

# *Mg<sup>2+</sup> Dependence of Halothane-induced Ca<sup>2+</sup> Release from the Sarcoplasmic Reticulum in Skeletal Muscle from Humans Susceptible to Malignant Hyperthermia*

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**Background:** Recent work suggests that impaired Mg<sup>2+</sup> regulation of the ryanodine receptor is a common feature of both pig and human malignant hyperthermia. Therefore, the influence of [Mg<sup>2+</sup>] on halothane-induced Ca<sup>2+</sup> release from the sarcoplasmic reticulum was studied in malignant hyperthermia-susceptible (MHS) or -nonsusceptible (MHN) muscle.

**Methods:** Vastus medialis fibers were mechanically skinned and perfused with solutions containing physiologic (1 mM) or reduced concentrations of free [Mg<sup>2+</sup>]. Sarcoplasmic reticulum Ca<sup>2+</sup> release was detected using fura-2 or fluo-3.

**Results:** In MHN fibers, 1 mM halothane consistently did not induce sarcoplasmic reticulum Ca<sup>2+</sup> release in the presence of 1 mM Mg<sup>2+</sup>. It was necessary to increase the halothane concentration to 20 mM or greater before Ca<sup>2+</sup> release occurred. However, when [Mg<sup>2+</sup>] was reduced below 1 mM, halothane became an increasingly effective stimulus for Ca<sup>2+</sup> release; e.g., at 0.4 mM Mg<sup>2+</sup>, 58% of MHN fibers responded to halothane. In MHS fibers, 1 mM halothane induced Ca<sup>2+</sup> release in 57% of MHS fibers at 1 mM Mg<sup>2+</sup>. Reducing [Mg<sup>2+</sup>] increased the proportion of MHS fibers that responded to 1 mM halothane. Further experiments revealed differences in the characteristics of halothane-induced Ca<sup>2+</sup> release in MHS and MHN fibers: In MHN fibers, at 1 mM Mg<sup>2+</sup>, halothane induced a diffuse increase in [Ca<sup>2+</sup>], which began at the periphery of the fiber and spread slowly inward. In MHS fibers, halothane induced a localized Ca<sup>2+</sup> release, which then propagated along the fiber. However, propagated Ca<sup>2+</sup> release was observed in MHN fibers when halothane was applied at an Mg<sup>2+</sup> concentration of 0.4 mM or less.

**Conclusions:** When Mg<sup>2+</sup> inhibition of the ryanodine receptor is reduced, the halothane sensitivity of MHN fibers and the characteristics of the Ca<sup>2+</sup> release process approach that of the MHS phenotype. In MHS fibers, reduced Mg<sup>2+</sup> inhibition of the ryanodine receptor would be expected to have a major influence on halothane sensitivity. The Mg<sup>2+</sup> dependence of the halothane response in MHN and MHS may have important clinical implications in circumstances where intracellular [Mg<sup>2+</sup>] deviates from normal physiologic concentrations.

**MALIGNANT hyperthermia (MH)** is a pharmacogenetic condition triggered by exposure to volatile anesthetics,

which results in muscle contracture and a potentially fatal increase in body temperature.<sup>1</sup> The condition has been studied extensively in pigs, where the underlying abnormality is known to be a single Arg615Cys mutation of the ryanodine receptor (RYR).<sup>2</sup> In human MH, the genetics are much more complex: There are both locus and allelic heterogeneity, with five susceptibility loci identified in addition to the RYR locus.<sup>3–5</sup> However, the RYR seems to have a major role in most families, and more than 30 mutations have now been identified.<sup>6</sup> Consistent with this, MH is associated with increased sensitivity to drugs such as caffeine, 4-chloro-M-cresol, and ryanodine, all of which induce sarcoplasmic reticulum (SR) Ca<sup>2+</sup> release by acting directly on the RYR.<sup>7–9</sup>

A number of recent studies have considered the possibility that abnormal regulation of the RYR by cytosolic Mg<sup>2+</sup> may be involved in MH. In normal muscle, Mg<sup>2+</sup> exerts a dual inhibitory effect by competing with Ca<sup>2+</sup> at the activation site on the RYR and binding to a low-affinity Ca<sup>2+</sup>/Mg<sup>2+</sup> inhibitory site.<sup>10,11</sup> Consequently, RYR gating is strongly inhibited at physiologic concentrations of free Mg<sup>2+</sup>, which may explain the low sensitivity of resting muscle to pharmacologic activators.<sup>12</sup> However, experiments on skinned muscle fibers and isolated RYRs incorporated into lipid bilayers suggest that the inhibitory action of Mg<sup>2+</sup> may be reduced in both porcine and human MH.<sup>13–16</sup>

In theory, reduced Mg<sup>2+</sup> inhibition might increase the sensitivity of the RYR to such an extent that Ca<sup>2+</sup> release is triggered by concentrations of volatile anesthetics within the range found during anesthesia. This possibility is supported by experiments on skinned muscle fibers from rats, showing that the potency of halothane's action on the SR increases markedly when Mg<sup>2+</sup> inhibition of the RYR is reduced by decreasing the free [Mg<sup>2+</sup>].<sup>17</sup> This raises a secondary issue, that changes in cellular [Mg<sup>2+</sup>] levels associated with pathologic conditions such as diabetes<sup>18</sup> might influence the probability or severity of an MH episode. However, it is not clear whether conclusions drawn from experiments on normal rat skeletal muscle can be extended to a more heterogeneous population of human fibers, where MH is associated with numerous mutations. Therefore, the aim of the current study was to characterize the effects of cytosolic Mg<sup>2+</sup> on halothane-induced Ca<sup>2+</sup> release from the SR in normal (MHN) and MH-susceptible (MHS) human skeletal muscle.

