Abnormal ryanodine receptor channels in malignant hyperthermia

Michael Fill,* Roberto Coronado,* James R. Mickelson,‡ Janeen Vilven,* Jianjie Ma,*
Blake A. Jacobson,‡ and Charles F. Louis‡§
*Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, Texas 77030; and
Departments of ‡Veterinary Biology and §Biochemistry, University of Minnesota, St. Paul, Minnesota 55108

ABSTRACT Previous studies have demonstrated a defect associated with the calcium release mechanism of sarcoplasmic reticulum (SR) from individuals susceptible to malignant hyperthermia (MH). To examine whether SR calcium release channels were indeed altered in MH, SR vesicles were purified from normal and MH susceptible (MHS) porcine muscle. The Ca^{2+} dependence of calcium efflux rates from $^{45}Ca^{2+}$-filled SR vesicles was then compared with the Ca^{2+} dependence of single-channel recordings of SR vesicles incorporated into planar lipid bilayers. The rate constants of $^{45}Ca^{2+}$ efflux from MHS SR were two to threefold larger than from normal SR over a wide range of myoplasmic Ca^{2+}. Normal and MHS single channels were progressively activated in a similar fashion by *cis* Ca^{2+} from pCa 7 to 4. However, below pCa 4, normal channels were inactivated by *cis* Ca^{2+}, whereas MHS channels remained open for significantly longer times. The altered Ca^{2+} dependence of channel inactivation in MHS SR was also evident when Ca^{2+} was increased on the *trans* side while *cis* Ca^{2+} was held constant. We propose that a defect in a low-affinity Ca^{2+} binding site is responsible for the altered gating of MHS SR channels. Such a defect could logically result from a mutation in the gene encoding the calcium release channel, providing a testable hypothesis for the molecular basis of this inherited disorder.

INTRODUCTION

Malignant hyperthermia (MH) is an inherited pharmacogenetic disorder characterized by an accelerated skeletal muscle metabolism, muscle rigidity, and rapidly rising body temperature (1, 2). MH is triggered in as many as one in 15,000 anesthetic procedures, and can be fatal if not treated promptly (3–5). Numerous reports indicate that regulation of myoplasmic Ca^{2+} is abnormal in MH susceptible (MHS) individuals, primarily due to an abnormality in the sarcoplasmic reticulum (SR) Ca^{2+} release mechanism (6–11). The defect appears closely associated with the ryanodine receptor, the protein that forms the SR Ca release channel (12–16), because binding of [^{3}H]ryanodine to MHS SR exhibits an altered Ca^{2+} dependence and a higher affinity for ryanodine than normal SR (17).

In this paper we show that ryanodine receptor channels from the SR of a homozygous recessive pig model of MH (18) remain open significantly longer than do channels from homozygous normal animals. This is due to the failure of MHS channels to inactivate in physiological Ca^{2+} gradients, and appears to result from an altered low-affinity Ca^{2+} binding site in the channel pore. This alteration may have resulted from a mutation in the gene encoding for the ryanodine receptor.

Address correspondence to Dr. C. F. Louis.

METHODS

Heavy SR vesicles from the longissimus dorsi muscle of MHS and normal pigs were prepared in the presence of a cocktail of protease inhibitors (PMSF, benzamidine, leupeptin, pepstatin, and aprotinin) (17) using discontinuous sucrose gradient centrifugation (19), and did not differ in Ca^{2+}-ATPase activity, phospholipid and cholesterol content, or the Coomassie blue staining pattern on SDS polyacrylamide gels (9, 17). Vesicles were stored at −80°C until used. All pigs were obtained from a herd maintained by Dr. William Rempel, of the Department of Animal Science at the University of Minnesota, for genetic studies of MH inheritance. The pigs were tested for MH susceptibility by a halothane challenge test (exposure to 3% halothane in oxygen by mask), with the MHS Pietrain strain (homozygous for the gene responsible for MH) responding to the test with limb muscle rigidity in <4 min exposure; the normal Yorkshire strain of pigs (homozygous for the normal allele) did not react to the halothane test.

