W. Klein, and F. Lehmann-Horn. 1988. Fura-2 detected myoplasmic calcium and its correlation with contracture force in skeletal muscle from normal and malignant hyperthermia susceptible pigs. *Pflügers Arch.* 411:648–653.

Lamb, G. D. 1993. Ca^{2+} inactivation, Mg^{2+} inhibition and malignant hyperthermia. *J. Muscle Res. Cell Motil.* 14:554–556.

Lamb, G. D., and D. G. Stephenson. 1991. Effect of Mg^{2+} on the control of Ca^{2+} release in skeletal muscle fibres of the toad. *J. Physiol. (Lond.).* 434:507–528.

Lamb, G. D., and D. G. Stephenson. 1992. Importance of Mg^{2+} in excitation-contraction coupling in skeletal muscle. *News Physiol. Sci.* 7:270–274.

Lamb, G. D., and D. G. Stephenson. 1994. Effects of intracellular pH and $[Mg^{2+}]$ on excitation-contraction coupling in skeletal muscle fibres of the rat. *J. Physiol. (Lond.).* 478:331–339.

Laver, D. R., T. M. Baynes, and A. F. Dulhunty. 1997. Magnesium-inhibition of ryanodine-receptor calcium channels: evidence for two independent mechanisms. *J. Membr. Biol.* 156:213–229.

Laver, D. R., and B. A. Curtis, 1996. Response of ryanodine receptor channels to Ca^{2+} steps produced by rapid solution exchange. *Biophys. J.* 71:732–741.

Laver, D. R., L. D. Roden, G. P. Ahern, K. R. Eager, P. R. Junankar, and A. F. Dulhunty. 1995. Cytoplasmic Ca^{2+} inhibits the ryanodine receptor from cardiac muscle. *J. Membr. Biol.* 147:7–22.

Lopez, J. R., L. Alamo, D. Jones, P. Allen, L. Papp, J. Gergely, and F. Sreter. 1986. $[Ca^{2+}]_i$ in muscles of malignant hyperthermia pigs determined in vivo with Ca^{2+}-selective microelectrodes. *Arch. Biochem. Biophys.* 247:294–301.

Ma, J., M. B. Bhat, and J. Zhao. 1995. Rectification of skeletal muscle ryanodine receptor mediated by FK506 binding protein. *Biophys. J.* 69:2398–2404.

MacLennan, D. H., and M. S. Phillips. 1992. Malignant hyperthermia. *Science.* 256:789–794.

Meissner, G. 1984. Adenine nucleotide stimulation of Ca^{2+}-induced Ca^{2+} release in sarcoplasmic reticulum. *J. Biol. Chem.* 259:2365–2374.

Meissner, G. 1994. Ryanodine receptor/Ca^{2+} release channels and their regulation by endogenous effectors. *Annu. Rev. Physiol.* 56:485–508.

Meissner, G., E. Darling, and J. Eveleth. 1986. Kinetics of rapid Ca^{2+} release from the sarcoplasmic reticulum. Effects of Ca^{2+}, Mg^{2+}, and adenine nucleotides. *Biochemistry.* 25:236–244.

Meissner, G., E. Rios, A. Tripathy, and D. A. Pasek. 1997. Regulation of skeletal muscle Ca^{2+} release channel (ryanodine receptor) by Ca^{2+} and monovalent cations and anions. *J. Biol. Chem.* 272:1628–1638.

Mickelson, J. R., L. A. Litterer, B. A. Jacobson, and C. F. Louis. 1990. Stimulation and inhibition of [^{3}H]ryanodine binding to sarcoplasmic reticulum from malignant hyperthermia susceptible pigs. *Arch. Biochem. Biophys.* 278:251–257.

Mickelson, J. R., and C. F. Louis. 1996. Malignant hyperthermia: excitation-contraction coupling, Ca^{2+} release channel, and cell Ca^{2+} regulation defects. *Physiol. Rev.* 76:538–592.

Miller, C., and E. Racker. 1976. Ca^{++}-induced fusion of fragmented sarcoplasmic reticulum with artificial planar bilayers. *Cell.* 9:283–300.