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## Materials and Methods

### *Solution Composition*

All chemicals were purchased from Sigma-Aldrich Ltd. (Gillingham, Dorset, United Kingdom). The basic solution contained 25 mM HEPES, 0.15 mM EGTA, 10 mM creatine phosphate, 5 mM adenosine 5' triphosphate, 100 mM KCl, and 0.002 mM fura-2. Creatine phosphate and adenosine 5' triphosphate were added as disodium salts. The free  $[Ca^{2+}]$  of the basic solution was adjusted to 120 nM by addition of  $CaCl_2$ . Free  $[Mg^{2+}]$  was altered to 1, 0.4, 0.2, and 0.1 mM by addition of  $MgCl_2$ . The free and bound concentrations of ions in the experimental milieu were calculated using an in-house computer program as necessary. Experiments were performed at room temperature ( $22 \pm 2^\circ C$ ), pH 7.0. Halothane was added from a stock solution prepared in dimethyl sulfoxide. In most experiments, where 1 mM halothane was used, the dimethyl sulfoxide concentration was 0.1%. When higher concentrations for halothane were applied (5–40 mM), the maximum dimethyl sulfoxide concentration was 2%. However, in control experiments, 2% dimethyl sulfoxide did not induce release of  $Ca^{2+}$  from the SR or modify other aspects of SR function (e.g., SR  $Ca^{2+}$  uptake or caffeine-induced release, results not shown). Throughout the experiments, the halothane solutions were kept in airtight syringes to prevent vaporization.

### *Preparation*

Samples of vastus medialis muscle were obtained by open biopsy from patients presenting for MH susceptibility testing at St. James's Hospital, Leeds, United Kingdom. Approximately 1 g of muscle was removed for the *in vitro* contracture test (IVCT). With institutional Research Ethics Committee approval and informed patient consent, an additional bundle (0.2 g) was used to provide material for studies on mechanically skinned muscle preparations. All procedures were conducted according to the Declaration of Helsinki. The IVCT provided the primary method of categorizing MHS or MHN muscle, according to the criteria for MH research of the European MH Group.<sup>19</sup> This ensures a high sensitivity and specificity of the MH diagnosis (98% and 94% respectively).<sup>20</sup> Most experiments were conducted on fibers classified as MHN or MHS. However, a small number of patients positive to halothane alone (MH equivocal) were included for comparison. Only one fiber was used from each sample/patient. Samples were obtained from a total of 115 patients (MHN, 71; MHS, 35; and MH equivocal, 9).

The smaller bundle of muscle used to prepare skinned fibers was placed in a "relaxing" solution approximating the intracellular milieu (see Solution Composition). Individual muscle fibers were isolated and then mechanically skinned with fine forceps. Vastus medialis is of mixed fiber type, and strontium sensitivity tests were routinely

conducted to classify the fibers as type 1 or type 2.<sup>21</sup> It was found that most of the preparations did not generate significant tension at pSr 5.2, suggesting that the majority of selected fibers were type 2. Any preparations generating significant tension at pSr 5.2 were not used in the current study.

### *Apparatus*

The apparatus for simultaneous measurement of  $[Ca^{2+}]$  and force from skinned muscle fibers is described in detail elsewhere.<sup>22</sup> Briefly, a mechanically skinned muscle fiber was mounted in a shallow bath with a glass coverslip base. A cylindrical Perspex column (3 mm diameter) was lowered to within a few micrometers of the stainless steel tubes (approximately 200  $\mu m$  diameter) used to attach the muscle. The volume of solution immediately below the column was approximately 1.4  $\mu l$ . Preparations were perfused by pumping solution at 0.8 ml/min *via* a narrow duct passing through the center of the column. Caffeine or halothane was applied rapidly using a syringe pump, which allowed each of eight channels to be controlled independently *via* a computer interface. The plastic syringes (5 ml), containing caffeine or halothane solutions, were connected *via* narrow cannulae to a series of injection ducts near the base of the column. The experimental bath was placed on the stage of an S 200 Nikon Diaphot inverted microscope (Nikon UK Limited, Kingston upon Thames, Surrey, United Kingdom). The muscle fiber was viewed *via*  $\times 40$  Fluor objective (Nikon CF Fluor, NA 0.75).

### *Ca<sup>2+</sup> Detection and Measurement*

In most experiments, the preparation was alternately illuminated with light of wavelengths 340 and 380 nm at a frequency of 45 Hz using a spinning wheel spectrophotometer (Cairn Research Limited, Faversham, Kent, United Kingdom). Changes in the spatially averaged  $[Ca^{2+}]$  within the visual field containing the preparation were indicated by the ratio of light intensities emitted at greater than 500 nm. In some experiments, sequential x-y images were obtained using confocal microscopy and cells were viewed using a  $\times 20$  lens (Nikon Plan Fluor DLL, NA 0.3). A confocal laser-scanning unit (BioRad, Microradiance, Herts, United Kingdom) was attached to the side port of the microscope. Fluo-3 excitation was achieved using the 488-nm line of an argon ion laser, and emitted fluorescence was measured at greater than 515 nm.