To determine the rate constant of calcium release, SR vesicles (1.5 mg/ml) were first passively loaded with $^{45}Ca^{2+}$ for 2 h at 22°C in 0.1 M KCl, 20 mM Pipes (pH 7.0), 5 mM $CaCl_2$ (containing 0.5 mCi/ml $^{45}Ca^{2+}$). Under these conditions both MHS and normal SR loaded in the range 50–75 nmol Ca/mg SR; there was no difference in the Ca loading of these two types of SR (9). Calcium release was initiated by rapid mixing (Update System 1000 Chemical/Freeze Quench Apparatus, Update Instruments Inc., Madison, WI) of the $^{45}Ca^{2+}$-loaded SR vesicles with solutions containing 0.1 M KCl, 20 mM Pipes (pH 7.0) and various EGTA plus nitriloacetic acid–containing media to obtain the indicated final Ca^{2+} concentrations (17). Calcium release was stopped at various times after the initial mixing (50–600 ms depending on the rate of release) by further rapid mixing with 0.1 M KCl, 20 mM Pipes, 10 μM ruthenium red, 10 mM $MgCl_2$, 10 mM EGTA (final concentrations), and the SR calcium content was determined after filtration of samples on 0.45-μm filters (Millipore/Continental Water Systems, Bedford, MA) (20). Rate constants of calcium release were determined from semilogarithmic plots of SR calcium content vs. time as described

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by Meissner et al. (20). The SR calcium content was in all cases corrected for the nonreleasable calcium component (18, 20). The driving force for Ca^{2+} release (E_{Ca}) was calculated from an Eyring rate model (21) restricted to conduction in single-file with three free energy peaks and two free energy wells. Parameters that fit current-voltage curves of release channels under the same conditions of efflux experiments are:

Ion	E_1	E_2	E_3	E_4	E_5
K	9.0	−1.0	4.0	−1.0	9.0
Ca	3.5	−10.0	2.0	−10.0	3.5

Where E_1, E_3, and E_5 are free energy peaks in RT units; E_2 and E_5 are free energy wells in RT units. Peaks and wells are located symmetrically within the electric field.

Planar lipid bilayers were formed by the Mueller-Rudin method in Delrin partitions with apertures of 300 μm. The decane-lipid mixture was composed of bovine brain phosphatidylethanolamine and phosphatidylserine each at 10 mg/ml (Avanti Polar Lipids, Inc., Birmingham, AL). The *cis* side was connected to the head-stage input of a model EPC 7 amplifier (List-Electronic, Eberstadt, FRG). The *trans* side was held at virtual ground. Data was stored on FM tape, played back, and filtered at a corner frequency of 2 KHz using an eight-pole Bessel filter, and digitized for computer analysis at 71 KHz using a 12-bit A/D converter. Recording solutions were 250 mM CsCl in the *cis* chamber (connected to the headstage amplifier), and 50 mM CsCl in the *trans* chamber (connected to ground). Buffer in both compartments was 20 mM MOPS, pH 7.2. In approximately 30% of vesicle fusions, release channels could be recorded in the absence of Cl^- channels and these cases were used for analysis. The total recording time was ~1,200 minutes from seven preparations each of normal and MHS SR.

RESULTS AND DISCUSSION

To examine whether ryanodine receptor channels were altered in MH, we purified SR vesicles from normal and MHS muscle and compared the Ca^{2+} dependence of $^{45}Ca^{2+}$ efflux rates with that of single-channel recordings in planar lipid bilayers. Fig. 1 shows that Ca^{2+}-induced Ca^{2+} release is more prominent in MHS SR because efflux rate constants are two- to threefold larger than from normal SR over a wide range of myoplasmic Ca^{2+}. The Ca^{2+} concentration required to activate the Ca^{2+} release rate by 50% was 0.30 ± 0.18 μM and 0.31 ± 0.04 μM, for three preparations each of MHS and normal SR, respectively; in both cases, maximal release rates were at ~6 μM Ca^{2+}. In these experiments the driving force for Ca^{2+} efflux decreased as the Ca^{2+} gradient decreased. A theoretical calculation of the decrease in driving force for Ca^{2+} (E_{Ca}) as a function of extravesicular Ca^{2+} and constant 5 mM intravesicular Ca^{2+} is shown in the inset of Fig. 1. E_{Ca} decreases continuously with myoplasmic Ca^{2+} and is essentially zero at concentrations > 100 μM. Thus, absolute efflux rates at high Ca^{2+} may be largely underestimated by this experimental approach.

