Anesthesiology
2000; 92:1799-806
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Hypersensitivity of Malignant Hyperthermia–susceptible Swine Skeletal Muscle to Caffeine Is Mediated by High Resting Myoplasmic $[Ca^{2+}]$

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Background: **Malignant hyperthermia (MH) is an inherited pharmacogenetic syndrome that is triggered by halogenated anesthetics and/or depolarizing muscle relaxants. MH-susceptible (MHS) skeletal muscle has been shown to be more sensitive to caffeine-induced contracture than muscle from nonsusceptible (MHN) subjects and is the basis for the most commonly used clinical diagnostic test to determine MH susceptibility.**

Methods: **We studied the effects of caffeine on myoplasmic free calcium concentration ($[Ca^{2+}]_i$) in MHN and MHS swine muscle fibers by means of Ca^{2+}-selective microelectrodes before and after K^+-induced partial depolarization.**

Results: **$[Ca^{2+}]_i$ in untreated MHN fibers was 123 ± 8 nM versus 342 ± 33 nM in MHS fibers. Caffeine (2 mM) caused an increase in $[Ca^{2+}]_i$ in both groups (296 ± 41 nM MHN *vs.* 1,159 ± 235 nM MHS) with no change in resting membrane potential. When either MHN or MHS, muscle fibers were incubated in 10 mM K^+ $[Ca^{2+}]_i$ transiently increased to 272 ± 22 nM in MHN and 967 ± 38 nM in MHS for 6–8 min. Exposure of MHN fibers to 2 mM caffeine while resting $[Ca^{2+}]_i$ was elevated induced an increment in $[Ca^{2+}]_i$ to 940 ± 37 nM. After 6–8 min of exposure to 10 mM K^+, $[Ca^{2+}]_i$ returned to control levels in all fibers, and the effect of 2 mM caffeine on resting $[Ca^{2+}]_i$ returned to control, despite continued partial membrane depolarization.**

Conclusions: **These results suggest that the increased "sensitivity" to caffeine of MHS swine muscle fibers is a nonspecific response related, at least in part, to the high resting $[Ca^{2+}]_i$ and not an increased caffeine sensitivity of the sarcoplasmic reticulum Ca^{2+} release channel *per se*. (Key words: Hyperpyrexia; potassium; xanthine.)**

MALIGNANT hyperthermia (MH) is a potentially fatal pharmacogenetic syndrome caused by exposure to halogenated volatile anesthetics and/or depolarizing muscle relaxants.[1] This syndrome has been associated with a dysfunction of intracellular calcium homeostasis in skeletal muscle.[2-4] In swine, susceptibility to the syndrome is caused by a single point mutation (Arg^{615} to Cys^{615}) in the sarcoplasmic reticulum (SR) Ca^{2+} release channel (RyR1).[5] In humans, it seems that susceptibility may have a multigenic origin[6,7]; however, more than 40% of individuals are known to have one of 15 point mutations in RyR1.[8] Both human and swine MH-susceptible (MHS) skeletal muscle has been shown to be more sensitive to caffeine-induced contracture than nonsusceptible (MHN) muscle. This difference is the basis for the most commonly used clinical diagnostic test to determine MH susceptibility.[9-11] Despite the fact that the caffeine and halothane contracture tests are the gold standard for diagnosing MH susceptibility, false-negative results have been reported in survivors of very well-documented MH episodes.[12,13] In addition, false-positive caffeine contracture tests have also been reported and are frequently associated with a variety of neuromuscular disorders, such as Duchenne muscular dystrophy, central core disease, and exertional rhabdomyolysis.[14-16] False-positive test results in patients with these diseases has not been a significant diagnostic problem, because they either have characteristic pathologic changes in their skeletal muscle that are not present in MHS individuals, or their

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Received from the Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela. Submitted for publication October 28, 1999. Accepted for publication February 10, 2000. Supported in part by grants from Laboratorio Elmor de Venezuela, Caracas, Venezuela (to Drs. López, Linares, and Contreras), and the Muscular Dystrophy Association, Tuscon, Arizona (to Dr. Allen).

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presenting clinical symptoms are different from those of MHS subjects. However, these findings do pose the question of whether there is a common pathway for the increased caffeine sensitivity among these various muscle diseases. One common feature that is shared by all of these disorders is an increased myoplasmic free calcium concentration ($[Ca^{2+}]_i$).[2,3,16]

In the present study, we examined the relationship between $[Ca^{2+}]_i$ and caffeine sensitivity of muscle fibers from MHS and MHN swine. We determined that by increasing resting $[Ca^{2+}]_i$, the caffeine sensitivity of MHN skeletal muscles is enhanced to the same extent as is usually found in MHS skeletal muscles.