### *Application of Caffeine or Halothane*

In all protocols, introduction of halothane was preceded by a series of responses induced by brief (500-ms) application of a solution containing 40 mM caffeine but lacking  $Mg^{2+}$  (40 mM caffeine–0  $Mg^{2+}$ ) at 4-min intervals. In control experiments, it was found that (1) application of 40 mM caffeine–0  $Mg^{2+}$  for 500 ms produced a

maximal  $\text{Ca}^{2+}$  release, *i.e.*, a higher caffeine concentration or more prolonged application did not increase the response, and (2) increasing the  $\text{Ca}^{2+}$  loading period beyond 4 min did not increase the response. This suggests that the releasable pool of SR  $\text{Ca}^{2+}$  reaches a steady state within 4 min. The caffeine-induced fluorescence transients provided an index of the SR  $\text{Ca}^{2+}$  content, and the consistency of the responses was used to gauge the viability of the preparation.  $\text{Mg}^{2+}$  was omitted from the caffeine solution because previous studies have shown that even high concentrations of caffeine do not fully activate the SR  $\text{Ca}^{2+}$  release mechanism unless  $[\text{Mg}^{2+}]$  is reduced to submillimolar concentrations.<sup>12</sup>

When halothane is rapidly applied for a brief period during perfusion, a higher concentration is required to induce a measurable SR  $\text{Ca}^{2+}$  release than if halothane is applied for a longer period (1–2 min) in the absence of perfusion.<sup>17</sup> This may occur for the following reasons: First, during constant perfusion,  $\text{Ca}^{2+}$  released from the SR can diffuse out of the skinned fiber, after which it is rapidly washed away. Consequently, even a large efflux of  $\text{Ca}^{2+}$  from the SR may be essentially undetectable if it occurs at a very low rate. In contrast, when the flow is stopped, any  $\text{Ca}^{2+}$  released from the SR accumulates in the limited volume of solution underneath the column, increasing the likelihood of detection. Second, in the absence of flow, any given efflux of  $\text{Ca}^{2+}$  from the SR results in a larger local increase in  $[\text{Ca}^{2+}]$  in the vicinity of the RYR, which may result in positive feedback *via*  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release. In intact skeletal muscle cells,  $\text{Ca}^{2+}$  transport across the sarcolemma occurs at a very low rate. Hence, the conditions in the absence of perfusion mimic that of the intact cell more closely than does continuous perfusion.

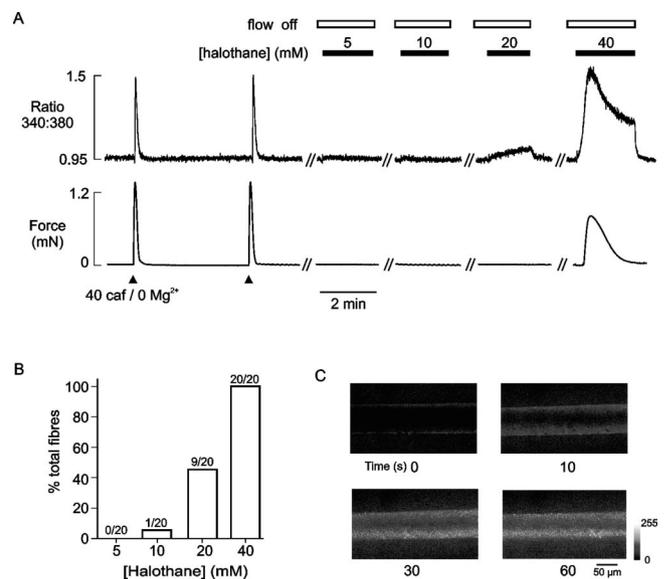
#### Data Recording and Analysis

In all experiments, the ratio and individual wavelength intensities signals were low-pass filtered (3 db at 30 Hz) and digitized for later analysis using a personal computer with a Data Translation (Basingstoke, Hants, United Kingdom) 2801A card. The  $\text{IC}_{50}$  values (95% confidence limits) were derived using the Hill equation fitted to the cumulative quantal (all-or-none) response data using Microcal Origin v6.0 (OriginLab Corporation, Northampton, MA).

## Results

#### Halothane Sensitivity of Normal Human Skeletal Muscle Fibers in the Presence of 1 mM $\text{Mg}^{2+}$

Figure 1A (top panel) shows a record of the fura-2 fluorescence ratio obtained from a mechanically skinned normal (MHN) muscle fiber. The preparation was initially perfused with a weakly  $\text{Ca}^{2+}$ -buffered solution containing 120 nM  $\text{Ca}^{2+}$  and 1 mM free  $\text{Mg}^{2+}$ . SR  $\text{Ca}^{2+}$



