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Halothane Effects on Human Malignant Hyperthermia Skeletal Muscle Single Calcium-release Channels in Planar Lipid Bilayers

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Malignant hyperthermia (MH) may be life-threatening when genetically predisposed individuals are administered triggering anesthetic agents that are believed to produce intracellular calcium release. To test this theory, the effects of halothane on normal and MH human skeletal muscle calcium-release channels were studied. Single calcium-release channels were incorporated from isolated sarcoplasmic reticulum membrane vesicles into a planar lipid bilayer, and halothane effects on the conductance and gating properties were measured by electrophysiologic techniques. Among the subjects studied, seven were MH-susceptible, and 13 channels were recorded from this group. Five subjects were negative for MH, and 10 channels were recorded from this group. Among the 13 channels recorded from the MH group, 7 were affected by halothane, which increased the probability of the channel to change from the inactive, closed state to an open state. This effect of halothane to increase open-state probability was associated with an overall increase in channel conductance. Thus, halothane affected the activation/inactivation process of the halothane-sensitive calcium-release channel from MH muscle as well as the gating properties of the MH calcium-release channel, as evidenced by the increased conductance. In 6 of the 13 channels recorded from MH muscle, halothane (2.2–17.6 μM) was without effect on these properties of the channel. Halothane (2.2–17.6 μM or 0.0057–0.0456 vol%) also had no measurable effect on the 10 channels from the negatively diagnosed subjects. Results of this study support a defect in the ryanodine-sensitive calcium-release channel from MH human muscle. Affected subjects may also synthesize normally functional channels, and in three of the seven MH subjects (5 channels recorded) no halothane-sensitive channels were observed, suggesting the possibility of some other MH-predisposing defect in these subjects. (Key words: Muscle, skeletal: calcium-release channel; ryanodine receptor protein. Temperature: malignant hyperthermia.)

MALIGNANT HYPERTHERMIA is a disease of skeletal muscle in which an abnormality in calcium regulation in the muscle cell predisposes to an anesthetic agent-induced hypermetabolic syndrome. Malignant hyperthermia susceptibility (MHS) in humans, pigs, and dogs is associated with abnormal contracture response to caffeine, halothane, and ryanodine in skeletal muscle biopsied from each of these species.[1–3] Dantrolene sodium, a direct-acting skeletal muscle relaxant, blocks calcium release and is efficacious in treatment of malignant hyperthermia (MH) in each susceptible species.[2,4,5]

An abnormal calcium-release channel protein in MHS pig sarcoplasmic reticulum (SR) membranes has been established.[6–9] The abnormal calcium-release channel in MH pig muscle, known as the ryanodine receptor protein (RyR), is a homotetrameric protein that bridges the transverse tubule and SR terminal cisternae membranes (fig. 1) and is believed to play a key role in excitation–contraction coupling by acting as the major pathway for calcium release from the terminal cisternae.[10] Each monomer (molecular weight = 450 K) is the product of a DNA segment found on human chromosome 19[11,12] and pig chromosome 6,[13] and the rabbit RyR gene has been cloned and sequenced.[14] Present evidence indicates that the gene predisposing MHS in humans is on chromosome 19 and in close proximity to the RyR gene.[11,12] In susceptible pigs, a single amino acid substitution in the mutant RyR is associated with the genetic predisposition to MH.[15]

Technological advances have made possible the study of ionic channels in artificially produced planar phospholipid bilayers.[16] A small diameter hole (200 μm) separates two chambers, and by coating a mixture of phospholipids over this hole, a planar bilayer, arranged similarly to those in biologic membranes, is formed (fig. 1). Single protein molecules can become incorporated into the lipid bilayer by fusion of channel-containing membrane vesicles, and if the protein is an ionic channel, then appropriate experimental conditions allow the measurement of ions (picoamperes current) flowing from one side of the bilayer to the other when the channel is open. Found with the use of this electrophysiologic methodology, an abnormality in calcium regulation of the MHS pig RyR channel was reported.[17] In the MHS human RyR channel, abnormal sensitivity to caffeine effects on the single protein molecule's channel opening events was discovered.[18] This finding suggests a relationship between caffeine effects on the intact muscle cell and those on the isolated single RyR calcium-release channel. Although abnormality in calcium inactivation was reported for RyR channels from MH pig muscle,[17] those from MHS human muscle do not have an abnormality in the calcium regulated site(s).[18] These studies strongly suggest functional alteration in single-channel MHS RyR protein molecules, indicating the like-