Mueller, P., D. O. Rudin, H. C. Tien, and W. C. Westcott. 1962. Reconstitution of cell membrane structure in vitro and its transformation into an excitable system. *Nature.* 194:979–981.

Nagura, S., T. Kawasaki, T. Taguchi, and M. Kasai. 1988. Calcium release from isolated sarcoplasmic reticulum due to 4,4′-dithiodipyridine. *J. Biochem.* 104:461–465.

O'Brien, P. J. 1986. Porcine malignant hyperthermia susceptibility: hypersensitive calcium-release mechanism of skeletal muscle sarcoplasmic reticulum. *Can. J. Vet. Res.* 50:318–328.

Ohta, T., M. Endo, T. Nakano, Y. Morohoshi, K. Wanikawa, and A. Ohga. 1989. Ca-induced Ca release in malignant hyperthermia-susceptible pig skeletal muscle. *Am. J. Physiol.* 256:C358–C367.

Otsu, K., M. S. Phillips, V. K. Khanna, S. De Leon, and D. H. MacLennan. 1992. Refinement of diagnostic assays for a probable causal mutation for porcine and human malignant hyperthermia. *Genomics.* 13:835–837.

Owen, V. J., N. L. Taske, and G. D. Lamb. 1997. Reduced Mg^{2+} inhibition of Ca^{2+} release in muscle fibres of pigs susceptible to malignant hyperthermia. *Am. J. Physiol.* 272:203–211.

Shomer, N. H., B. Fruen, J. R. Mickelson, and C. F. Louis. 1995. Mg^{2+} regulation of the malignant hyperthermia-susceptible (MHS) Ca^{2+} release channel. *Biophys. J.* 68:A173.

Shomer, N. H., C. F. Louis, M. Fill, L. A. Litterer, and J. R. Mickelson. 1993. Reconstitution of abnormalities in the malignant hyperthermia-susceptible pig ryanodine receptor. *Am. J. Physiol.* 264:125–135.

Shomer, N. H., J. R. Mickelson, and C. F. Louis. 1994a. Caffeine stimulation of malignant hyperthermia-susceptible sarcoplasmic reticulum Ca^{2+} release channel. *Am. J. Physiol.* 267:1235–1261.

Shomer, N. H., J. R. Mickelson, and C. F. Louis. 1994b. Ion selectivity of porcine skeletal muscle Ca^{2+} release channels is unaffected by the Arg^{616} to Cys^{615} mutation. *Biophys. J.* 67:641–646.

Smith, J. S., T. Imagawa, J. Ma, M. Fill, K. P. Campbell, and R. Coronado. 1988. Purified ryanodine receptor from rabbit skeletal muscle is the calcium-release channel of sarcoplasmic reticulum. *J. Gen. Physiol.* 92:1–26.

Soler, F., F. Fernandez-Belda, and J. C. Gomez-Fernandez. 1992. The Ca^{2+} release channel in junctional sarcoplasmic reticulum: gating and blockade by cations. *Int. J. Biochem.* 24:903–909.

Timerman, A. P., E. Ogunbumni, E. Freund, G. Wiederrech, A. R. Marks, and S. Fleischer. 1993. The calcium release channel of sarcoplasmic reticulum is modulated by FK-506-binding protein. *J. Biol. Chem.* 268:22992–22999.

Tinker, A., and A. J. Williams. 1992. Divalent cation conduction in the ryanodine receptor channel of sheep cardiac muscle sarcoplasmic reticulum. *J. Gen. Physiol.* 100:479–493.

Tripathy, A., and G. Meissner. 1996. Sarcoplasmic reticulum lumenal Ca^{2+} has access to cytosolic activation and inactivation sites of skeletal muscle Ca^{2+}. *Biophys. J.* 70:2600–2615.

Tripathy, A., L. Xu, G. Mann, and G. Meissner. 1995. Calmodulin activation and inhibition of skeletal muscle Ca^{2+} release channel (ryanodine receptor). *Biophys. J.* 69:106–119.