Materials and Methods

Intact intercostal muscle biopsy specimens were obtained from 10 Yorkshire swine (MHN) and 15 Poland China swine (MHS). Susceptibility to MH was determined by polymerase chain reaction and halothane challenge as previously described.[1,5] Muscle biopsy specimens were removed during general anesthesia using nontriggering agents (thiopental 15/20 mg/kg for induction, N_2O/O_2, and supplemental thiopental for maintenance) as approved by the Animal Care committee at the Instituto Venezolano de Investigaciones Científicas.

The biopsy samples were dissected into several small muscle bundles containing five to eight intact fibers. Intact muscle bundles were mounted horizontally in a Plexiglas temperature-controlled chamber (International Plastic, Miami, FL) (37 ± 1.5°C) placed on stage of an inverted triocular microscope. Each tendon was fastened to a stainless-steel hook connected to micromanipulators. The experimental chamber was perfused with swine physiologic solution (see below) equilibrated and constantly bubbled with a mixture of 95% O_2 and 5% CO_2, pH 7.3, at 37°C.

Striation spacing and sarcomere oscillations (irregular movements of single sarcomeres) were monitored using a laser light diffraction technique.[17] Muscle fibers were illuminated perpendicular to their axis by a He-Ne laser beam (632 nm). Intensity of the first- and second-order diffraction pattern was analyzed using a photodiode. Signals from the photodiode were displayed on a Tektronix 11201 digitizing oscilloscope (Tektronix International, Portland, OR) and stored on diskettes. Striation spacing of muscle fibers was adjusted to 2.3 μm and confirmed in at least four different places along a muscle fiber. Analysis of diffraction patterns can provide information about distribution of sarcomere length in a muscle fiber, which reflects their state of activation under different experimental conditions.

Calcium-Selective Microelectrodes

Double-barrel calcium microelectrodes were prepared from thin-walled 1.2- and 1.5-mm OD borosilicate glass capillaries (WP Instruments, New Haven, CT) with internal filaments. Before pulling, the capillaries were washed with HCl and distilled water and then dried at 150°C for 3 h. Then one capillary of each size was glued together using fast-setting araldite. After the epoxy had set, the paired capillaries were then softened in a vertical puller (Narishige PE-2) and then twisted 360° with respect to each other. The two capillaries were then pulled simultaneously to a short taper with a total outside tip diameter of approximately 0.6 μm (determined using scanning electron microscopy). The 1.5-mm OD barrel was silanized by exposing it to dimethyldichlorosilane vapor, while air pressure was applied to the 1.2-mm OD barrel to prevent its silanization. The silanized barrel was backfilled 24 h later with the neutral carrier ETH 1001 (Fluka, Ronkontioma, NY) and 48 h later with pCa 7 solution (tip resistance 10^{10}–10^{11} Ω). The 1.2-mm OD barrel was backfilled with 3 M KCl and had a resistance between 10 and 15 MΩ. After 24 h in N_2, calcium-selective microelectrodes were calibrated in solutions of known $[Ca^{2+}]$ at 37°C.[4] Only Ca^{2+} microelectrodes that gave an almost Nernstian response between pCa3 and 7 (30.5 mV per pCa unit at 37°C) were used. Interference from H^+, K^+, and Mg^{2+} on the calibration of these electrodes was negligible (*e.g.*, Na^+ 20 mM, Mg^{2+} 1 mM, and K^+ 120 mM did not alter the response at pCa7). The response time of the calcium microelectrodes varied between 3 and 5 s for a change from pCa7 to pCa3.

Calcium Measurements

Single muscle fibers (2.3 μm sarcomere length) from MHN and MHS swine were impaled with flexible doubled-barreled Ca^{2+}-selective microelectrode.[4] A polished lucite pedestal with petroleum jelly on the top and a fire polished glass rod mounted in a micromanipulator were placed under and against the muscle fibers to provide support for the fiber when it was impaled with doubled-barreled Ca^{2+}-selective microelectrode and to minimize movement during sarcomere shortening. The potential from the 3-M KCl barrel (Vm) and the potential from the Ca^{2+} barrel (VCae) were recorded *via* a high impedance amplifier (Word Precision Instruments FD-223; New Ha-

