Abnormal human sarcoplasmic reticulum Ca^{2+} release channels in malignant hyperthermic skeletal muscle

Michael Fill,* Enrico Stefani,* and Thomas E. Nelson‡

*Department of Molecular Physiology and Biophysics, One Baylor Plaza, Baylor College of Medicine, Houston, Texas 77030; and
‡Department of Anesthesiology, University of Texas Health Science Center, Houston, Texas 77030 USA

ABSTRACT Single sarcoplasmic reticulum (SR) Ca^{2+} release channels were reconstituted from normal and malignant hyperthermic (MH) human skeletal muscle biopsies (2–5-g samples). Conduction, gating properties, and myoplasmic Ca^{2+} dependence of human SR Ca^{2+} release channels were similar to those in other species (rabbit, pig). The MH diagnostic procedure distinguishes three phenotypes (normal, MH-equivocal, and MH-susceptible) on the basis of muscle contracture sensitivity to caffeine and/or halothane. Single channel studies reveal that human MH muscles (both MH phenotypes) contain SR Ca^{2+} release channels with abnormally greater caffeine sensitivity. Muscles from MH-equivocal and MH-susceptible patients appear to contain channels with the same abnormality. Further, our data (n = 115, 21 channels, 11 patients) reveals that human MH muscles (both phenotypes) may contain two populations of SR Ca^{2+} release channels, possibly corresponding to normal and abnormal isoforms. Thus, whole cell phenotypic variation (MH-equivocal vs. MH-susceptible) arises in muscles containing channels with similar caffeine sensitivity suggesting that human MH does not arise from a single defect. These results have important ramifications concerning (*a*) correlation of functional and genetic MH studies, (*b*) identification of other, yet to be determined, factors which may influence MH expression, and (c) characterization of normal SR Ca^{2+} release channel function by exploring genetic channel defects.

INTRODUCTION

Malignant hyperthermia is an inherited skeletal muscle disorder triggered by commonly used anesthetic agents. It can be lethal unless immediately recognized and treated (1–3). The MH syndrome is characterized by accelerated metabolism, skeletal muscle rigidity, and rapidly rising body temperature. The frequency of MH is ~1 in 100,000 adult anesthetic procedures (1 in 15,000 for children). An abnormality in the regulation of the SR Ca^{2+} release mechanism is thought to be involved in MH pathogenesis (4–8). Recent molecular genetic studies suggest that human MH mutations are located in the gene that codes for the ryanodine receptor (RyR) protein (9, 10), the putative SR Ca^{2+} release channel. Identification of the rabbit RyR as the SR Ca^{2+} release channel (11), sequencing of the RyR protein (12, 13), and putative localization of human MH mutations in the RyR gene (9) provide a stimulating prelude to our study in which we identify functional defects in the human MH Ca2+ release (RyR) channel.

We show that conductance, gating properties, and myoplasmic Ca^{2+} dependence of normal and MH human SR Ca^{2+} release channels are similar to rabbit and normal pig channels. A recent study using a porcine model of MH reported that MH pig SR Ca^{2+} release channels have abnormal myoplasmic Ca^{2+} dependence (14). In this study, we demonstrate that human MH muscles contain SR Ca^{2+} release channels with abnormal caffeine sensitivity. Comparison of molecular (single channel) and whole cell (contracture) data indicate that (*a*) two MH phenotypes (distinguished by caffeine/halothane contracture sensitivity) arise in muscles containing channels with similar caffeine sensitivity and (*b*) human MH muscles (both phenotypes) contain two populations of SR Ca^{2+} release channels, possibly normal and abnormal isoforms. Defining functional abnormalities in human MH muscle is a necessary step in the identification of genes containing human MH mutations.

MATERIALS AND METHODS

Heavy SR membrane fractions were prepared as previously described (15–17). Excess human tissue from vastus lateralis biopsies from consenting MH diagnostic patients was used. Human (and animal) protocols and procedures were approved by the appropriate institutional review boards. Heavy SR membranes were stored at −85°C until used in bilayer experiments.