To further define the basis for the increased Ca release rate from MHS SR, and its regulation by Ca^{2+}, we examined recordings of single release channels incorporated into planar lipid bilayers. The polarity of the inserted channels was found to be the same as in previous studies (22), i.e., the myoplasmic side facing into the *cis* solution and the intravesicular side facing into the *trans* solution. A gradient of Cs^+ instead of Ca^{2+} was used as current carrier (250 mM CsCl on the *cis* side and 50 mM CsCl on the *trans* side) (*a*) to avoid the use of large Ca^{2+} gradients which decrease the activity of release channels (15); (*b*) to eliminate the interference from SR K^+ channels, because these are actually blocked by Cs^+ (23); and (*c*) because Cs^+ has a higher conductance than Ca^{2+} or Na^+ through release channels (g_{Cs}/g_{Ca} = 2) (14) and thus is useful in improving the signal to noise ratio. SR Cl channels were separated from release channels on the basis of reversal potential (E_{Cl^-} = +37 mV; E_{Cs^+} = −37 mV).

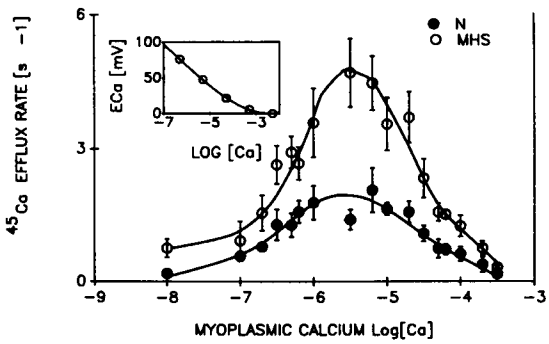

FIGURE 1 Ca^{2+}-dependence of Ca^{2+} release from MHS (*solid circles*) and normal (*open circles*) SR vesicles. Points represent means ± SE for three preparations each of MHS and normal SR. Inset shows the driving force for Ca^{2+} release (E_{Ca}) as a function of extravesicular Ca^{2+} and constant 5 mM intravesicular Ca^{2+}. E_{Ca} is the difference between the Nernst potential for the Ca^{2+} gradient and the reversal potential for the release channel.

The effect of four regulators of SR Ca^{2+} release channels, namely ATP and ryanodine (activators), and Mg^{2+} and ruthenium red (inhibitors) (20, 22) are shown in Fig. 2. In the control periods before additions (10 μM *cis* free Ca^{2+}, +20 mV holding potential), MHS channels had a 12% higher conductance than normal channels (438 pS ± 34, n = 6 for MHS; 383 pS ± 24, n = 7 for normal; $P < 0.01$). ATP increased open probability in both cases by approximately threefold above control (3.1-fold ± 1.2, n = 4 for MHS; 2.9-fold ± 1.2, n = 4 for normal). Ryanodine modified gating of normal and MHS channels in the characteristic way, by decreasing conductance and increasing mean open time (14). Likewise, ruthenium red and millimolar Mg^{2+} blocked all activity seen in controls. These results confirmed that Ca^{2+} release channels in normal and MHS SR are functional, and display pharmacological characteristics already recognized in SR of rabbit skeletal muscle (20, 22).