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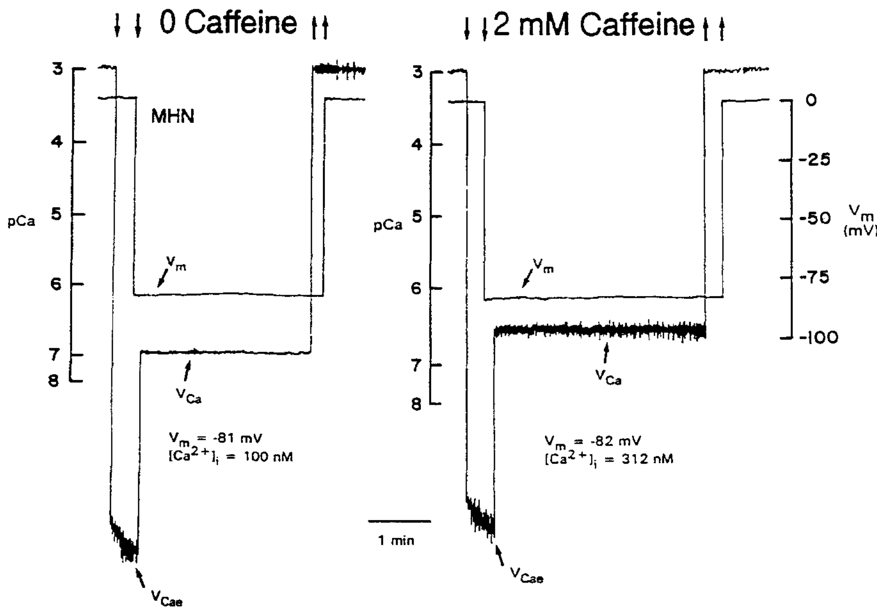

Fig. 1. Effects of caffeine on myoplasmic free calcium concentration ($[Ca^{2+}]_i$) in malignant hyperthermia–nonsusceptible (MHN) swine. Resting membrane potential (Vm) and $[Ca^{2+}]_i$ were recorded simultaneously using a double-barreled microelectrode in a single MHN muscle fiber in swine physiologic solution. VCae represents the potential recorded with the calcium-selective barrel. VCa represents the $[Ca^{2+}]_i$ obtained after the electronic subtraction of Vm (the potential recorded by the 3 mM KCl–filled barrel) from VCae potential. Vm was −81 mV and $[Ca^{2+}]_i$ was 100 nM (*left*). Addition of 2 mM caffeine to the bath elevated the $[Ca^{2+}]_i$ to 312 nM, with no significant change in Vm (*right*). Measurements of Vm and $[Ca^{2+}]_i$ before and after caffeine was added to the bath were conducted in same muscle fiber. Downward and upward arrows indicate impalement and withdrawal of the double-barreled Ca^{2+} microelectrode from the muscle fiber. (*Left* and *right*) The calibration bars for the Ca^{2+}-selective microelectrode and the resting membrane potential, respectively, are shown. The meaning of VCae, VCa, Vm, and the downward and upward arrows is the same for figures 2–4.

ven, CT). The Vm potential was subtracted electronically from the VCae potential to give a differential signal (VCa) that represents the resting myoplasmic Ca^{2+} concentration. VCa potential was filtered to improve the signal-to-noise ratio. Calibration was repeated after every five Ca^{2+} measurements; however, the properties of these Ca^{2+}-selective microelectrodes remained almost constant for the whole experiment in every case.

Solutions

Swine physiologic solution had the following composition: 135 mM NaCl, 3 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 18 mM $NaHCO_3$, 1 mM NaH_2PO_4, and 5 mM glucose. The pH was 7.3 when it was aerated with a mixture of 5% CO_2 and 95% O_2. High K^+ solution (10 mM) was made by replacing Cl^- by methanesulfonate to maintain a constant $[K^+] \times [Cl^-]$ and avoid muscle fiber swelling from osmotic changes. Caffeine solution was made by adding 50 mM caffeine to the physiologic solution to achieve the desired concentration.

Statistical Analysis

All the values are expressed as mean ± SD of the number (n) of fibers that met the criteria for an acceptable reading for that condition. Statistical difference was determined by Student *t* test. Data were considered statistically significant at $P < 0.05$.

Results

$[Ca^{2+}]_i$ in MHN and MHS Skeletal Muscle

$[Ca^{2+}]_i$ was determined successfully in 15 fibers from MHN and 17 fibers from MHS muscles. $[Ca^{2+}]_i$ in MHN muscle was 123 ± 8 nM (range, 100–131 nM), whereas in MHS muscle it was 342 ± 33 nM (range, 263–391 nM).