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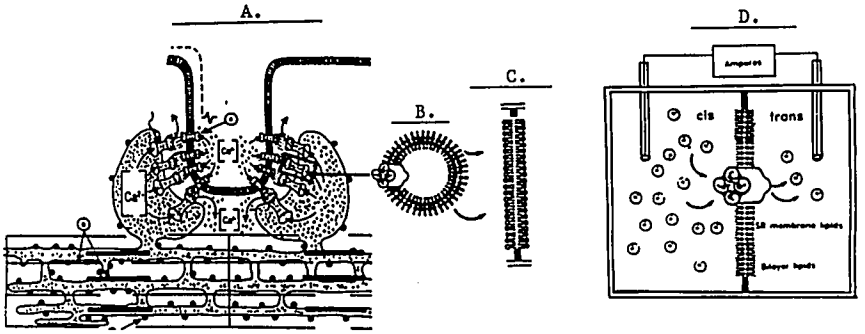

FIG. 1. *A:* Structures of the skeletal muscle cell. The hatched membrane is sarcolemma that invaginates to form the transverse tubule with terminal sacs of the sarcoplasmic reticulum (SR) on either side of the T-tubule. The small box is drawn around the calcium-release channel of the SR, and when the muscle is homogenized, the membranes reseal to form vesicles. *B:* A membrane vesicle containing a calcium-release channel which, when in contact with artificial bilayer (*C*), can fuse to form a channel in the bilayer that conducts cesium ions under appropriate conditions. Cesium ions are conducted from the *cis* to the *trans* chamber (*D*), and the current is measured, amplified, and recorded for subsequent analysis.

lihood that a mutation in this protein is associated with MHS in humans and pigs.

Halothane produces abnormal contracture of biopsied skeletal muscle and triggers MH in susceptible species, but its effects on the functional properties of the RyR calcium-release channel have not been investigated. We tested a hypothesis that halothane would alter the conductance and gating properties of the human skeletal muscle RyR calcium-release channel and that one or more effects would be expressed abnormally in channels from MHS muscle.

Materials and Methods

Vastus lateralis muscle was biopsied from patients referred to the MH diagnostic center because of a clinical episode suspect for MH or because of a family history. Fascicles were contracture-tested and diagnostically classified on the basis of caffeine and halothane sensitivity.[19] Individual skeletal muscle fascicles were mounted in an isometric contracture tension-measuring chamber maintained at 37° C, and after optimizing field-stimulating voltage and sarcomere length for maximal twitch, two different pharmacologic tests were performed using three fascicles per test. These contracture tests to caffeine (0.5–32 mM) and to halothane 3% (volume/volume) followed methods established by the North American MH Diagnostic Group.[19] Normal human skeletal muscle fascicles develop 1.0 g of isometric contracture tension at caffeine concentrations equal to or > 4.0 mM and contracture response to halothane 3% that is < 0.7 g of tension. In the present study we have used muscle only from unequivocally diagnosed MH-susceptible or MH–not-susceptible (MHN) patients. By previous criteria[1] these are referred to as phenotypes H (MH-susceptible) and N (MH–not-susceptible).

Extra muscle was obtained from consenting subjects for the isolation of SR membrane vesicles by a protocol approved by the Committee for the Protection of Human Subjects at my institution. Heavy SR membranes were prepared as previously described[18] except that protease inhibitors were present at the following concentrations throughout SR membrane isolation: phenylmethylsulfonylfluoride, 200 μM; aminobenzamidine, 200 μM; aprotinin, 2 mg/ml; pepstatin, 2 μg/ml, leupeptin, 2 μg/ml; soybean trypsin inhibitors, 10 μg/ml; and iodoacetamide, 20 μM. Planar lipid bilayers were formed by painting a 25-mg/ml decane mixture of phospholipids (palmitoyl-oleoyl-phosphatidyl-ethanolamine: palmitoyl-oleoyl-phosphatidylcholine, 7:3) across a 200-μm diameter aperture. Salt-agar $Ag/AgCl_2$ electrodes were used to measure the current flowing from one chamber (*cis*) to the other (*trans*) chamber, both separated by the lipid bilayer. *Cis* and *trans* refer to chambers on either side of the bilayer; *cis* is cytoplasmic, and *trans* is intraluminal for the SR membrane. SR membrane vesicles were applied directly to the *cis* side of the bilayer, and fusion and recording were performed in solutions containing 250 mM *cis* $CsCH_3SO_4$ (50 mM *trans*), 10 mM CsHEPES (*p*H 7.4), and CaEGTA, 1 mM (*p*Ca = 5.2). Cesium was used for the conducting ion because it blocks potassium channels[20] and is readily conducted by the RyR calcium-release channel.[10] All lipid bilayer experiments were done at a temperature of 25° C.