Human vastus lateralis biopsies were performed as part of the MH diagnostic procedure (MH Diagnostic Center, Department of Anesthesiology, University of Texas, Health Science Center, Houston, TX). Individual skeletal muscle fascicles (average: 78 mg and 15 mm long) were isolated and secured in a temperature controlled (37°C) tension monitoring apparatus. The diagnostic test was based on criterion established by the North American MH Diagnostic Group (18) and by our laboratory (19). These tests distinguish three phenotypes: Normal, MH-equivocal (MHE), and MH-susceptible (MHS). The diagnostic criterion are described below. Normal human skeletal muscle fascicles generate 1.0 g of tension at caffeine concentrations equal to or > 4.0 mM and generate < 0.7 g of tension in the presence of 3% halothane. MHS muscles generate 1.0 g at caffeine concentrations < 4.0 mM and/or have abnormal contracture (> 1.0 g) triggered by 3% halothane.

0006-3495/91/05/1085/06 $2.00

The MHE diagnosis is based on another contracture test, i.e., caffeine contracture sensitivity in the presence of 1% halothane. MHE muscle responds normally to caffeine in the absence of halothane and normally to 3% halothane. In the presence of 1% halothane, MHE muscle generates 1.0 g of tension at caffeine concentrations < 0.8 mM.

Bilayer experiments were performed as described in detail elsewhere (20). Briefly, planar lipid bilayers were formed by the Mueller-Rudin technique across a 250-μm diam aperture. Bilayer forming solution contained a 7:3 miture (50 mg/ml in decane) of palmitoyl-oleoyl-phosphatidylethanolamine and palmitoyl-oleoyl-phosphatidylcholine. Lipids were obtained from Avanti Polar lipids (Pelham, AL). Salt-agar Ag/AgCl² electrodes were used. Membranes (3–20 mg/ml) were applied directly to one side of the bilayer (defined as *cis*, other side defined as *trans*). Fusion and recording were performed (unless specified) in standard solutions containing 250 mM *cis* Cs-CH_3SO_4 (50 mM *trans*), 10 mM Cs-Hepes (pH 7.4), pCa 5. After SR membrane fusion, bilayers remained stable at 0 mV for up to 1.5 h. Membrane potentials are referenced to *trans*. Polarization (50–100 mV) of the bilayer significantly decreased bilayer lifetime (< 30 min). Thus, single channel data were sampled at positive potentials (50–100 mV) during short (100–500 ms) repetitive (0.5–0.05 Hz) steps from 0 mV. Routinely, single channel activity (e.g., open probability) was assessed under each experimental condition from at least 64 voltage steps (corresponding to roughly 10,000 open events under control conditions). Most single channel data were analyzed using automated single channel detection. When necessary, detection threshold was set at 50% of the average channel amplitude determined from total amplitude histograms (21). Approximately 60% of channel incorporations involved multiple channels. Bilayers which contained more than two channels were immediately reformed. Bilayers containing channels displayed time-dependent changes in open probability (Po) were also discarded (~5% of reconstituted channels). Po experiments were performed only on bilayers which contained a single channel. Some conduction, pharmacology, and survey experiments (those not involving Po determinations) were performed on bilayers which contained two channels. The bilayer pulsing technique (*a*) provided a reliable automated sampling protocol, (*b*) allowed reproducible and consistent measurements of single channel properties, and (*c*) extended the experimental lifetime of the bilayer.

Data acquisition software and hardware (pClamp, Tl-1 interface; Axon Instruments, Burlingame, CA) were computer interfaced. Data were digitized at 5–10 kHz, filtered at 3–4 kHz, and stored on floppy disk. Analysis software was provided by Dr. T. VanDongen and Dr. A. Brown (Baylor College of Medicine, Houston, TX) and supplemented by commercial programs (pClamp; Axon Instruments).