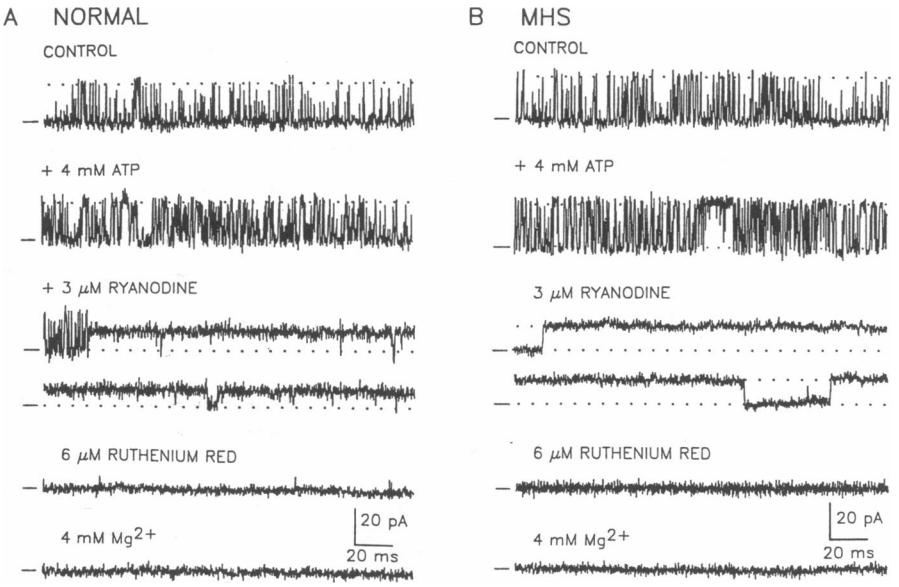

FIGURE 2 Ca^{2+} release channels from normal and MHS SR. Holding potential in all records was +20 mV. Ca^{2+} was present at contaminant levels (6–11 μM). Lines to the left of each record indicate baseline. All additions, including SR protein, were to the *cis* chamber. Records labeled control, ATP, and ryanodine are from the same experiment, whereas records labeled ruthenium red and Mg^{2+} are from separate experiments. (*A*) Normal channel; (*B*) MHS channel. Desired free Ca^{2+} was obtained using Ca^{2+}-EGTA buffers and confirmed with Ca^{2+} electrode and Ca^{2+} dye methods.

The Ca^{2+} dependence of normal and MHS channels as a function of *cis* Ca^{2+} is shown in Fig. 3. Open probabilities, P_o (Fig. 3, top), were computed in the range of *cis* pCa 7 to pCa 2, while keeping *trans* Ca^{2+} constant at 10 μM. At *cis* 10 μM Ca^{2+}, P_o of normal and MHS channels did not differ significantly (0.211 ± 0.130; 0.207 ± 0.116, respectively). Normal and MHS channels were progressively activated by *cis* Ca^{2+} from pCa 7 to 4, which was similar to the concentration range required to activate Ca^{2+}-induced $^{45}Ca^{2+}$ release from SR vesicles (Fig. 1). However, below pCa 4, normal channels were inactivated by *cis* Ca^{2+}, whereas MHS channels remained open for significantly longer times. For instance, normal channels are essentially closed at 1 mM *cis* Ca^{2+}, whereas in MHS channels, the same activity seen at 10 μM is also observed in 1 mM *cis* Ca^{2+}. The Ca^{2+} level at which channel activity was 50% of that recorded at 10 μM *cis* Ca^{2+} differed by an order of magnitude, ~200 μM in normal and 2.5 mM Ca^{2+} in MHS channels.

The open time histograms at three *cis* Ca^{2+} concentrations is shown in the bottom of Fig. 3. The lifetime of normal and MHS channels at 10 μM *cis* Ca^{2+} was best described by a single exponential distribution with similar mean open times of 1.1 ms (1,743 events) and 1.0 ms (1,255 events), respectively. At 1 mM Ca^{2+}, histograms of normal channels remained monoexponential (mean lifetime of 0.4 ms, 434 events), whereas those of MHS channels showed in addition to the brief events with a lifetime of 1.5 ms, a tail distribution of longer events with a mean lifetime of 13.9 ms (294 events). Evidently, MHS channels not only failed to inactivate in high *cis* Ca^{2+}, but also remained open for longer times.