Effects of Caffeine on $[Ca^{2+}]_i$

It is known that MHS muscle fibers respond with higher sensitivity to caffeine than normal muscle.[9–11] The addition of 2 mM caffeine to the bathing solution induced an increase in $[Ca^{2+}]_i$ in both MHN and MHS fibers with no change in resting membrane potential. Figure 1 illustrates the effects of 2 mM caffeine on $[Ca^{2+}]_i$ in a representative MHN fiber. In these muscle fibers there was an increase in $[Ca^{2+}]_i$ from 123 ± 8 nM (n = 15) to 296 ± 41 nM (n = 12; range, 221–354 nM; table 1). This increase in $[Ca^{2+}]_i$ was associated with an oscillatory activation of individual sarcomeres that was detected by a change in the intensity of the diffraction pattern. Oscillations started immediately after fibers were transferred to the caffeine solution and lasted for approximately 30 min.

Figure 2 shows the effects exposure to 2 mM caffeine on $[Ca^{2+}]_i$ in a typical MHS muscle fiber. In these fibers,

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Table 1. Effects of Caffeine and K^+ on $[Ca^{2+}]_i$ in MHN and MHS Muscle Fibers

	Resting	Caffeine (2 mM)	K^+ (10 mM)
MHN	123 ± 8 (n = 15)	296 ± 41 (n = 12)	272 ± 22 (n = 12)
MHS	342 ± 33 (n = 17)	1,159 ± 235 (n = 13)	967 ± 38 (n = 11)

MHN = malignant hyperthermia nonsusceptible; MHS = malignant hyperthermia susceptible.

there was an increase in $[Ca^{2+}]_i$ from 342 ± 33 nM (n = 17) to 1,159 ± 235 nM (n = 13; range, 745–1,579 nM; table 1). In MHS muscle fibers, 2 mM caffeine caused an oscillatory activation of individual sarcomeres in some fibers, but in the majority of MHS fibers (10 of 13), the increase in $[Ca^{2+}]_i$ originated a sustained sarcomere shortening. After the washout of caffeine, $[Ca^{2+}]_i$ returned to precaffeine levels in both MHN and MHS muscle fibers. These findings demonstrate the already-observed differences in the caffeine sensitivity between muscle fibers from MHN and MHS individuals. Similar changes in $[Ca^{2+}]_i$ were observed when MHN and MHS fibers were incubated with other methylxanthines, *e.g.*, 1.7-dimethylxanthine and 1-methyl-3-isobutylxanthine (data not shown), showing the effect was not specific to caffeine alone.

Effects of $[K^+]_e$ on $[Ca^{2+}]_i$ in MHN and MHS Muscles

It is known that increasing $[K^+]$ in the external bathing solution above 5 mM induces a partial depolarization of the surface membrane, which then causes an elevation of $[Ca^{2+}]_i$ in skeletal muscle.[18–20] When either MHN or MHS muscle fibers were incubated in an external solution containing 10 mM K^+ (keeping the $[K^+]$ $[Cl^-]$ product constant), a transient increase in $[Ca^{2+}]_i$ that was associated with the partial depolarization was seen in both muscle preparations. In 12 MHN fibers, 10 mM K^+ induced a depolarization from −82 ± 1.3 mV to −73 ± 1.6 mV, and $[Ca^{2+}]_i$ increased to 272 ± 22 nM (range, 240–314 nM; table 1). In 11 MHS muscles, 10 mM K^+ produced a depolarization from −82 ± 1.2 mV to −74 ± 0.9 mV, and $[Ca^{2+}]_i$ increased to 967 ± 38 nM (range, 903–1,040 nM). These results reveal the expected dependence of calcium release on the membrane potential[18–20] and show the enhancement of this calcium release in MHS muscle in response to a given membrane depolarization. After 6–8 min of exposure to the 10-mM K^+ solution, resting $[Ca^{2+}]_i$ slowly returns to control levels in both types of fibers, despite the fact that the membrane remains depolarized. This confirmed the hypothesis that intracellular Ca^{2+} release in response to membrane depolarization is both voltage- and time-dependent.[21]

Effect of Caffeine on $[Ca^{2+}]_i$ in Partial Depolarized MHN Fibers

Incubation of the partially depolarized MHN fibers in 2 mM caffeine during the time that $[Ca^{2+}]_i$ was elevated induced an increment in $[Ca^{2+}]_i$ of a similar magnitude to that observed in MHS fibers. Figures 3A–C show a typical experiment in a muscle fiber obtained from MHN swine. The fiber was first exposed to 10 mM K^+ that induced a partial depolarization of the plasma membrane and an elevation $[Ca^{2+}]_i$ from 125 nM to 282 nM. The addition of 2 mM caffeine to the 10-mM K^+ solution further elevated $[Ca^{2+}]_i$ to 998 nM, similar to what we observed in polarized MHS fibers. In 20 MHN fibers

