Enhanced response to caffeine and 4-chloro-\textit{m}-cresol in malignant hyperthermia-susceptible muscle is related in part to chronically elevated resting $[\text{Ca}^{2+}]_i$

José R. López,1,2 Nancy Linares,1 Isaac N. Pessah,3 and Paul D. Allen2

1Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela; 2Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women’s Hospital, Boston, Massachusetts; and 3Department of Molecular Biosciences, University of California, Davis, California

Submitted 23 June 2004; accepted in final form 8 October 2004

Malignant hyperthermia (MH) is a potentially fatal pharmacogenetic syndrome caused by exposure to halogenated volatile anesthetics and/or depolarizing muscle relaxants. We have measured intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) using double-barreled, $\text{Ca}^{2+}$-selective microelectrodes in myoballs prepared from skeletal muscle of MH-susceptible (MHS) and MH-nonsusceptible (MHN) swine. Resting $[\text{Ca}^{2+}]_i$ was approximately twofold in MHS compared with MHN quiescent myoballs ($232 \pm 35$ vs. $112 \pm 11$ nM). Treatment of myoballs with caffeine or 4-chloro-\textit{m}-cresol (4-CmC) produced an elevation in $[\text{Ca}^{2+}]_i$ in both groups; however, the concentration required to cause a rise in $[\text{Ca}^{2+}]_i$ was four times lower in MHS than in MHN skeletal muscle cells. Incubation of MHS cells with the fast-complexing $\text{Ca}^{2+}$ buffer BAPTA reduced $[\text{Ca}^{2+}]_i$, raised the concentration of caffeine and 4-CmC required to cause an elevation of $[\text{Ca}^{2+}]_i$, and reduced the amount of $\text{Ca}^{2+}$ release associated with exposure to any given concentration of caffeine or 4-CmC to MHN levels. These results suggest that the differences in the response of MHS skeletal myoballs to caffeine and 4-CmC may be mediated at least in part by the chronic high resting $[\text{Ca}^{2+}]_i$ levels in these cells.

Calcium homeostasis; 1,2-bis(2-aminophenoxy)ethane-$N,N',N''$,N$'''$-tetraacetic acid

Malignant hyperthermia (MH) is a potentially fatal pharmacogenetic myopathy of humans and several large mammals, including swine, dog, and horse. It can be induced by volatile anesthetics and/or depolarizing muscle relaxants (1, 33, 37). While in swine susceptibility to the syndrome has been associated with a single point mutation (Arg615Cys) in the $\text{Ca}^{2+}$ release channel at the sarcoplasmic reticulum (ryanodine receptor 1, RyR1) (13, 32), in humans at least 42 MH mutations have been identified at 34 different RyR1 residues and two MH mutations at one residue in the skeletal dihydropyridine receptor (DHPR) (21, 33, 37). In both humans and swine, this syndrome is associated with dysregulation of intracellular $\text{Ca}^{2+}$ homeostasis in skeletal muscle (28, 30) and dyspedic myotubes expressing MH mutations (50). Exposure of MH-susceptible (MHS) humans or swine to volatile anesthetics and/or depolarizing muscle relaxants triggers an MH episode that is characterized by hypermetabolism, muscle rigidity, increased heart rate, and finally elevated body temperature. These clinical manifestations are associated with a nonphysiological elevation of myoplasmic $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) at the cellular level ($[\text{Ca}^{2+}]_i$) (17, 26). In addition, it was previously shown that skeletal muscle from MHS individuals and animals have a lower pharmacological threshold and an exaggerated response at submaximal concentrations of caffeine (22, 45) and 4-chloro-\textit{m}-cresol (4-CmC) (44, 48) than do those with MH-nonsusceptible (MHN) muscle. This enhanced sensitivity is widely used as part of the clinical diagnosis of MH susceptibility in humans and has been confirmed experimentally in swine (16, 21). The molecular and cellular basis for heightened sensitivity to pharmacological agents in MHS has remained unclear (33, 37). Evidence of chronically elevated resting $[\text{Ca}^{2+}]_i$, has been presented on the basis of direct measurements with $\text{Ca}^{2+}$-selective electrodes in isolated human and swine skeletal muscle fibers (28, 30). However, the extent to which high $[\text{Ca}^{2+}]_i$ contributes to sensitizing responses to caffeine and 4-CmC is not known.