The altered Ca^{2+} dependence of channel inactivation in MHS SR was also evident when Ca^{2+} was increased on the *trans* side while *cis* Ca^{2+} was held constant at physiological levels. In Fig. 4, channels were first incorporated in symmetrical 10 μM free Ca^{2+} and afterwards *trans* free Ca^{2+} was raised to the indicated value. Control activity in symmetrical 10 μM Ca^{2+} was deliberately chosen to be high (channels with $P_o < 0.2$ were rejected) so that the decrease in P_o in normal channels in high *trans* Ca^{2+} could be adequately measured. Previous studies have demonstrated that millimolar *trans* Ca^{2+} inactivates K^+ movement through rabbit SR Ca channels (15). With Cs^+ as the permeant ion, high *trans* Ca^{2+} resulted in the permanent closure of three of five normal pig SR channels; one of these cases is shown in Fig. 4. That not all normal channels inactivated completely in high *trans* Ca^{2+} may explain why channels can be recorded using large *trans* Ca^{2+} gradients (22). It is thus possible that two types of ryanodine receptor channels may be present in the SR, a predominant *trans* Ca^{2+}-dependent inactivating type, and a less frequent *trans* Ca^{2+}-dependent noninactivating type. The P_o in *cis* 10 μM/*trans* 940 μM Ca^{2+} was 0.23 ± 0.29 for five normal channels, and

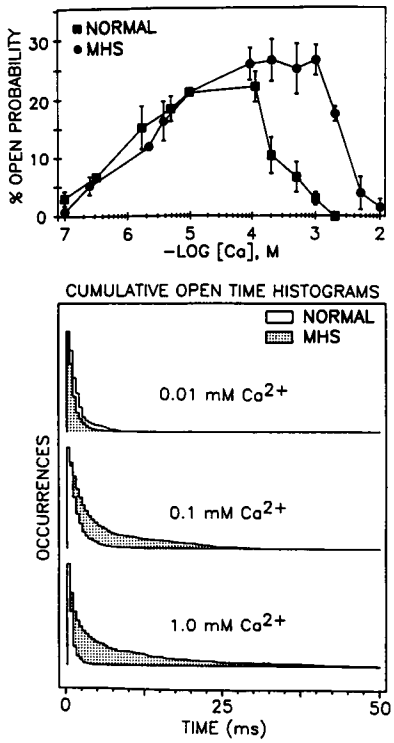

FIGURE 3 Ca^{2+} dependence of normal and MHS release channels. (*Top*) P_o is plotted as a function of the free *cis* [Ca^{2+}]. Free *trans* [Ca^{2+}] was held constant at 10 μM. The P_o at each [Ca^{2+}] was determined from 10 to 12 recordings (1,000 ms each) taken at 6-s intervals. To compare normal and MHS channels, the data was normalized to the value at *cis* 10 μM Ca^{2+} (which did not differ) by dividing by the P_o at *cis* 10 μM Ca^{2+} and multiplying by 20. Each point represents the mean ± SD of four to seven experiments. Data was collected from two different preparations of MHS and normal SR. (*Bottom*) Histograms of cumulative events (percent of open events of duration time t or longer plotted as a function of t) for representative normal and MH channels.

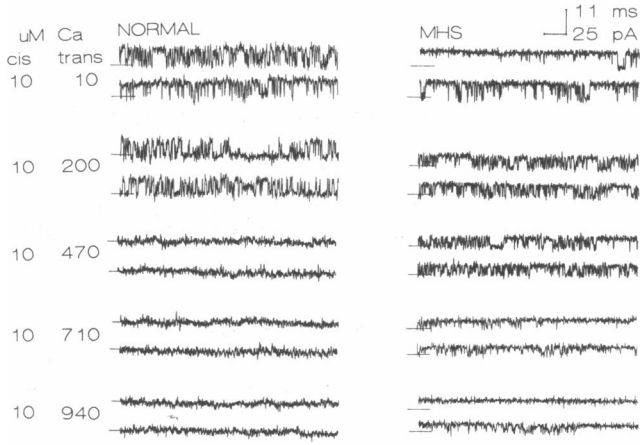

FIGURE 4 Effect of *trans* Ca^{2+} on normal and MHS release channels. Normal and MHS channels are shown at the indicated free Ca^{2+} at +20 mV, after incorporation in symmetrical 10 μM Ca^{2+} (top records). P_o was measured from 10–20 segments, each of 1,000 ms duration. In the records shown, average P_o at 940 μM *trans* Ca^{2+} was <0.001 for normal and 0.75 for MHS. Lines indicate baseline current.