RESULTS AND DISCUSSION

In this study, we present a methodology to reconstitute the SR Ca^{2+} release channel in planar bilayers from small samples (2–5 g) of human biopsied skeletal muscle. This method is based on the observation that purified rabbit RyR channel mediates a fast gating, large monovalent conductance (11). Since cesium virtually blocks the conductance of other SR cation channels (22) and is highly conductive through the RyR channel (11), cesium was used to isolate the conductance of single human SR Ca^{2+} release channels. The cesium conducting human SR Ca^{2+} release channel was pharmacologically identified by its ryanodine, ruthenium red, Ca^{2+}, and caffeine sensitivity.

Conduction and gating properties of normal human, rabbit, and pig SR Ca^{2+} release channels are illustrated in Fig. 1. Pig channels were included in the interspecies comparison since MH has been extensively studied using porcine MH models (4–7, 14). Single channel records

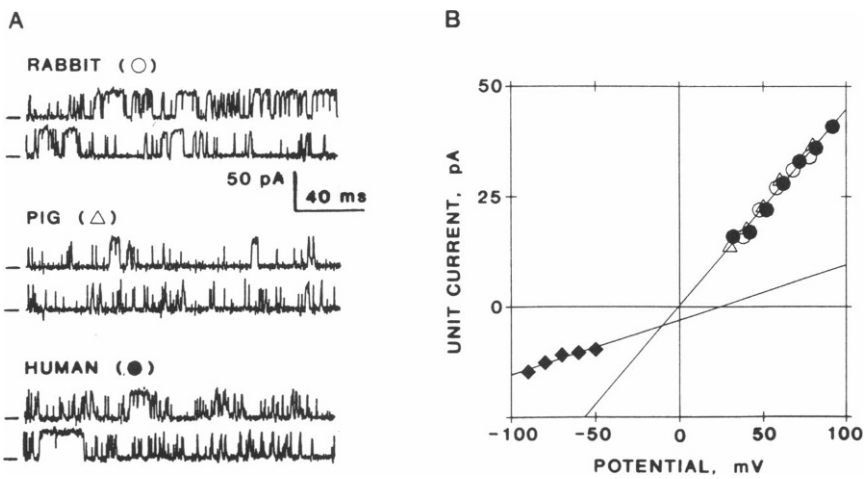

FIGURE 1 Interspecies comparison of single SR Ca^{2+} release channels. Channels (conducting cesium) were pharmacologically identified as the RyR. (*A*) Single channel records (*opening upward, baseline marked*) from rabbit, pig, and human skeletal muscle. Channels from all three species have similar gating characteristics. Solutions contained: 250 mM Cs-CH_3SO_4 symmetrical, 10 mM Cs-Hepes (pH 7.4), pCa 5. The holding potential was +80 mV. (*B*) Current/voltage relationships from rabbit (*open circles*), pig (*open triangles*), and human (*solid circles*) under same conditions. The cesium slope conductance is 470 pS. Filled diamonds depict conductance after an ionic exchange (50 mM Ba^{2+} for 250 mM Cs^+ *trans* with removal of all *cis* Cs^+). The shift in reversal potential indicates an estimated PBa/PCs of 7.8.

(Fig. 1 *A*) were collected at +80 mV under identical ionic conditions (symmetrical 250 mM Cs-CH_3SO_4). Single channel open events are shown as upward deflections from the baseline (marked). The channel gating in all three species was well described by two open and two closed states (see human data, Table 1). The current/voltage relationships for rabbit (*open circles*), pig (*open triangles*), and human (*filled circles*) SR Ca^{2+} release channels are shown in Fig. 1 *B*. Data from all three species were well described by slope conductance of 479 pS. A reversal potential shift due to addition of barium (50 mM *trans, filled diamonds*) reveal that the Cs^+ conducting channels were divalent selective (PBa/PCs estimate 7.8). Channel gating, divalent slectivity, and slope conductance of rabbit, pig, and human SR Ca^{2+} release channel were nearly indistinguishable.