**Fig. 1.** (A) A normal (malignant hyperthermia–nonsusceptible) skeletal muscle fiber was perfused with a mock intracellular solution containing 120 nM  $\text{Ca}^{2+}$  and 1 mM free  $\text{Mg}^{2+}$ . A series of control responses (two shown) was initiated by brief application of a solution containing 40 mM caffeine and 0  $\text{Mg}^{2+}$  (40 caf/0  $\text{Mg}^{2+}$ ) at 4-min intervals (*left*). During the period indicated by the break in the trace (//), the preparation was perfused for a further 4 min to allow the sarcoplasmic reticulum to reaccumulate  $\text{Ca}^{2+}$ , and perfusion was then stopped. The solution within the bath was then rapidly replaced with one containing 5 mM halothane, and perfusion was stopped again. The perfusion was then restarted, and the protocol was repeated in the same preparation but with 10 mM, 20 mM, and 40 mM halothane. (B) Accumulated data summarizing the proportion of normal fibers responding at each halothane concentration. (C) Confocal x–y images using the same protocol shown in A. Representative frames are shown 0, 10, 20, and 60 s after exposure to 40 mM halothane in the presence of 1 mM free  $\text{Mg}^{2+}$ .

release was induced by brief application of 40 mM caffeine–0  $\text{Mg}^{2+}$  at 4-min intervals. After several responses to caffeine–0  $\text{Mg}^{2+}$ , the preparation was perfused for a further 4 min, allowing the SR to fully reload with  $\text{Ca}^{2+}$ . Perfusion was then stopped, and the solution was replaced rapidly with one containing 5 mM halothane. This protocol was then repeated in the same preparation with 10, 20, and 40 mM halothane.

In this MHN fiber, exposure to 5 or 10 mM halothane did not induce a detectable release of  $[\text{Ca}^{2+}]$  from the SR. After introduction of 20 mM halothane, the  $[\text{Ca}^{2+}]$  within the bath increased slowly and peaked at approximately 20% of the preceding caffeine response. However, this increase in  $[\text{Ca}^{2+}]$  was insufficient to generate tension. On introduction of 40 mM halothane,  $[\text{Ca}^{2+}]$  increased more rapidly and reached a higher concentration, before decreasing to a new steady state level. The response to 40 mM halothane was consistently associated with a transient tension transient. When the flow was resumed,  $[\text{Ca}^{2+}]$  within the bath rapidly returned to the control level. As in previous studies,<sup>17</sup> the halothane-induced  $\text{Ca}^{2+}$  efflux and subsequent force production was abolished completely by previous exposure to ru-

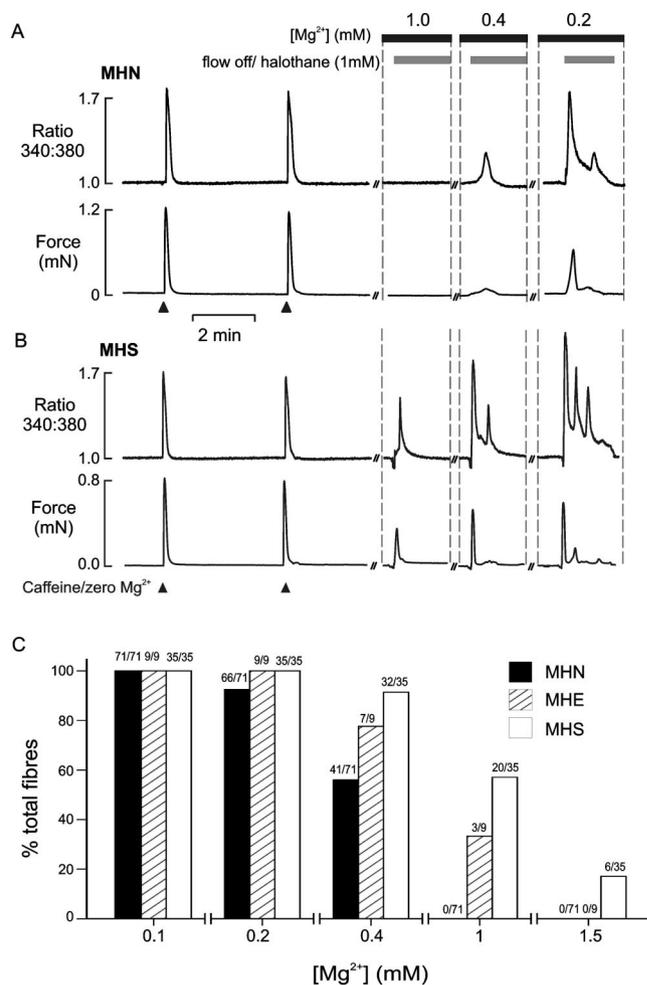
thenium red, indicating that release occurs *via* the RYR (not shown). Figure 1B shows cumulative data illustrating the proportion of MHN fibers in which SR  $\text{Ca}^{2+}$  release occurred at each halothane concentration. In 5% of MHN fibers, a detectable increase in  $[\text{Ca}^{2+}]$  occurred in the presence of 10 mM halothane. However, in most fibers, it was necessary to increase the halothane concentration to 20 mM or greater before SR  $\text{Ca}^{2+}$  release was detected.

Figure 1C shows confocal x-y images obtained from an MHN fiber 0, 10, 30, and 60 s after introduction of 20 mM halothane, using the protocol shown in figure 1A. Ten seconds after introduction of halothane, an increase in  $[\text{Ca}^{2+}]$  was apparent at the edges of the fiber. After 30 s and then 60 s, the increase in  $[\text{Ca}^{2+}]$  became more uniform as SR  $\text{Ca}^{2+}$  efflux spread progressively toward the center of the fiber. Under these conditions, the increase in  $[\text{Ca}^{2+}]$  was consistently slow and diffuse. Localized  $\text{Ca}^{2+}$  release or propagated  $\text{Ca}^{2+}$  waves were never detected ( $n = 5$ ).