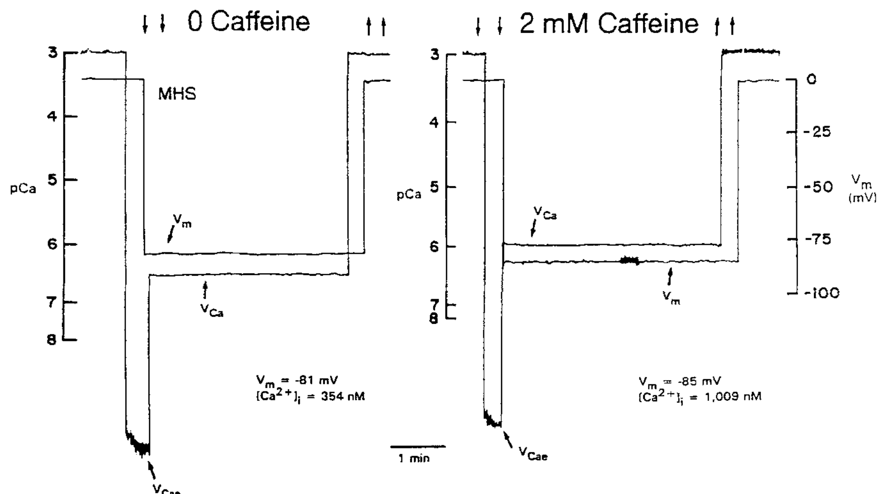

Fig. 2. Effects of caffeine on myoplasmic free calcium concentration ($[Ca^{2+}]_i$) in malignant hyperthermia–susceptible (MHS) swine. Simultaneous recording, using double-barreled microelectrodes, of Vm and $[Ca^{2+}]_i$ in a MHS fiber before and after caffeine 2 mM was added. In swine, physiologic solution Vm was −81 mV and $[Ca^{2+}]_i$ was 354 nM. The addition of 2 mM induced an elevation of $[Ca^{2+}]_i$ to 1,009 nM, which was associated to muscle fiber shortening. There was no change in resting membrane potential. Measurements of Vm and $[Ca^{2+}]_i$ before and after caffeine was added to the bath were conducted in same muscle fiber. (*Left* and *right*) The calibration bars for the Ca^{2+}-selective microelectrode and the membrane potential, respectively, are shown.

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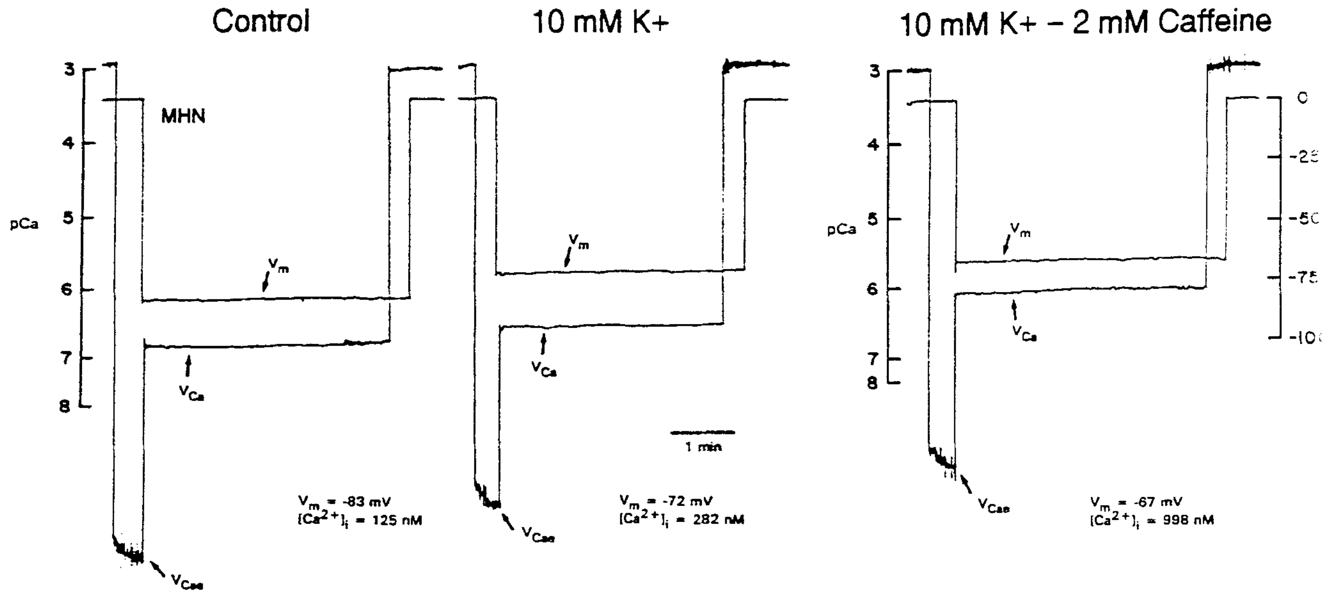