A generally supported hypothesis is that MH arises from a defect in regulation of myoplasmic Ca^{2+} concentration, probably involving the SR Ca^{2+} release mechanism (4–8). A recent study has demonstrated in a porcine model that MH muscles do contain abnormal SR Ca^{2+} release channels (14). Thus, our investigation of human SR Ca^{2+} release channels began by examining some fundamental properties of normal and MH-susceptible (MHS) channels including, conductance, stationary Po, channel gating, and the action of ryanodine. In the standard solution (see Methods) normal and MH channels did not have significantly different conductance (Fig. 2 *A*, Table 1) or average Po (Table 1). Further, the gating of MHS and MHE channels (two open and two closed states) correspond well to the averages of normal human channels (Table 1). The action of 2 μM ryanodine on normal and MH human release channels was also very similar. The effect of ryanodine on a human channel from a MH patient is illustrated in Fig. 2 *B*. Ryanodine dramatically increased

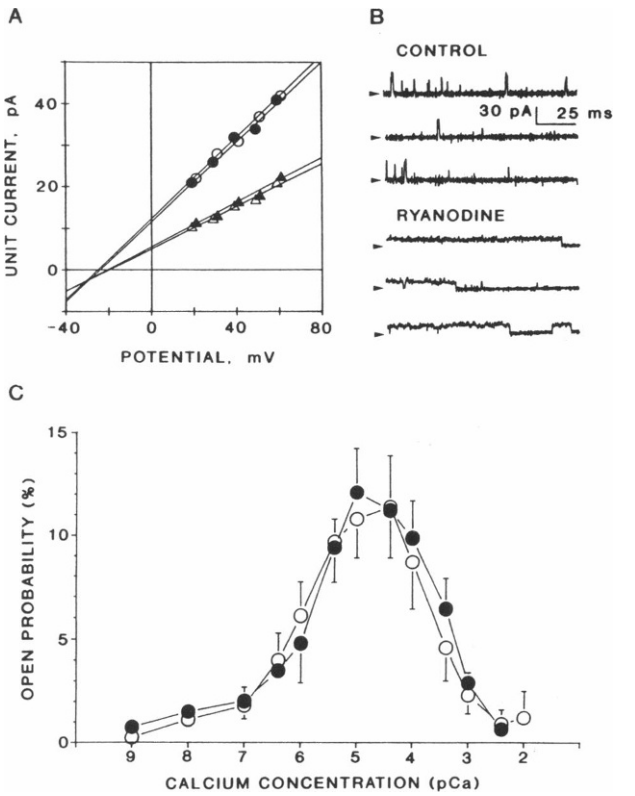

FIGURE 2. Comparison of human SR Ca^{2+} release channels from normal and MHS muscle. (*A*) Current/voltage relationships. Under control conditions, normal (*open circles*) and MHS (*solid circles*) channels had a slope conductance of 488 and 479 pS, respectively. Addition of 2 μM ryanodine to the same channels (normal = open triangles, MHS = solid triangles) decreased conductance to 255 and 268 pS, respectively. (*B*) Single channel records (holding potential 40 mV) from the same MHS patient demonstrating the action of ryanodine on channel gating. Ryanodine dramatically increased single channel Po while decreasing single channel conductance. Baselines (zero current levels) are marked at left. (*C*) Calcium dependence of single channel activity. The free myoplasmic (*cis*) [Ca^{2+}] was titrated by adjusting the Ca/EGTA ratio. Normal human (*solid symbols*) and MHS human (*open symbols*) channels had very similar calcium dependence. Each point represents mean (±S.E.) of data from two to six channels from two normal and three MHS diagnostic patients.

TABLE 1 Values are means and standard deviations

	Normal	MHE	MHS
Po	0.084 ± 0.056 (11/5)	0.098 ± 0.081 (9/3)	0.066 ± 0.043 (9/4)
gCs	480.6 ± 24.1 (11/4)	471.3 ± 27.6 (7/3)	467.2 ± 39.9 (8/4)
τo1	0.153 ± 0.082 (3/3)	0.216 ± 0.109 (3/3)	0.241 ± 0.138 (3/3)
τo2	3.101 ± 0.624 (3/3)	4.394 ± 0.865 (3/3)	2.615 ± 0.932 (3/3)
τc1	0.453 ± 0.191 (3/3)	0.330 ± 0.129 (3/3)	0.474 ± 0.207 (3/3)
τc2	30.76 ± 8.37 (3/3)	32.77 ± 9.157 (3/3)	33.01 ± 8.34 (3/3)

Number of determinations (in parenthesis) are number of channels over number of patients. Po = open probability, gCs = cesium conductance in standard solution. τo1 and τo2 are the two open time constants. τc1 and τc2 are the closed time constants. Time constant values were generated by fitting log (8 bin/decade) open and closed time histograms. Under control conditions (pCa 5), there were not significant (T test; p values > 0.05) differences in conduction or gating of normal, MHE, and MHS channels.