#### Effects of Cytosolic $\text{Mg}^{2+}$ on $\text{Ca}^{2+}$ Release Induced by 1 mM Halothane in MHN and MHS Fibers

Further experiments were conducted to assess the responsiveness of MHS and MHN fibers to a lower halothane concentration within the clinically relevant range,<sup>23,24</sup> under conditions where  $\text{Mg}^{2+}$  inhibition of the RYR was reduced. In figure 2A, a series of control responses (two shown) was initiated in an MHN fiber by brief application of 40 mM caffeine-0  $\text{Mg}^{2+}$  at 4-min intervals (left). The flow was then stopped for 1 min (not shown) before rapid introduction of 1 mM  $\text{Mg}^{2+}$ -1 mM halothane. As expected, 1 mM halothane did not induce a detectable release of  $\text{Ca}^{2+}$  in the presence of 1 mM  $\text{Mg}^{2+}$ . This protocol was repeated in the same preparation, with either 0.4 or 0.2 mM  $[\text{Mg}^{2+}]$  present in the halothane solution. In this example, 1 mM halothane induced a small SR  $\text{Ca}^{2+}$  release and an associated tension response at 0.4 mM  $\text{Mg}^{2+}$ . However, when  $[\text{Mg}^{2+}]$  was reduced to 0.2 mM, 1 mM halothane induced a much larger  $\text{Ca}^{2+}$  and force transient. The amplitude of the fluorescence transient peaked at approximately the same level as the preceding control response to 40 mM caffeine-0  $\text{Mg}^{2+}$ .

Figure 2B shows the same protocol applied to an MHS muscle fiber. In this example, 1 mM halothane induced a transient increase in both  $[\text{Ca}^{2+}]$  and force in the presence of 1 mM  $[\text{Mg}^{2+}]$ . The onset of the  $\text{Ca}^{2+}$  transient was delayed, and the amplitude peaked at approximately 70% of the preceding control responses to 40 mM caffeine-0  $\text{Mg}^{2+}$ . In the presence of 0.4 or 0.2 mM  $\text{Mg}^{2+}$ , the initial increase in  $[\text{Ca}^{2+}]$  was larger and more rapid in onset and tended to repeat cyclically in the continued presence of halothane. At 0.2 mM  $\text{Mg}^{2+}$ , the  $[\text{Ca}^{2+}]$  within the bath remained increased throughout exposure to halothane. However, on resumption of flow,

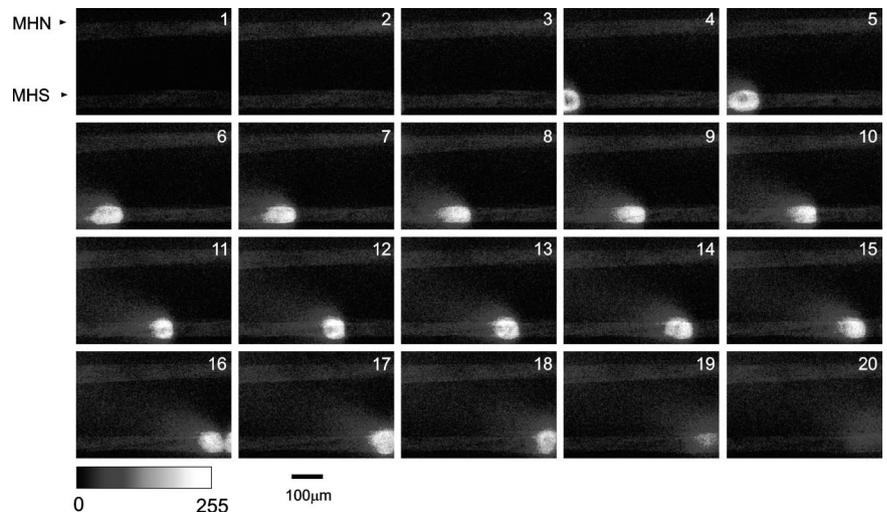


**Fig. 2.** (A) A normal (MHN) skeletal muscle fiber was perfused with a solution containing 120 nM  $\text{Ca}^{2+}$  and 1 mM  $\text{Mg}^{2+}$ . A series of control responses (two shown) was initiated by brief application of a solution containing 40 mM caffeine and 0  $\text{Mg}^{2+}$ . During the period indicated by the break in the trace (//), the preparation was perfused for a further 4 min to allow the sarcoplasmic reticulum to reload with  $\text{Ca}^{2+}$ , and perfusion was then stopped. The  $[\text{Mg}^{2+}]$  was then changed to either 1.5, 1, 0.4, 0.2, or 0.1 mM for a further minute (not shown) before rapidly introducing 1 mM halothane (at the same  $[\text{Mg}^{2+}]$ ). (B) The same protocol applied to a malignant hyperthermia-susceptible (MHS) fiber. (C) Cumulative data for MHN (filled), MHS (open), and malignant hyperthermia-equivocal (MHE) fibers (hatched). All values are expressed as a percentage of the total number of fibers: MHN, 71; MHS, 35; and MHE, 9 (1 fiber/patient).

$[\text{Ca}^{2+}]$  returned rapidly to control levels as halothane was washed away.