Fig. 3. Effects of caffeine in partially depolarized malignant hyperthermia–nonsusceptible (MHN) muscle fiber with elevated myoplasmic free calcium concentration ($[Ca^{2+}]_i$). Simultaneous recording, using a double-barreled microelectrodes, of Vm and $[Ca^{2+}]_i$ in a MHN fiber in swine physiologic solution (*left*), 10 mM K^+ solution (*middle*), and K^+ 10 mM and caffeine 2 mM (*right*). In control, Vm was −83 mV and $[Ca^{2+}]_i$ was 125 nM. The incubation of a MHN muscle fiber in 10 mM K^+ solution induced a partial depolarization (Vm= −72 mV) that was associated with an elevation of $[Ca^{2+}]_i$ to 282 nM. Exposure of this partially depolarized muscle fiber to caffeine 2 mM while $[Ca^{2+}]_i$ was elevated (approximately 2 times control) induced a further release reaching a value of 998 nM. Measurements of Vm and $[Ca^{2+}]_i$ before and after K^+ and caffeine solution was added to the bath were conducted in same muscle fiber. (*Left* and *right*) The calibration bars for the Ca^{2+}-selective microelectrode and the resting membrane potential, respectively, are shown.

partially depolarized by K^+, $[Ca^{2+}]_i$ increased from 126 ± 7 nM (range, 106–135 nM) to 265 ± 18 nM (range, 220–295 nM), and exposure to caffeine 2 mM produced a further elevation of $[Ca^{2+}]_i$ to 940 ± 37 nM (range, 878–998 nM; table 2). This increase in $[Ca^{2+}]_i$ is not significantly different from that seen after polarized MHS fibers were exposed to 2 mM caffeine. After 10 min, when $[Ca^{2+}]_i$ returned to control levels (122 ± 6 nM; range, 115–131 nM; n = 10), the response to 2 mM caffeine also returned to control levels (304 ± 15 nM; range, 289–331 nM; n = 10), despite continued partial membrane depolarization (72 ± 1.3 mV; fig. 4). Similar responses were seen in MHS fibers, but in each case the increase in their $[Ca^{2+}]_i$ response was greater than in MHN fibers. In 14 MHS fibers, partial depolarization elevated $[Ca^{2+}]_i$ from 322 ± 22 nM (range, 287–356 nM) to 905 ± 38 nM (range, 834–986 nM; n = 11). After caffeine incubation, $[Ca^{2+}]_i$ increased to 2,860 ± 782 nM (range, 1,789–3,789 nM; n = 6; table 2). The determination of $[Ca^{2+}]_i$ was very difficult in these MHS fibers because of the sustained sarcomere shortening, which caused displacement of the flexible double-barreled Ca^{2+}-selective microelectrode in some fibers despite the use of fiber-stabilizing devices.

Table 2. Effects of Caffeine on $[Ca^{2+}]_i$ in Partially Depolarized MHN and MHS Muscle Fibers

	Resting	K^+ (10 mM)	K^+ (10 mM) + Caffeine (2 mM)
MHN	126 ± 7 (n = 20)	265 ± 18 (n = 20)	940 ± 37 (n = 20)
MHS	322 ± 22 (n = 14)	905 ± 38 (n = 11)	2,860 ± 782 (n = 6)

MHN = malignant hyperthermia nonsusceptible; MHS = malignant hyperthermia susceptible.

Discussion

The present study demonstrates that MHS fibers are more sensitive to caffeine-induced Ca^{2+} release than MHN muscle fibers. Second, it shows that exposure of MHN and MHS fibers to 10 mM K^+ solution causes a partial membrane depolarization with an associated transient increase in $[Ca^{2+}]_i$ (6–8 min) that was greater in MHS than in MHN fibers. Third, it shows that the transient elevation in $[Ca^{2+}]_i$ in MHN fibers, which is associated with partial membrane depolarization, increased their caffeine sensitivity to a level similar to that ob-