Po (~0.1–0.8) and stabilized the channel in a subconductance state. The ryanodine-modified conductances of normal, MHE, and MHS human channels were not significantly different (Fig. 2 *A*). This dual action of ryanodine (increase Po, stabilized subconductance) is identical to that described for the purified and native channels from rabbit muscle (11).

A single channel study on a porcine MH model demonstrated that SR Ca^{2+} release channels from MH muscles have abnormal myoplasmic Ca^{2+} dependence (14). Thus, we searched for a similar abnormality in human MH muscles. The myoplasmic Ca^{2+} dependence

of normal (*filled circles*) and MHS (*open circles*) human SR Ca^{2+} release channels are nearly identical (Fig. 2 *C*). Single channel open probability is normalized to control Po measured in the standard solution at the beginning of each tritation. These experiments on human channels correspond directly to the experiments on MH pig channels. We believe the apparent lack of a Ca^{2+} regulatory defect in MHS human channels may reflect subtle but important genetic differences between MH in pig and human. It is also important to note that our human experiments and the pig experiments (14) describe the steady state action of Ca^{2+} on channel activity. These experiments do not address the possibility that the time course of Ca^{2+} activation or inactivation are abnormal in MH channels.

The human MH diagnosis is, in part, based on abnormal contracture response to caffeine (see Methods). Because caffeine is a potent modulator of the SR Ca^{2+} release channel, we explored the caffeine sensitivity of human MH channels. The MH diagnostic contracture test distinguishes three phenotypes, normal (N), MH-equivocal (MHE), and MH-susceptible (MHS). Human skeletal muscle biopsies were divided into two portions, one portion was used for a diagnostic contracture test, the other was used to prepare microsomes for single channel experiments. Thus, contracture and single channel data were obtained from each individual biopsy. Sample diagnostic contracture data from normal, MHE, and MHS patients are shown in Fig. 3. Two contraction phenomena are monitored in the contracture test, isometric tension and twitch (stimulated at 0.1 Hz). The basal isometric tension is the diagnostically important parameter. Caffeine contracture threshold (CCT), indicated by the initial rise in basal isometric tension, was 8.0 mM in the normal human muscle (Fig. 3 *A*). The CCT was 4.0 and 1.0 mM for the MHE and MHS muscles (Fig. 3, *B* and *C*), respectively. The halothane contracture data is included to illustrate further differences between the three phenotypes. Single channel data from the same three patients (N, MHE, and MHS) are paired with the corresponding contracture data. The normal human SR Ca^{2+} release channel (Fig. 3 *A*) was not significantly activated by 1 mM caffeine. Single channels from the MHE and MHS patient (Fig. 3, *B* and *C*), however, were significantly activated by 1 mM caffeine. At the single channel level, channels from MHE and MHS patients appear to be abnormally sensitive to caffeine.

This study includes data collected on 11 diagnostic patients (3 normal, 4 MHE, and 4 MHS). Contracture data was collected in triplicate. Several channel reconstitution experiments were performed on each patient. The caffeine-dose dependencies of contracture and single channel Po are plotted (means ± S.E.) in Fig. 4. At the whole cell level (Fig. 4 *A*), the three phenotypes