Accumulated data obtained using this protocol is shown in figure 2C. The percentage of fibers responding to 1 mM halothane is represented on the abscissa and the free  $[\text{Mg}^{2+}]$  on the ordinate. Data are included for 71 normal, 35 MHS, and 9 MH-equivocal fibers. The results show that none of the MHN fibers responded to 1 mM halothane in the presence of 1 mM  $\text{Mg}^{2+}$ . In contrast,  $\text{Ca}^{2+}$  release occurred in 57% MHS fibers under similar conditions. In the presence of 0.4 mM  $\text{Mg}^{2+}$ , 58% of MHN fibers and 91% of MHS fibers responded to halothane. At 0.2 mM and 0.1 mM  $\text{Mg}^{2+}$ , almost all MHS and

**Fig. 3.** A series of 20 sequential confocal x-y images obtained from a normal (MHN, *top*) and a malignant hyperthermia-susceptible (MHS, *bottom*) fiber mounted in parallel. At the beginning of the sequence, 1 mM halothane was introduced in the presence of 1 mM free  $Mg^{2+}$ , and perfusion was stopped. Conditions were as described for figure 1, except  $40 \mu M$  fluo-3 replaced fura-2. After a short delay, halothane caused a propagating  $Ca^{2+}$  wave in the MHS fiber, whereas no response occurred in the MHN fiber. Frames were collected at 0.47 Hz.



MHN fibers responded to halothane. The sensitivity of fibers classified as MH-equivocal lay between that of the MHS and MHN fibers. The accumulated data also show responses obtained at a free  $[Mg^{2+}]$  of 1.5 mM, where 1 mM halothane induced SR  $Ca^{2+}$  release in 17% of MHS fibers. The  $IC_{50}$  for the inhibitory action of  $Mg^{2+}$  on response to 1 mM halothane was 0.39 (0.38–0.4) mM in MHN fibers and 1.09 (0.99–1.19) mM in MHS fibers. These values are significantly different ( $P < 0.05$ ).

During the experiments shown in figure 2B, visual observation of the preparation suggested that when  $Ca^{2+}$  release was induced by 1 mM halothane in MHS fibers, it took the form of a wave (apparent as localized sarcomere shortening), which propagated along the fiber. Therefore, further experiments were conducted using confocal microscopy to investigate the properties of halothane-induced  $Ca^{2+}$  release under these conditions. Figure 3 shows sequential x-y images obtained during an experiment in which single skinned MHN (top) and MHS (bottom) fibers were mounted in parallel within the bath. The first image was obtained just after a solution containing 1 mM halothane–1 mM  $Mg^{2+}$  was rapidly introduced into the bath and the perfusion was stopped. After a short delay, a localized  $Ca^{2+}$  release occurred on the left of the MHS fiber, which then propagated across the preparation (left to right) before gradually extinguishing. In contrast, no response was apparent in the MHN fiber. Similar results were obtained in four other preparations.

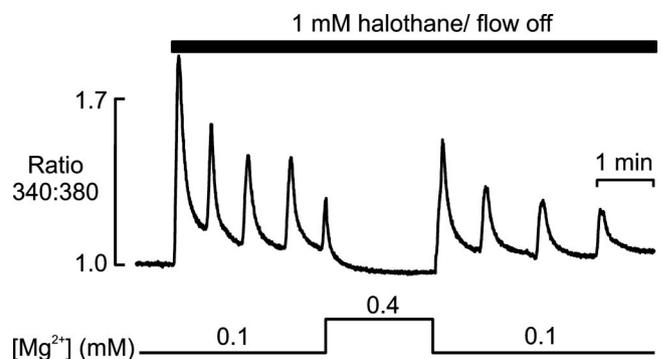
#### *Initiation and Termination of Halothane-induced $Ca^{2+}$ Release by Changing Cytosolic $[Mg^{2+}]$ in MHN Fibers*

In figure 4, an MHN fiber was initially exposed to a solution containing 0.1 mM  $Mg^{2+}$  under “stop-flow” conditions. The bath solution was then rapidly withdrawn and exchanged for one containing 0.1 mM  $Mg^{2+}$ –1 mM halothane. The introduction of halothane was associated with a rapid increase in  $[Ca^{2+}]$  within the preparation,