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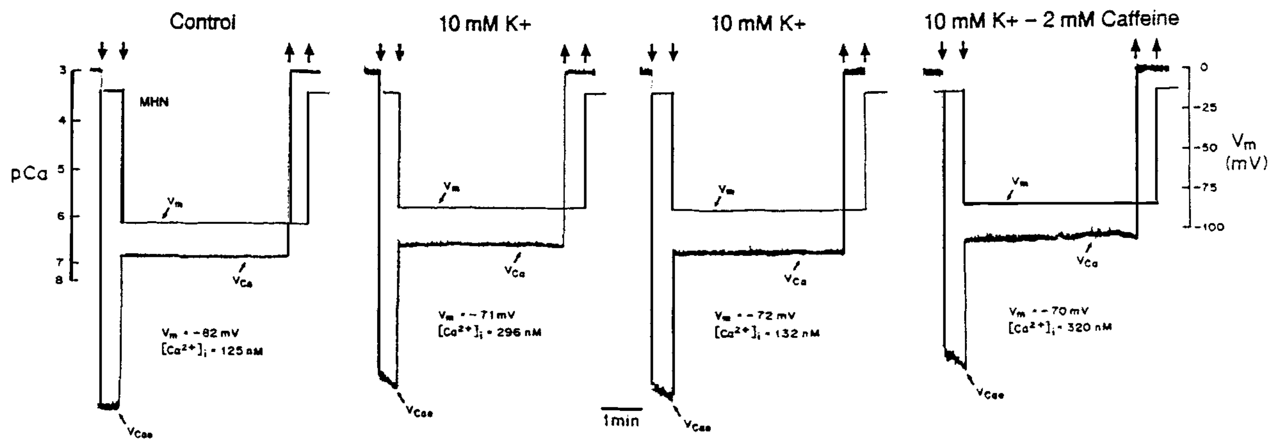

Fig. 4. Effects of caffeine in partially depolarized malignant hyperthermia–nonsusceptible (MHN) muscle fiber with a physiologic myoplasmic free calcium concentration ($[Ca^{2+}]_i$). Simultaneous recording, using a double-barreled microelectrodes, of Vm and $[Ca^{2+}]_i$ in a MHN in swine physiologic solution *(first panel)*, 10 mM K^+ solution *(second panel)* after$[Ca^{2+}]_i$ has returned to control levels *(third panel)* and after the application of caffeine 2 mM *(fourth panel)*. In control, Vm was −82 mV and $[Ca^{2+}]_i$ was 125 nM. The incubation of a MHN muscle fiber in K^+ 10 mM solution induced a partial depolarization (Vm = −71 mV) that was associated with a transient elevation of $[Ca^{2+}]_i$ to 296 nM, which returned to 130 nM after 8 min. Exposure of this still partially depolarized muscle fiber to 2 mM caffeine after $[Ca^{2+}]_i$ returned to control levels caused an elevation of $[Ca^{2+}]_i$ to 320 nM, which was the same as was seen in control polarized cells. All of the measurements for Vm and $[Ca^{2+}]_i$ were conducted in same muscle fiber. (*Left* and *right*) Calibration bars for the Ca^{2+}-selective microelectrode and the resting membrane potential, respectively, are shown.

served in polarized MHS muscle fibers. Fourth, it demonstrates that the increased caffeine sensitivity caused by the temporary increase in $[Ca^{2+}]_i$ associated with partial depolarization returned to normal when $[Ca^{2+}]_i$ decreased to control levels, despite continued partial membrane depolarization.

It has been previously shown in skeletal muscle that caffeine produces transient contractures with no change in the electrical properties of the muscle plasma membrane.[22] It also potentiates twitch tension,[23] induces intracellular Ca^{2+} release at subthreshold concentrations (for contracture),[20] and increases the release of calcium from isolated fragments of SR.[24] Single channel recordings have revealed that caffeine activates the SR Ca^{2+} release channel by increasing the number and the duration of open channel events without changing the unitary conductance.[25] In addition, it has been demonstrated that caffeine does not change the voltage dependence or time course of charge movement.[26]

An increased caffeine sensitivity of MHS fibers is the basis of the clinical diagnostic test currently used to determine MH susceptibility.[9-11] The results of the present study demonstrate that caffeine induces a larger release of intracellular calcium in MHS (3.4 times greater than resting level) than in MHN (2.4 times greater than resting level) fibers. The calcium released by caffeine in MHN muscle fibers sometimes caused an oscillatory activation of individual sarcomeres. It is known that caffeine can trigger asynchronous contraction of single sarcomeres at concentrations that are subthreshold for contracture. These local sarcomere contractions, in turn, may or may not be converted into more organized waves.[27] This oscillatory sarcomere pattern has been attributed to a cyclic release and uptake of calcium by the SR and might represent a subthreshold process in excitation-contraction coupling that precedes a full activation of the muscle fiber.[27,28] In MHS fibers, the calcium released by caffeine always produced a sustained muscle shortening in addition to its oscillatory activation of individual sarcomeres.