0.71 ± 0.17 for 3 MHS channels ($P < 0.03$), where each channel was studied in a different SR preparation. The P_o values were computed from a pool of 32 1-s segments in the case of normal, or from 31 1-s segments in the case of MHS SR. This characteristic failure of MHS channels to inactivate in high *trans* Ca^{2+} appeared as the most likely explanation for the higher rate of $^{45}Ca^{2+}$ efflux observed in MHS SR (Fig. 1). On the other hand, the unit currents decreased by ~55% in MHS and 12% in normal at the highest Ca^{2+} concentration tested (10.5 ± 0.8 pA and 16.2 ± 0.18 pA, respectively, at +20 mV). Clearly, *trans* Ca^{2+} decreased the open channel current to a much greater extent in the MHS than in the normal channel suggesting that Ca^{2+} ions, as they pass through the pore, bind more tightly to the MHS than to the normal channel (14). However, because the turnover rates of ion transport are so high even for the MHS channel in high Ca^{2+} (~6×10^7 s^{-1}), it is unlikely that the rate of ion passage through open channels is in fact the rate determining step of $^{45}Ca^{2+}$ efflux in Fig. 1.

Previous studies suggested that the higher Ca^{2+} permeability of MHS SR vesicles could not be readily explained by an increase in the density of release channels. Thus, the amount of the 400–450 kD protein in Coomassie blue stained gels, and the B_{max} of the ryanodine receptor in the presence of 6 μM Ca^{2+}, was similar for MHS and normal SR (17). We have performed further ryanodine binding experiments in the presence of 6 μM Ca^{2+} plus 10 mM ATP, which promotes optimal binding of ryanodine (12, 17), and find that MHS and normal SR have a similar receptor density under these conditions (B_{max} = 14.8 ± 1.5 pmol/mg, and 15.9 ± 0.9 pmol/mg). That the higher Ca^{2+} permeability of MHS SR vesicles is due to an altered Ca^{2+} dependence of ryanodine receptor channels appears even more likely. Because ryanodine binds with a higher affinity to the open state than to the closed state of the release channel (12, 24), a prolonged open state of the MHS channel could readily explain the higher binding affinity of ryanodine to MHS SR (17). Furthermore, the lower intravesicular Ca^{2+} threshold for initiation of Ca^{2+} release from MHS SR reported by others (10, 11) may also be a result of a diminished ability of intravesicular (*trans*) calcium to inactivate MHS channels. We suggest that a defect in a low-affinity Ca^{2+} binding site is responsible for the altered gating of MHS channels, and envision this site as occupying the interior of the ion conduction pathway because it is accessible from both sides of the SR membrane. Furthermore, it must have a low affinity for Ca^{2+} because in symmetrical 10 μM Ca^{2+}, that is in the absence of a Ca^{2+} gradient,

there was no significant difference in open probability between normal and MHS channels (Fig. 2).

Because the site of action of both halothane (25) and caffeine (26) appears to be the ryanodine receptor channel, the defect we have observed in the Ca^{2+} release–ryanodine receptor channel could also underlie the increased sensitivity of MHS muscle to contractures induced by halothane and/or caffeine (1, 2, 6, 7). Such a defect could logically result from a mutation in the gene encoding the ryanodine receptor, providing an explanation for the molecular basis of this inherited disease. This makes the MHS SR a valuable model in the study of intracellular Ca^{2+} regulation and the molecular mechanism of excitation-contraction coupling.

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