The purpose of the present study was to address the question of whether the enhanced intracellular $\text{Ca}^{2+}$ release at submaximal concentrations of caffeine and 4-CmC (44, 45, 48) in MHS cells is related to chronic elevation in resting $[\text{Ca}^{2+}]_i$, in affected muscle cells. To do so, resting $[\text{Ca}^{2+}]_i$ of MHS myoballs was decreased nearly to MHN levels by loading them with the $\text{Ca}^{2+}$ chelator 1,2-bis(2-aminophenoxy)ethane-$N,N',N'',N'''$-tetraacetic acid (BAPTA) and then reexamining the responses to caffeine and 4-CmC. Our results suggest that the enhanced intracellular $\text{Ca}^{2+}$ release at submaximal concentrations of caffeine and 4-CmC was closely associated with the high resting $[\text{Ca}^{2+}]_i$, observed in these cells.

Materials and Methods

Muscle cell preparations. Muscle biopsies were obtained from the hindlimb muscles of newborn (4–8 days) Yorkshire (MHN; $n = 4$) and Poland China (MHS; $n = 5$) swine. Susceptibility to MH was determined using polymerase chain reaction genotyping as previously described (33, 37). Hindlimb muscle was removed while the animals were anesthetized with the nontriggering agents thiopental (15 mg/kg) and propofol (200 $\mu$g.g$^{-1}$.min$^{-1}$). After muscle collection, the anesthetized animal was euthanized by administering a bolus injection

Address for reprint requests and other correspondence: J. R. López, Dept. of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women’s Hospital, Harvard Medical School, 75 Francis St., Boston, MA 02115 (E-mail: lopez@zeus.bwh.harvard.edu).

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of KCl. MHN and MHS muscle cells were dissociated according to the technique described by Yasin et al. (52). Skeletal muscle myoballs were cultured according to the method previously described by Boldin et al. (5). The myoballs used in this study were cultured for 9 days, had a diameter between 65 and 85 μm, and were similar to those used by others to study the electrophysiology of cultured muscle cells (5, 6, 49).

**Ca**2+-selective microelectrodes. Double-barreled, **Ca**2+-selective microelectrodes were prepared from thin-walled 1.2- and 1.5-mm outside diameter (OD) borosilicate HCl-washed glass capillaries as described previously (25, 27). The 1.5-mm OD tube was silanized by exposing it to dimethyldichlorosilane vapor, and then, 24 h later, the tip was back-filled with the neutral carrier ETH 1001 (Fluka, Ronkonkoma, NY). The remainder of the barrel was back-filled with pCa7 solution after 48 h. The 1.2-mm OD barrel was back-filled with 3 M KCl just before the measurements were performed. The tip resistances were measured by passing a current pulse of 1 pA through an individual barrel while the electrode tip was in standard bathing solution. For the **Ca**2+-selective microelectrodes, the resistances ranged from 5 to 8 × 107 MΩ, and for the membrane potential microelectrodes, the resistances ranged from 10 to 15 MΩ. At low [**Ca**2+], these microelectrodes showed quantitative variations, despite the fact that they were constructed in similar fashion using the same batch of sensors. Therefore, each Ca**2+**-selective microelectrode was individually tested by exposure to a series of calibrating solutions of known [**Ca**2+] at 37°C as described previously (30), but with the addition of 1 mM Mg2+ to each solution to mimic intracellular ionic conditions (25). Calibration curves were constructed by plotting pCa (−log10 [**Ca**2+]) against the **Ca**2+ electrode potential, and only those **Ca**2+ microelectrodes that produced a Nernstian response between pCa3 and pCa7 (30.5 mV/pCa unit at 37°C) were used. Because the response of these electrodes is not linear from pCa7 to pCa8, the absolute value of [**Ca**2+]i is less accurate in this range. These microelectrodes retain their responsiveness for periods of 24–36 h. The use of the microelectrodes was not limited by aging but rather by blunting of the tip with repeated penetrations. Therefore, individual **Ca**2+-selective microelectrodes were used for a maximum of six determinations of resting [**Ca**2+]i, after which the calibration curve between pCa6 and pCa8 was repeated. If the two calibration curves did not agree within 2.5 mV in the relevant range of the calibration curve, the data from that microelectrode were discarded. We determined directly that the **Ca**2+ sensitivity of the **Ca**2+-selective microelectrodes was not affected by changes in pH (7.4–6.4), changes in Mg2+ over the range of free Mg2+ concentrations expected to be found in muscle cells, or changes in any of the drugs used in the present study. The electrode potential of the voltage microelectrode (resting membrane potential) was 0 mV, as determined at room temperature using a high-impedance amplifier (>1011 MΩ (model FD-223, WPI, Sarasota, FL)). The potential of the voltage microelectrode (Vm) was subtracted electronically from the potential of the **Ca**2+ electrode (V**Ca**) to obtain the differential signal (VCa) representing the resting myoplasmic **Ca**2+ concentration. Vm and V**Ca** potentials were filtered using a low-pass filter (LPF-30-WPI, WPI) at 10–30 KHz, acquired at a frequency of 1,000 Hz with Axiograph software (version 4.6; Axon Instruments, Foster City, CA), and stored for further analysis. Two criteria (cell polarization and signal stability) were used as key elements to accept or to reject individual [**Ca**2+]i measurements performed in MHN and MHS myoballs. Thus resting [**Ca**2+]i data from MHN and MHS myoballs were retained only for polarized myoballs (membrane potential −60 mV or more negative) in which Vm remained stable for at least 45 s.