A pulsed-voltage protocol was used for sampling of single-channel data. The holding membrane potential was O mV, and a 50 mV (*cis*) polarization was applied for 200 ms at a frequency of 1.4 Hz. Recordings of 64 episodes, each 250 ms in duration, at a sampling rate of 10 kHz were obtained twice for controls prior to and once after each addition of halothane. No filtering of the data, other than intrinsic (5 kHz), occurred. In a separate experiment, channels from each of two MH-susceptible patients were used to measure halothane effects on unitary conductance. Recordings were obtained at 30, 40, 50, and 60 mV with the holding potential at O mV, and from these data the

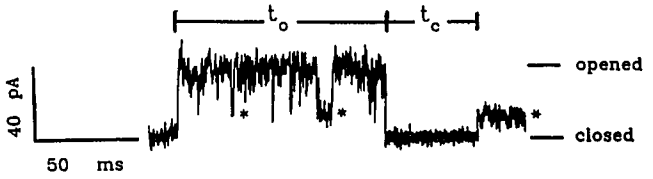

FIG. 2. A section from a single-channel recording illustrating some properties of the channel and how these are evaluated. The baseline represents zero current when the channel is completely closed, and the uppermost trace is the maximum current (picoamperes) measured and represents an open state of the channel. Asterisks label different levels at which the channel can conduct, illustrating gating properties. Based on selected threshold current levels above which the channel is considered to be opened, the open state time (t_o) is measured and averaged to estimate the mean open state time for the channel. Similarly, if the current level does not exceed the threshold value, the channel is assumed to be in a closed state, and these measured closed state times (t_c) are averaged across recordings to determine the mean open state time. Recording conditions are detailed in Materials and Methods.

slope of current *versus* voltage plots were used to estimate conductance in picoSiemans ($1/\text{ohms} \times 10^{-12}$). Data acquisition software and hardware (pClamp, TL-1 Interface, Axon Instruments, Burlingame, CA) were computer-interfaced. Analysis software was provided by Dr. T. Van Dongen and Dr. A. Brown at the Baylor College of Medicine. The basis for this analysis is illustrated, partially, in figure 2.

Halothane was added to the *cis* chamber from a 28 mM stock solution in ethanol as 0.5-μl increments, each producing 2.2 μM in the gas phase (volume = 2.14 ml) and 2.65 μM in the liquid phase (volume = 3.5 ml) at equilibrium. The phospholipid bilayer:liquid partition coefficient for halothane has been estimated to be approximately 150,[21] so that small changes in liquid halothane concentration would have very little effect on the very small bilayer. After each addition of halothane, the *cis* chamber was covered and stirred for 1 min. A gas concentration of 2.2 μM halothane is equivalent to 0.0076 times the minimum alveolar concentration of halothane (*i.e.*, 0.0076 × MAC) at which 50% of human patients are surgically anesthetized. Recordings of 64 episodes, each of 250-ms duration, at a sampling rate of 10 kHz were obtained twice for controls prior to halothane and once after each addition of halothane. When the bilayer remained intact, halothane was added to produce cumulative concentrations of 2.2, 4.4, 6.6, 8.8, and 17.6 μM in the gas phase. These concentrations correspond to 0.0057, 0.0114, 0.0171, 0.0228, and 0.0456 vol%, respectively. Throughout the text halothane concentrations will be expressed as the gas concentration in micromolar units.

The MHS group comprised seven subjects from whom 13 single-channel recordings were obtained. For the MHN group, 10 single-channel recordings were obtained, representing five different subjects. Analysis of variance and the Newman-Keuls method for multiple comparisons were used for statistical testing of the data.

Results

The *in vitro* contracture response of biopsied skeletal muscle provided unequivocal diagnostic contracture results (table 1). Each of the seven patients diagnosed as MHS had abnormal contracture sensitivity to caffeine (average caffeine specific concentration [CSC] values range from 1.83 to 3.96 mM) and to halothane (average contractures range from 0.67 to 2.98 g) that is interpreted by the North American MH Diagnostic Group as diagnostic for MHS. Similarly, each of the MHN patients had caffeine sensitivity (average CSC values range from 3.84 to 10.15 mM) and halothane sensitivity (average contractures range from 0 to 0.08 g) that fall outside the range of values interpreted as MHS.