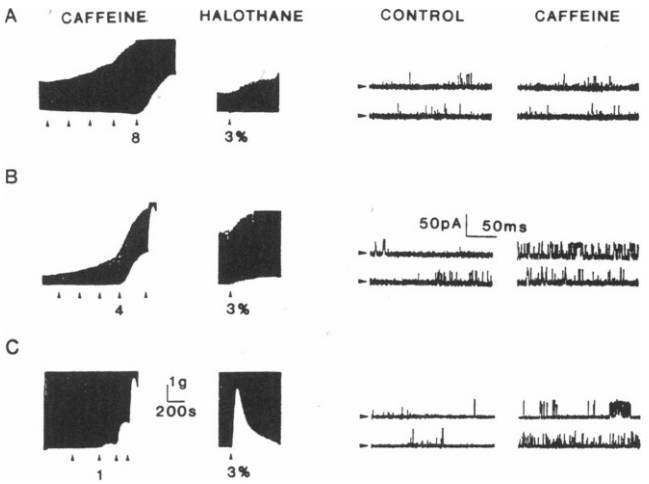

FIGURE 3 Comparison of contracture data and single channel data. At left, diagnostic contracture data illustrating the contracture response to caffeine (dose-dependence) and 3% halothane. Two contractile phenomena evident, twitch and basal isometric tension. The diagnostic contracture test is based on isometric tension changes. Arrow heads indicate application of caffeine (0.5, 1, 2, 4, 8 mM left to right) and halothane. At right, single channel data (holding potential 40 mV) collected in the standard recording solution (control) and after the addition of 1 mM caffeine. Open events are upward deflections. Baselines (zero current levels) marked. (*A*) Contracture and single channel data collected from a normal patient (N-SS1). Threshold caffeine concentration (in millimolar) are marked. (*B*) Contracture and single data collected from a MHE patient (K-MW1). Only the threshold caffeine concentration is marked. Caffeine additions made in the same sequence as above. (*C*) Contracture and single channel data collected from a MHS patient (H-BT1).

(normal, MHE, and MHS) can clearly be distinguished by caffeine contracture sensitivity. At the single channel level (Fig. 4 *B*), smaller caffeine doses were necessary to elicit an effect. Single channel data is plotted as percent change in Po compared with control Po obtained for each channel during an initial period in the standard solution. The caffeine sensitivity of MHE (*open triangles*) and MHS channels (*open circles*) was significantly greater than that of normal channels (*filled circles*). Note that the MHE and MHS phenotypes cannot be distinguished at the single channel level on the basis of caffeine sensitivity. This indicates that the phenotypic variation in caffeine contracture sensitivity (Fig. 4 *A*, MHE and MHS curves) arises in muscles containing SR Ca^{2+} release channels with comparable caffeine sensitivity (Fig. 4 *B*, MHE and MHS curves). This observation leads to the suggestion that other, yet to be identified, factors or abnormalities may influence phenotypic expression of MH in humans.

The relatively low frequency (occurrence) of MH and its complex inheritance pattern suggests that MH patients are probably heterozygous for MH mutations (10).

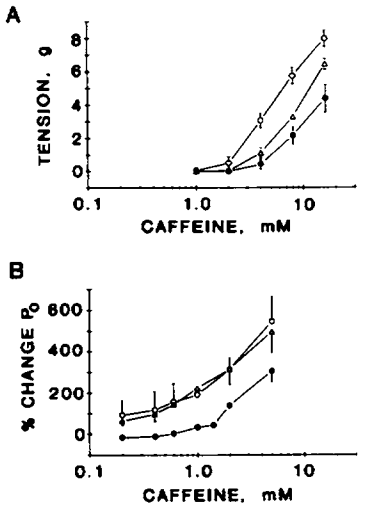

FIGURE 4 Comparison of caffeine dose dependencies at whole cell (contracture) and molecular (single channel) levels. Data compiled from 11 diagnostic patients (3 normal, 4 MHE, and 4 MHS). Data points represent mean (±S.E.) of 4–9 determinations. (*A*) Caffeine dose dependency of peak isometric contracture height. Normal (*open circles*), MHE (*open triangles*), and MHS (*open circles*) muscles were clearly distinguishable by caffeine sensitivity. (*B*) Caffeine dose dependency of single channel Po (plotted as % change Po relative to an initial caffeine-free control period). Normal (*solid circles*) channels were clearly distinguishable from MHE (*open triangles*) and MHS (*open circles*) channels. Channels from MHE and MHS muscles have nearly identical caffeine dose dependencies.