Among the possible mechanisms that could cause this difference in intracellular calcium release induced by caffeine in MHN and MHS fibers, two have been postulated as the most likely cause of the difference. One is that there is the specific alteration in the MHS RyR1 inherently increases its sensitivity to caffeine.[29] The second is that this increased sensitivity is secondary to some other factor associated with the MH phenotype, such as an increased resting $[Ca^{2+}]_i$. In this regard, Shomer *et al.*[30] have shown that, although caffeine significantly increases mean open time of both MHS and MHN RyR1 channels in lipid bilayers, there were no difference in channel properties between the two groups.

Our findings confirm previous observations that the release of calcium by the SR can be mediated by membrane depolarization[18-21] and show for the first time that

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the amount of calcium release is greater in MHS than MHN muscle for the same depolarizing pulse. Thus, extracellular K^+ induced the same magnitude of membrane depolarization (−9 mV in MHN and −8 mV in MHS) but produced an elevation of $[Ca^{2+}]_i$ by a factor of 2.1 in MHN and 2.8 in MHS muscle fibers. These results are in agreement with studies that have demonstrated that MHS fibers have a lower K^+ contracture threshold than MHN fibers,[31,32] although $[Ca^{2+}]_i$ was never measured in these previous studies. One possible explanation for this could be that transduction signal from the transverse tubular system membrane to the SR calcium release in response to a given membrane depolarization is magnified in MHS fibers. Interestingly, $[Ca^{2+}]_i$ in partially depolarized MHN fibers during the first 6-8 min was similar to that seen in polarized MHS fibers. In this state, when they were exposed to 2 mM caffeine, their $[Ca^{2+}]_i$ was elevated to a similar concentration to that seen when polarized MHS fibers are exposed to 2 mM caffeine. Thus, changing the $[Ca^{2+}]_i$ in MHN fibers to a similar level to that seen in polarized MHS fibers, it was possible to mimic their increased caffeine sensitivity. There was a direct relationship between the degree of elevation of $[Ca^{2+}]_i$ in MHN fibers and the magnitude of the Ca^{2+} release that followed a 2-mM caffeine challenge. Thus, fibers with the higher $[Ca^{2+}]_i$ after exposure to K^+ solution show the greater response to caffeine and those with the lower $[Ca^{2+}]_i$ after exposure to K^+ solution show the smaller response to caffeine. Therefore, it seems that it is the elevation of resting $[Ca^{2+}]_i$ and not a change in the inherent RyR1 caffeine sensitivity that accounts for the enhanced response to caffeine observed in MHS fibers. This enhanced sensitivity to caffeine induced by K^+ depolarization has also been observed in frog skeletal muscle.[19,33] It is the increase in $[Ca^{2+}]_i$ and not the depolarization itself that enhances the caffeine effect on intracellular release of calcium in MHN fibers, because when $[Ca^{2+}]_i$ returned to control levels in the still-depolarized MHN fibers, caffeine sensitivity returned to normal. The cause for the spontaneous reduction of $[Ca^{2+}]_i$ after a potassium-induced release of calcium in skeletal muscle has been interpreted to be part of an inactivation process that stops the release of calcium from the SR rather than being a consequence of a depletion of calcium in the SR.[34,35]

It is also interesting to point out that the caffeine contracture test for MH diagnosis is conducted in cut muscle fibers. This membrane injury eventually leads to significant membrane depolarization. Geffner *et al.*[36] have shown that in muscle fibers partially depolarized with subcontracture, concentrations of K^+ rapidly developed tension when exposed to 1 mM caffeine, which alone would not cause contracture. Thus, the depolarization present in the cut fibers used in caffeine contracture tests can cause a further elevation of an already high $[Ca^{2+}]_i$. This might act as an additional contributing factor in the sensitivity of MHS fibers to caffeine.

Finally, these results have been obtained in swine MHS muscle fibers in which the susceptibility to the syndrome is caused by a single point mutation in the SR Ca^{2+} release channel (RyR1)[5] and could be specific to this mutation. However, in humans, MH susceptibility seems to have a multigenic origin,[8] and recent experiments conducted in intact muscle fibers obtained from six control subjects and four survivors of well-documented cases of MH show similar responses to those reported here (López and Allen, unpublished results, September 1999).

We conclude that the increased sensitivity of MHS muscle fibers to caffeine seems not to be due to a specific alteration in the caffeine sensitivity of the SR Ca^{2+}-release channel *per se*, but is a nonspecific response related to a high resting $[Ca^{2+}]_i$ found in MHS fibers, and this might explain why a positive response may also be seen in other myopathies where an increased $[Ca^{2+}]_i$ is part of the phenotype.

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