TABLE 1. Characteristics of Malignant Hyperthermia Diagnostic Muscle Contracture Responses and Halothane Sensitivity of Single Channels for Individual Subjects

	CSC (mM)		Halothane, 3% Contracture (g)		Ca-Release Channel Ratio of Halothane Sensitive / Halothane Insensitive
	MHS	MHN	MHS	MHN	MHS
	2.37		2.42		1/0
	2.84		2.98		1/0
	1.83	5.35	2.60	0.08	0/2
	2.79	5.63	0.67	0.03	0/2
	2.16	5.10	2.53	0.08	0/1
	3.96	3.84	2.0	0.03	3/0
	2.14	10.15	0.76	0	1/2
Mean	2.58	6.01	1.99	0.044	
SE	± 0.26	± 1.07	± 0.34	± 0.015	
n	(7)	(5)	(7)	(5)	

Each subject value is the average of three different muscle fascicles. CSC = the caffeine specific concentration at which 1 g isometric contracture was produced by the muscle; MHS and MHN = malignant hyperthermia–susceptible and not susceptible, respectively.

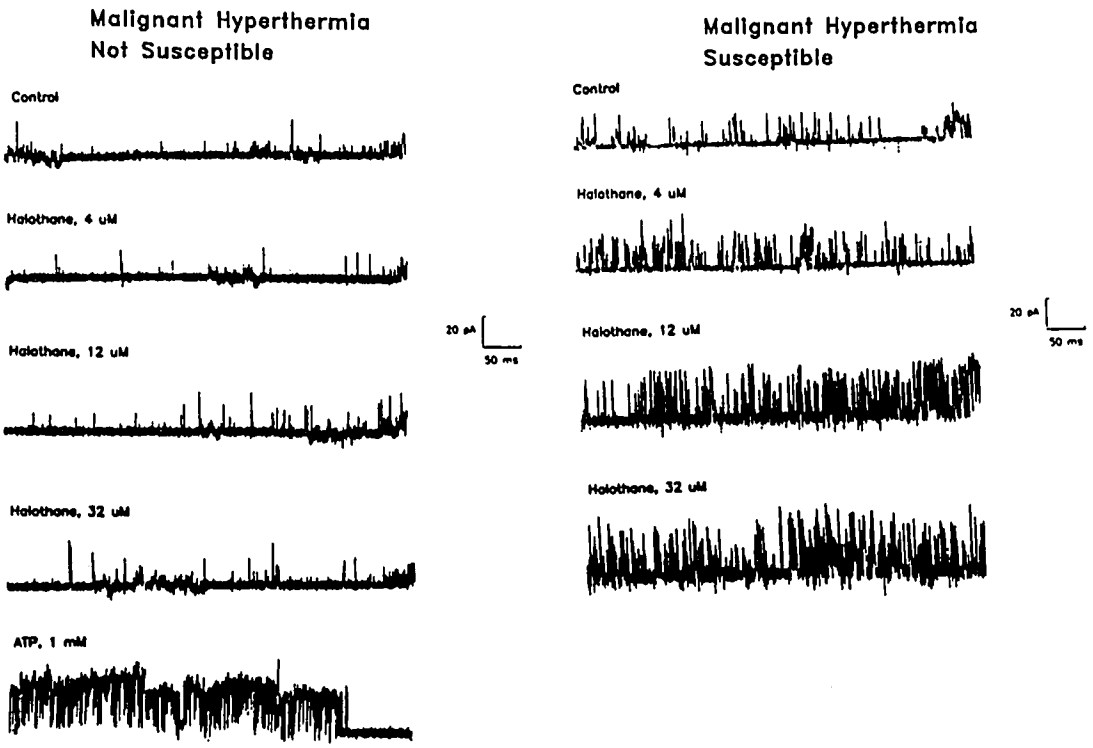

FIG. 3. Single-channel records from MHN and MHS human skeletal muscle sarcoplasmic reticulum. The lower, thick trace is the baseline, closed channel with openings represented by upward spikes. *Left:* A channel from MHN muscle is not affected by halothane 4–32 μM, whereas addition of ATP produces a marked increase in channel openings. *Right:* A channel from MHS muscle has a marked increase in the number of channel openings at each concentration of halothane.