**Caffeine and 4-CmC experiments.** Individual MHN or MHS myoballs were impaled with the double-barreled **Ca**2+ electrode to measure Vm and V**Ca** and then were exposed sequentially to each of the three caffeine or 4-CmC concentrations tested. Each exposure lasted for 60–70 s. After recording Vm and V**Ca** for at least 50 s at each concentration, the **Ca**2+-selective microelectrode was withdrawn. For each concentration tested, the caffeine or 4-CmC solution was washed out for 5 min, and then the cell was reimpaled and retested with the next-highest concentration.

**BAPTA loading.** In a set of pilot experiments, MHN and MHS myoballs were impaled with double-barreled **Ca**2+ microelectrodes and then observed while exposed for different times (range, 5–30 min) to concentrations ranging from 2 to 50 μM membrane-permeable AM of the **Ca**2+ selective chelator BAPTA (BAPTA-AM; Molecular Probes, Eugene, OR) (46, 47). The resulting reduction in resting [**Ca**2+]i was directly monitored using a **Ca**2+-selective microelectrode with the aim of obtaining ideal BAPTA incubation time and loading concentration that could reduce [**Ca**2+]i in MHS cells to a concentration as close as possible to the [**Ca**2+]i observed in untreated MHN cells (range, 80–130 nM) and that did not reduce [**Ca**2+]i in MHN myoballs to <80 nM. Despite our efforts (different incubation times and BAPTA concentrations, large number of cells tested), we did not find a BAPTA protocol that consistently lowered [**Ca**2+]i, to the desired concentrations. Factors such as initial [**Ca**2+]i, chelator membrane permeability, concentrations of intracellular esterases, hydrolysis of the AM esters, decrease in intracellular BAPTA concentration due to active transport out of the cell or to leak from the cells, and accumulation inside intracellular compartments made it difficult to find an experimental protocol that decreased [**Ca**2+]i to the desired levels every time. However, we found that incubation with 10 μM BAPTA for 10 min reduced [**Ca**2+]i to the desired concentration in 39% of both MHN- and MHS-BAPTA-treated myoballs. If the [**Ca**2+]i in MHN- and MHS-BAPTA-treated myoballs was not in the preestablished range after the BAPTA incubation, the cell was discarded.

For actual data collection, we first directly measured [**Ca**2+]i in all myoballs using a **Ca**2+-selective electrode and then exposed the myoballs to 10 μM BAPTA-AM for 10 min. Once steady-state [**Ca**2+]i had been achieved, the electrode was removed and extracellular medium was exchanged several times with BAPTA-AM-free solution to remove all remaining extracellular BAPTA. The washout period lasted 5 min, after which the myoballs were impaled again for continuous monitoring of [**Ca**2+]i, before and during challenge with either caffeine or 4-CmC (see Caffeine and 4-CmC experiments).

**Solutions.** Swine Ringer solution was of the following composition (in mM): 135 NaCl, 2.5 KCl, 2.5 CaCl2, 1 MgCl2, 18 NaHCO3, 1.5 NaH2PO4, and 5 glucose, pH 7.2–7.3 (aerated with 95% O2–5% CO2). Caffeine was dissolved in H2O, and 4-CmC and BAPTA were dissolved in 0.1% dimethyl sulfoxide (DMSO). At this concentration, DMSO had no significant effect on either Vm or [**Ca**2+]i before and during challenge with either caffeine or 4-CmC (see Caffeine and 4-CmC experiments).
Statistics. All values are expressed as means ± SD of the number (n) of skeletal myoballs used experimentally. Statistical differences were determined using one-way ANOVA for multiple paired comparisons, with P < 0.05 considered statistically significant.