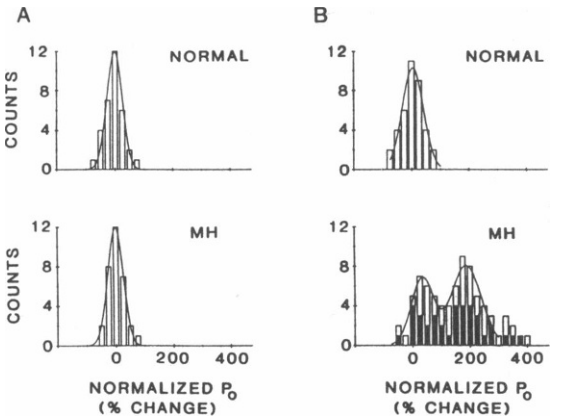

FIGURE 5 Data point scatter about normalized means suggests that human MH muscles may contain two populations of SR Ca^{2+} release channel. Smooth curves represent fitted normal distributions (least squares method). (*A*) Ca^{2+} dependency data. The mean Po value normal and MHS channels at each Ca^{2+} concentration was subtracted from all data points. Data points (all concentrations) were then pooled and binned. Ca^{2+} dependency data points were monotonically distributed about their means in both normal and MH muscle. (*B*) Caffeine dependency data. Data transformation same as above. Caffeine dependence data points were monotonically distributed in normal muscles, but were bimodally distributed in MH muscles. Data from MHE (*solid portion of bar*) and MHS (*open portion of bar*) muscles were combined.

Thus, human MH muscles may contain normal and abnormal (those containing MH mutations) copies of the gene coding for the SR Ca^{2+} release channel. If true, then human MH muscles may contain normal and abnormal isoforms of the SR Ca^{2+} release channel. This possibility was explored by pooling single channel data into two sets (Ca^{2+} dependence and caffeine) and examining how individual determinations were distributed about the mean. The Ca^{2+} dependence set is shown in Fig. 5 *A*. The percent change Po (at all Ca^{2+} concentrations) was monotonically distributed for both normal and MH channels. The caffeine set is shown in Fig. 5 *B*. The percent change Po (at all caffeine concentrations) was monotonically distributed for normal channels, but was bimodally distributed for MH channels. The filled bars (Fig. 5 *B, bottom*) correspond to the contribution MHE channels (remaining open portion to MHS channels). The bimodality is evident for both MHE and MHS channels and suggests that human MH muscles (both phenotypes) contain two populations of channels. The bimodality may arise from the presence of two channel isoforms (normal or abnormal) or possibly from a single isoform with variable caffeine sensitivity.

In this study, we present a methodology to study single SR Ca^{2+} release channels from human skeletal muscle biopsies from MH diagnostic patients. This methodology is not specialized to study MH and could be applied to study other human skeletal muscle disorders. In this study, we identify a human MH defect in the SR Ca^{2+} release channel, the RyR protein. Our data, therefore, is consistent with genetic studies suggesting that the RyR gene may contain mutations associated with the human MH syndrome (9). Our data also demonstrates that phenotypic variation (MHS vs. MHE) in caffeine sensitivity arises in muscles containing channels with similar caffeine sensitivity. This result leads to the provocative suggestion that MH does not result from a single defect in the RyR, but may arise from a combination of defects. Thus, other factors, which have yet to be identified, may influence phenotypic expression of MH in humans. Further, single channel caffeine data supports the hypothesis that human MH muscles (both MH phenotypes) contain normal and abnormal copies of the SR Ca^{2+} release channel.

Defining functional MH abnormalities at the molecular (single channel) level will have important ramifications in the identification of the gene(s) containing MH mutations(s) and eventually the development of a noninvasive genetically based MH diagnostic procedure. Additionally, our understanding of SR Ca^{2+} release channel

function in normal muscle will be significantly advanced by defining the functional and genetic abnormalities which underlie MH pathogenesis.

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