In the channels from MHN muscle, halothane did not alter the open state probability (Po) whereas halothane increased Po in some of the channels from MHS muscle. Even though halothane did not activate channels from MHN muscle, these channels could be readily activated by addition of ATP 1 mM. These effects are illustrated by the recordings in figure 3.

The following were compared between the channels from MHS and MHN human skeletal muscle (table 2): control values for Po; the mean values for amplitude of single channel conductance (picoamperes); and average mean open and closed times. Channels from MHS muscle were subdivided into those that had halothane-induced increase in Po (MHS) and those that were insensitive to halothane effects (MHI). No statistically significant difference existed for average time for open and closed state values between channels from MHS *versus* MHN muscle. The average Po value for MHN and MHI channels was significantly different ($P < 0.05$) from the average Po for MHS channels (table 2). The mean amplitude for MHS controls (15.98 ± 1.4) was significantly ($P < .05$) greater than for control mean amplitudes of the MHI (14.19 ± 1.2

TABLE 2. Comparison of Single-channel Characteristics Between MHS and MHN Groups

	Mean Open Time (ms)	Mean Closed Time (ms)	Amplitude (pA)	Open State Probability (Po)
MHN	0.576 ± 0.108 (11)	10.346 ± 1.28 (11)	10.925 ± 0.71* (11)	0.0589 ± 0.014* (10)
MHS	0.537 ± 0.095 (7)	7.833 ± 1.72 (7)	15.978 ± 1.44 (7)	0.1262 ± 0.032 (7)
MHI	0.272 ± 0.052 (6)	11.510 ± 2.34 (6)	14.192 ± 1.23† (6)	0.0338 ± 0.015* (6)

Each value is mean ± SE with number of observations in parentheses.
MHN = malignant hyperthermia–not-susceptible; MHS = malignant hyperthermia–susceptible; MHI = malignant hyperthermia–insensitive.

* Statistically different from MHS, $P < 0.05$.
† Statistically different from MHN, $P < 0.05$.

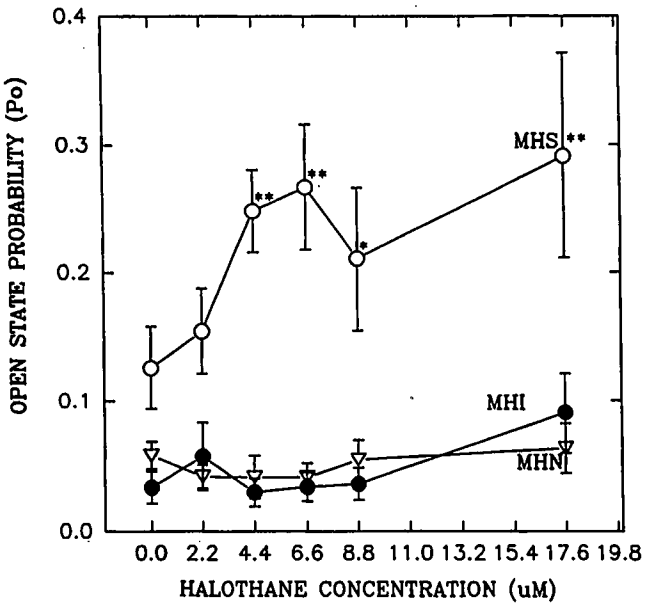

FIG. 4. The effect of halothane on the probability of the calcium-release channel to change from a closed, nonconducting state to an open, conducting state. A value of 0.5 would indicate that the channel is in an open state 50% of the time. Halothane concentration is that of equilibrated gas phase. MHS = channels from malignant hyperthermia–susceptible human muscle; MHN = channels from muscle not susceptible to malignant hyperthermia, and MHI = channels from malignant hyperthermia muscle that is insensitive to halothane. Asterisks indicate statistically significant differences from the control at $**P < 0.01$ or $*P < 0.05$.