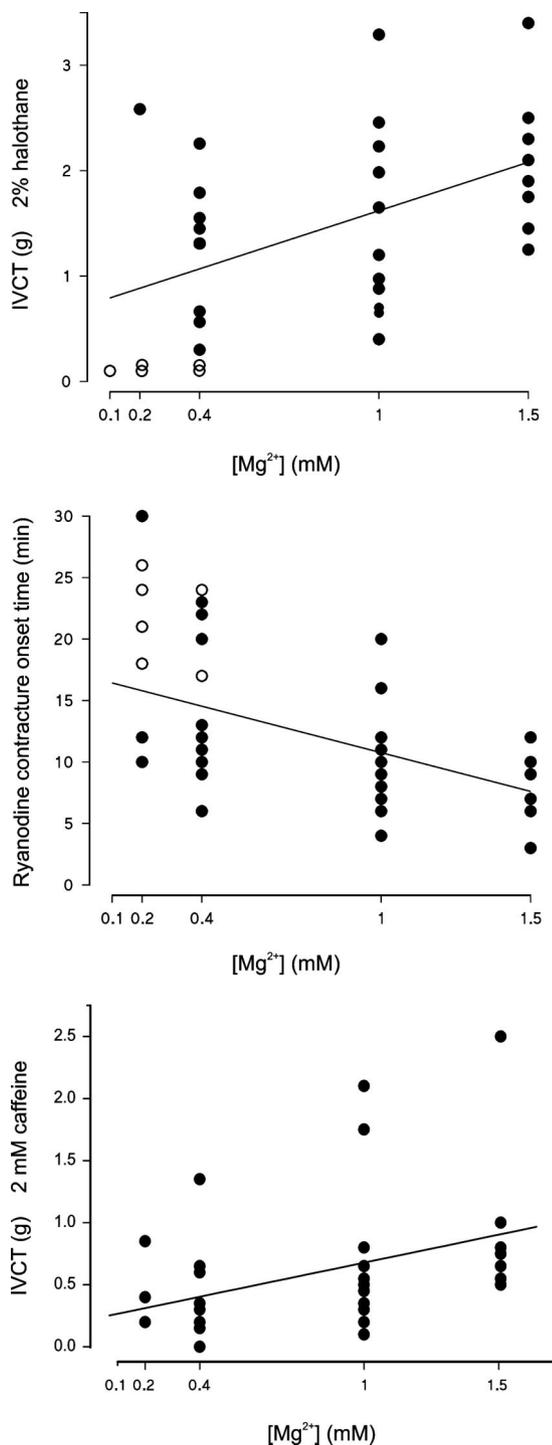
followed by repetitive  $Ca^{2+}$  waves (observed as sarcomere shortening). While the amplitude of the waves approached a steady state after approximately 1 min, there was a maintained increase in resting  $[Ca^{2+}]$  within the bath. When the  $[Mg^{2+}]$  was increased to 0.4 mM during the upstroke of a  $Ca^{2+}$  wave, there was an immediate cessation of spontaneous activity, and the resting  $[Ca^{2+}]$  returned to baseline levels. Cyclic SR  $Ca^{2+}$  release resumed on reintroduction of 0.1 mM  $Mg^{2+}$ . Similar results were seen in four other fibers tested.

#### *Relation between IVCT Results and the Threshold $[Mg^{2+}]$ for Halothane-induced $Ca^{2+}$ Release*

Given that the skinned fibers were obtained from a larger bundle of muscle used for MH diagnosis, it is possible to determine the relation between the IVCT and the skinned fiber data. In figure 5, the maximum  $[Mg^{2+}]$  at which a response to 1 mM halothane occurred in each skinned fiber is indicated on the abscissas, *i.e.*, the



**Fig. 4.** A mechanically skinned normal fiber was initially perfused with a solution containing 120 nM  $Ca^{2+}$  and 0.1 mM free  $Mg^{2+}$ . After a 4-min period of equilibration, 1 mM halothane was rapidly introduced, and the flow was stopped. This resulted in a release of  $Ca^{2+}$  from the sarcoplasmic reticulum, which repeated cyclically. Increasing the free  $[Mg^{2+}]$  from 0.1 mM to 0.4 mM resulted in a complete cessation of  $Ca^{2+}$  release. When the free  $[Mg^{2+}]$  was decreased again to 0.1 mM, cyclic sarcoplasmic reticulum  $Ca^{2+}$  release resumed.



**Fig. 5.** Correlations between the response of intact muscle fibers to caffeine, halothane, or ryanodine (*abscissa*) and the maximum  $[Mg^{2+}]$  (*ordinate*) at which a response to halothane was observed in skinned fibers from the same biopsy sample are shown. With skinned fibers, the higher the  $[Mg^{2+}]$  was at which a response to 1 mM halothane was observed, the more sensitive the preparation was. The data on intact fibers were obtained as part of the *in vitro* contracture test (IVCT) procedure, and absolute force developed in response to 2 mM caffeine (A) or 2 mM halothane (B) is shown. The onset time of the contracture in response to ryanodine is also given (C). Both normal (open circles) and malignant hyperthermia-susceptible fibers (closed circles) are shown. All correlations were statistically significant ( $P < 0.05$ ). The correlation coefficients were 0.44 (A),  $-0.48$  (B), and 0.41 (C).

higher the  $[Mg^{2+}]$  is, the more halothane-sensitive the fiber is. In figures 5A and B, the ordinates indicate the absolute force generated during the IVCT in response to 2% halothane or 2 mM caffeine. The onset time for contracture after introduction of ryanodine was also routinely measured (fig. 5C): In this case, a shorter onset time indicates a higher halothane sensitivity. All correlations were significant ( $P < 0.05$ ), indicating that the “ $Mg^{2+}$  threshold” in skinned fibers is closely related to the functional characteristics of intact fibers, which predispose to MH.