pA) and MHN (10.92 ± 0.71 pA) channels. Incremental concentrations of halothane from 2.2 to 17.6 μM had no effect on the Po of channels from MHN muscle (fig. 4). In contrast, 7 of 13 channels from MHS muscle had increases in Po at one or more of these concentrations of halothane (fig. 4). Among channels from MHS muscle, the maximum effective halothane concentrations ranged from 4.4 to 17.6 μM, while the maximum change in Po as percent of the control value ranged from 96.6 to 131.5%. The mean open time of calcium-release channels from MHN muscle was not changed by increasing concentrations of halothane used (fig. 5), whereas mean open time of the channels from MHS muscle increased ($P < 0.05$) when the concentration of halothane was 17.6 μM in the bilayer solution (fig. 5). At a concentration of 17.6 μM halothane, the changes in mean open times were −20.0% and +91.8% of controls for MHN and MHS channels, respectively. The mean closed time for MHN channels was not significantly changed by halothane, and the change at 16.7 μM halothane was −5.98% of the control value (fig. 6). The mean closed time for channels from MHS muscle decreased with each increase in halothane concentration with the maximum change of −63.0% occurring at 17.6 μM halothane (fig. 6). However, only the changes at 4.4 and 6.6 μM were significantly ($P < 0.05$) different from the control value.

In order to determine if halothane was altering unitary conductance of the channel, current *versus* voltage data

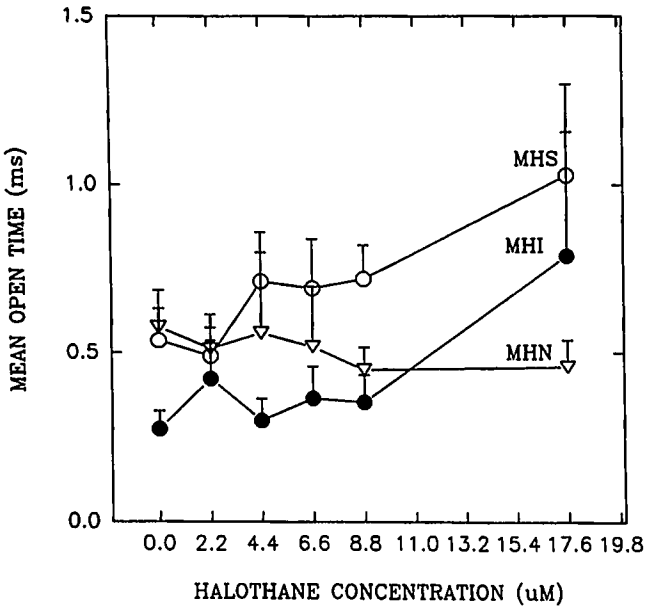

FIG. 5. The effect of halothane on the mean open state time of calcium-release channels from MHS and MHN skeletal muscle. MHI = channels from MHS muscle in which the open-state probability was not affected by halothane. Halothane concentration is equilibrated gas phase concentration.

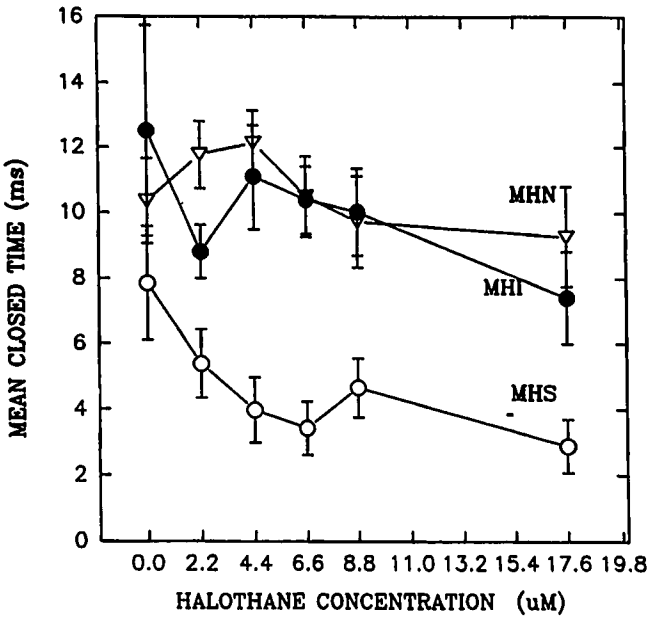

FIG. 6. The effect of halothane on the mean closed-state time of calcium-release channel from MHS and MHN skeletal muscle. MHI = channels from MHS muscle in which halothane had no effect on open state probability. Halothane is concentration in equilibrated gas phase.

was obtained on a single channel from each of two MHS patients over a wider range of halothane concentrations (fig. 7). At halothane concentrations ranging from 2.2 to 16 μM there was a marked increase in conductance by each channel. In one channel (fig. 7), halothane concentrations greater than 50 μM decreased conductance below the control value (fig. 7). The concentrations of halothane (2.2–16 μM) that increased conductance of these channels from MH human muscle are in the same concentration range at which halothane is increasing the Po (fig. 4).

An automated single-channel analysis program was used to measure the number of single-channel openings at two different amplitude levels. Selecting levels of conductance, at 12 and 24 pA, measurement of the number of events at each of these levels revealed a marked difference between MHN and MHS channels (fig. 8). Of the total number of MHN single-channel openings, 94% of these occurred with a conductance of 12 pA (fig. 8). In contrast, only 79% of the total openings of the MHS channels were at the 12-pA level of conductance (fig. 8). Of the MHS channel openings, 19% were occurring at a conductance of 24 pA, whereas only 7% of the MHN channel openings were at this higher level of conductance (fig. 8). No statistically significant effect of halothane on these conductance levels was observed.

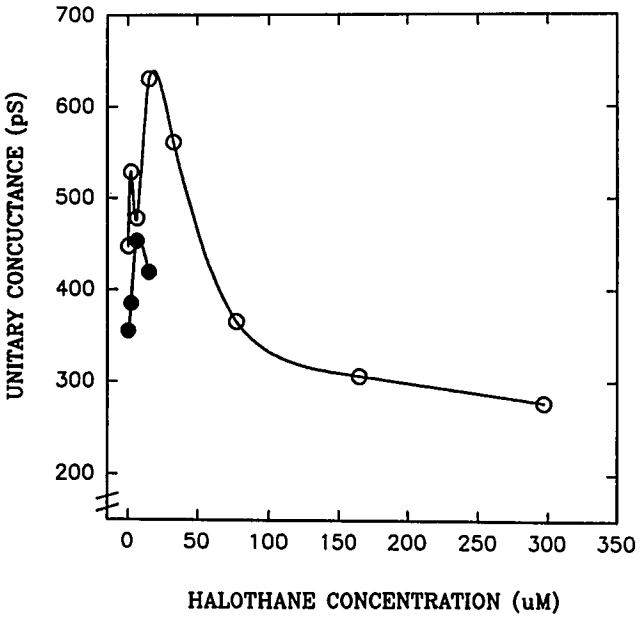

FIG. 7. The effect of halothane on unitary conductance of single calcium-release channels from two MHS patients. For each channel, each data point represents the unit conductance determined from the current measured at three different (30, 40, 50 mV) bilayer potentials from a holding potential of 0 mV. The slope of current (picoamperes) *versus* voltage (microvolts) produced the unitary conductance value ($1/\text{ohms} \times 10^{-12}$) = picoSiemans (pS).

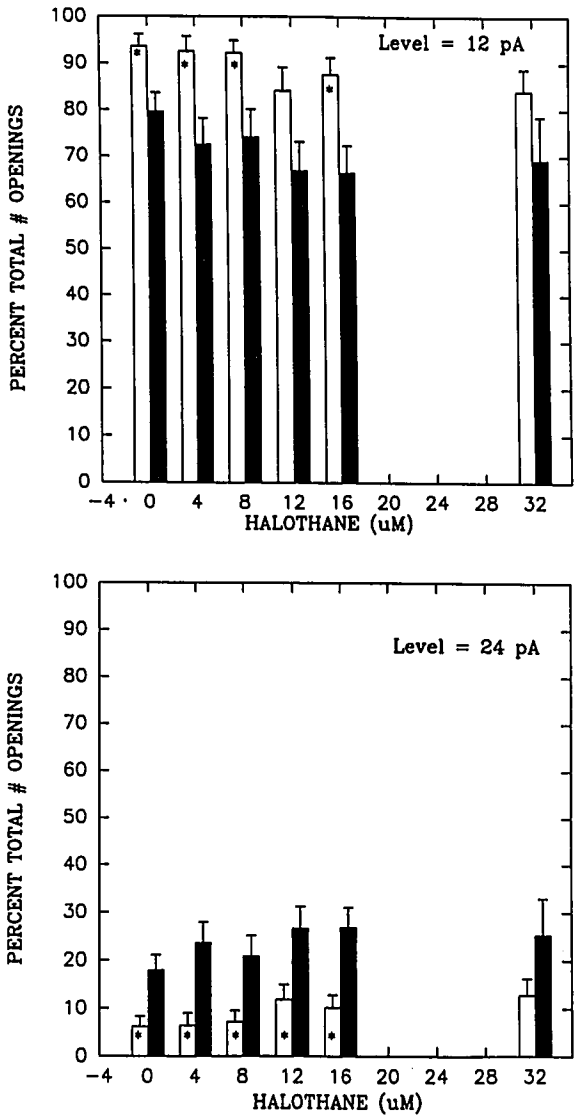

FIG. 8. The effect of halothane on the number of MHS and MHN channel openings to 12- or 24-pA levels of conductance. Open bars represent channels from MHN human skeletal muscle, and filled bars represent channels from MHS muscle. *MHN statistically significantly ($P < 0.05$) different from the corresponding MHS value.