## Discussion

### *Properties of Halothane-induced $Ca^{2+}$ Release in MHN and MHS Fibers*

Unlike other cytosolic modulators of RYR function,  $Mg^{2+}$  has a key role in controlling muscle activation: Current evidence suggests that the physiologic SR  $Ca^{2+}$ -release process involves a decrease in the  $Mg^{2+}$  affinity of the RYR, triggered by an interaction with the dihydropyridine receptor.<sup>25–27</sup> Furthermore, in normal resting muscle, the low open probability of the RYR and the absence of regenerative  $Ca^{2+}$ -induced  $Ca^{2+}$  release is entirely attributable to the inhibitory action of  $Mg^{2+}$ : This explains why a reduction in free  $[Mg^{2+}]$  to approximately 50  $\mu M$  results in RYR activation and subsequent SR  $Ca^{2+}$  depletion.<sup>25</sup>

In the current study, potent inhibition of RYR gating by cytosolic  $Mg^{2+}$  may explain why such a high concentration of halothane ( $\geq 20$  mM) was required to induce  $Ca^{2+}$  release in MHN fibers (fig. 1A). Furthermore, when  $Ca^{2+}$  release did occur with high concentrations of halothane, the diffuse increase in  $[Ca^{2+}]$  and the absence of propagating  $Ca^{2+}$  waves was consistent with RYR activation in circumstances where  $Ca^{2+}$ -induced  $Ca^{2+}$  release was strongly inhibited (fig. 1B). Conversely, the repetitive, propagating  $Ca^{2+}$  waves observed in MHS fibers (figs. 2 and 3) or in MHN fibers at reduced concentrations of  $Mg^{2+}$  (fig. 4) suggest the presence of an active  $Ca^{2+}$ -induced  $Ca^{2+}$  release mechanism, whereby localized  $Ca^{2+}$  release can trigger further release from neighboring RYRs.

### *Role of Altered $Mg^{2+}$ Regulation of RYR MH*

Functional studies on isolated RYRs and skinned muscle fibers have led to the suggestion that impaired  $Mg^{2+}$  regulation of the RYR may be the primary abnormality underlying MH in porcine muscle.<sup>13,14</sup> The situation might be expected to be much more complex in human MH, where multiple RYR mutations have been identified. However, there is emerging evidence that impaired  $Mg^{2+}$  regulation of the RYR also occurs in human MH.<sup>15,28</sup> This does not mean that the underlying mutations are necessarily in the regions of the RYR associated

with  $Mg^{2+}$  binding, but rather that reduced  $Mg^{2+}$  inhibition of the channel is a common outcome. This accepted, then the current study suggests that a reduction in the inhibitory influence of  $Mg^{2+}$  in MHS fibers could have a major influence on the response to halothane: As shown in figures 2A and B, the halothane sensitivity of MHN fibers and the characteristics of the  $Ca^{2+}$  release process (slow, diffuse) can be changed to that of MHS fibers (rapid, propagating) by a moderate decrease in cytosolic  $[Mg^{2+}]$ . Based on the cumulative data (fig. 2C), it seems that the MHN phenotype can be changed to the MHS phenotype by a decreasing in the free  $[Mg^{2+}]$  to approximately 0.4 mM.

### Possible Clinical Significance

During anesthesia, the halothane concentration in arterial blood may reach 1.2 mM during induction.<sup>23,24</sup> Under the conditions used in the current study, it was necessary to increase halothane to a much higher concentration ( $\geq 20$  mM) before SR  $Ca^{2+}$  release was apparent in most MHN fibers. In contrast, in MHS fibers,  $Ca^{2+}$  release occurred at much lower concentrations of halothane, within the clinically relevant range (fig. 2). This difference is to be expected, given that the IVCT classifies fibers as MHN or MHS based on differential sensitivity to caffeine and halothane. However, the results also show that when the cytosolic  $[Mg^{2+}]$  is reduced, (1) MHN fibers respond to low concentrations of halothane and (2) MHS fibers become even more responsive to low concentrations of halothane. This may have significance in clinical situations where the cytosolic  $[Mg^{2+}]$  changes. For example, conditions such as diabetes,<sup>18</sup> heart failure,<sup>29</sup> and treatment with diuretics<sup>30</sup> can result in reduced plasma and cellular  $[Mg^{2+}]$ . Conversely,  $Mg^{2+}$  concentrations can increase in renal failure<sup>31</sup> or after infusion of magnesium sulfate during operative procedures.<sup>32</sup> Based on the current study, even a small decrease in cytosolic  $[Mg^{2+}]$  would be expected to increase the probability of MH developing in susceptible individuals and the severity of the response. Conversely, an increase in plasma  $[Mg^{2+}]$  might be expected to reduce the likelihood of an MH episode. Interestingly, previous work on MH-susceptible pigs showed that previous infusion of magnesium sulfate (100 mg/kg) attenuated the increase in intracellular  $[Ca^{2+}]$  and the associated limb rigidity that would normally have occurred on introduction of 2% halothane.<sup>33</sup> However, in this case, further work is required to establish how rapidly and to what extent changes in plasma  $[Mg^{2+}]$  can influence the free cytosolic  $[Mg^{2+}]$  before an intracellular mechanism can be assumed.

### Conclusions

The current data show for the first time that the characteristics of  $Ca^{2+}$  release process differ between MHN

and MHS fibers in the presence of physiologic concentrations of free  $Mg^{2+}$ . Furthermore, we show that the phenotypic response of MHN fibers can be changed to that of MHS fibers by a reduction in the free  $[Mg^{2+}]$  to approximately 0.4 mM. These results support previous suggestions that reduced  $Mg^{2+}$  inhibition of the RYR could be a contributory factor in MH. Furthermore, the  $Mg^{2+}$  dependence of the halothane response in MHN and MHS may have important clinical implications in circumstances where intracellular  $[Mg^{2+}]$  deviates from normal physiologic concentrations.

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