Discussion

The incorporation of a single RyR molecule into a planar lipid bilayer and electrophysiologically recording the single channel properties of this molecule have provided a unique exploration of the MH abnormality. This methodology has previously shown that a defect exists in the RyR molecule isolated from human MH skeletal muscle.[18] Our initial studies[18] recorded the effects of caffeine on the gating and conductance properties of human RyR channels and showed that calcium-release channels from

human MH muscle have greater sensitivity to the effects of caffeine to increase the Po. This caffeine effect at the single RyR molecule level reflected the intact MH muscle cell's greater contracture sensitivity to caffeine, a method used to diagnose MHS. In the present study, I have shown similar abnormal sensitivity of the RyR channel from human MH muscle to halothane, which reflects the abnormal contracture-producing effects of halothane on the intact MH muscle cell. These functional studies provide direct evidence for an abnormality in the RyR molecule in MH human skeletal muscle. The abnormal contracture-producing effects of caffeine and halothane on MH skeletal muscle have become the hallmark for MH in humans, resulting in the search to determine how these agents affect the increased myoplasmic calcium that triggers the MH syndrome.

The present discovery in human MH muscle of abnormal functional alterations induced by caffeine and halothane on what is considered to be the major calcium-release channel for skeletal muscle[10] may provide a molecular basis for the MH abnormality. Other data tend to substantiate this provision. Two independent investigations have concluded that the gene predisposing to MH in humans is located on chromosome 19 and in close proximity to the locus for the RyR gene.[11,12] Ryanodine, an alkaloid with high affinity and specificity for binding to the RyR, produces contracture in skeletal muscle, and the sensitivity to these contracture producing effects is greater in human MH muscle.[22] The effect of ryanodine on the RyR calcium-release channel in planar lipid bilayers is to bind the opened channel and lock it in a substate of lower conductance.[23] Such an effect in the intact muscle cell would create an increased flux of calcium into the myoplasm and results in contractures when ryanodine is present. In MH skeletal muscle, this effect is, in some way, exaggerated.

Halothane appeared to affect two different mechanisms regulating the MH RyR calcium-release channel. The effect of halothane to increase the probability of the channel to change from a nonconducting, closed state to an open, conducting state indicates an effect on the regulation of the channel activation–inactivation processes. The other halothane effect was to increase the level of conductance of the MH channel, a process involving gating of the channel among the various conductance states.[24] The two processes, *i.e.*, activation–inactivation and conductance, were affected at similar concentrations of halothane. Exact mechanisms by which the calcium-release channel is activated or inactivated or by which the gating process is regulated remain unknown. It has been established that multiple ligand binding sites exist for the RyR and that some of these affect the channel's functional properties. Caffeine,[18] calcium, and ATP[25] activate the channel, increasing Po. The effect of calcium is concentration-dependent, 1–10 μM activating and >100 μM inactivating the channel. A preliminary investigation of calcium activation–inactivation in human calcium-release channels found no differences between channels from MH and normal muscle.[18] Ryanodine, in micromolar concentrations, locks the channel in an open substate level of conductance.[22] Calmodulin, magnesium, and ruthenium red inactivate the channel.[24] Each of these ligand binding sites may represent some form of regulatory site for the calcium-release channel, and one or more could be genetically altered in MH.

That only 7 of 13 channels from MH muscle exhibited halothane sensitivity suggests that more than one type of channel exists. One possible explanation for the presence of more than one type of channel is that the MH muscles are heterozygous for the RyR gene, resulting in the production of both normal and MHS RyR channels. Because MH syndromes and positive contractures may have multiple etiologies due to a variety of genetic defects, it is not unreasonable that in muscle from three of the MH subjects tested, no halothane-sensitive calcium release channels were observed. Indeed, of all five channels recorded from three of the MH subjects, none was halothane-sensitive. Also, a lipid abnormality has been suggested for MH,[26] and since lipid segments of the native SR membrane are incorporated with the RyR into the bilayer, our study does not rule out the lipogenic theory. The number of observations in this study is inadequate, however, to resolve these issues.

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