RESULTS

[Ca\(^{2+}\)]\(_i\) in MHN and MHS myoballs. Figure 1, A and B, shows superimposed recordings of a simultaneous recording of \(V_m\) and [Ca\(^{2+}\)]\(_i\) from a MHN and a MHS myoball. With satisfactory impalements in quiescent myoballs, there was no difference in the \(V_m\) value between MHN and MHS; however, [Ca\(^{2+}\)]\(_i\) was twice as high in MHS as in MHN. On average, \(V_m\) was \(-63 ± 6\) mV (n = 30) in MHN vs. \(-65 ± 7\) mV (n = 30) in MHS myoballs, and [Ca\(^{2+}\)]\(_i\) was 112 ± 11 nM (n = 30) in MHN vs. 232 ± 35 nM (n = 30) in MHS myoballs. This difference in resting [Ca\(^{2+}\)]\(_i\) displayed by MHS myoballs isolated from neonatal pigs, carrying a homozygous RyR1 Arg615Cys mutation, was similar to the dysfunction in intra-

AJP-Cell Physiol • VOL 288 • MARCH 2005 • www.ajpcell.org

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of 5 min between challenges (see MATERIALS AND METHODS). A challenge of MHS myoballs with 0.5 and 1 mM caffeine induced a significant elevation of \([Ca^{2+}]_i\), from 232 ± 32 nM (n = 15) to 339 ± 54 nM (n = 15; P < 0.001) and 464 ± 90 nM (n = 15; P < 0.001), respectively. However, there was no elevation in \([Ca^{2+}]_i\), observed in MHN myoballs after exposure to these concentrations of caffeine [from 112 ± 11 nM to 111 ± 13 nM (n = 15; P > 0.05) and 114 ± 11 nM (n = 15; P > 0.05)]. Caffeine (2 mM) caused an elevation of \([Ca^{2+}]_i\), in both populations of cells, although the magnitude of the response to this concentration of caffeine was significantly greater in MHS than in MHN myoballs (747 ± 135 nM in MHS myoballs, n = 15, vs. 242 ± 73 nM in MHN myoballs, n = 15; P < 0.001). Exposure to caffeine did not modify \(V_m\) in groups of cells at any dose level.

Incubation in 4-CmC also induced significant elevations of \([Ca^{2+}]_i\) in both MHN and MHS myoballs without any effect on \(V_m\). Exposure of MHS myoballs sequentially to 1, 5, and 10 μM 4-CmC with a 5-min washout between concentrations produced a concentration-dependent elevation of \([Ca^{2+}]_i\), from 231 ± 39 nM (n = 15) in the absence of 4-CmC to 325 ± 56 nM (n = 15, P < 0.001), 520 ± 129 (n = 15, P < 0.001), or 847 ± 112 (n = 15, P < 0.001) when 1, 5, and 10 μM 4-CmC was added, respectively. On the other hand, 1 and 5 μM 4-CmC did not significantly alter \([Ca^{2+}]_i\) in MHN myoballs [from 111 ± 11 nM (n = 15) to 115 ± 10 nM (n = 15, P > 0.05) and 111 ± 10 nM (n = 15, P > 0.05)], and as observed for the highest-dose caffeine challenge, the increase in \([Ca^{2+}]_i\), observed after addition of 10 μM 4-CmC to MHN cells (268 ± 63 nM, n = 15) was significantly smaller (P < 0.001) than the response in MHS myoballs.

**BAPTA ameliorates pharmacogenic sensitivity in MHS.** We next examined our hypothesis that the exaggerated responses to caffeine and 4-CmC of MHS cells are related, at least in part, to the higher \([Ca^{2+}]_i\) observed in MHS compared with MHN myoballs. To do this, \([Ca^{2+}]_i\) was reduced by loading the myoballs for 10 min with 10 μM BAPTA-AM (46, 47), which caused a reduction in steady-state \([Ca^{2+}]_i\), of MHS myoballs to near-MHN levels and a reduction of \([Ca^{2+}]_i\) in MHN myoballs to no less than 80 nM (Fig. 2, A and B). In fact, in large number of attempts in which MHN and MHS myoballs (n = 132) were treated with BAPTA at the “ideal condition,” only 23 MHN and 28 MHS myoballs were considered acceptable on the basis of the preestablished \([Ca^{2+}]_i\) range. As expected on the basis of the K_d of BAPTA (110 nM), its effect in reducing \([Ca^{2+}]_i\) was greater in MHS than in MHN myoballs, with observed decreases from 241 ± 35 nM (n = 28) to 115 ± 28 nM (n = 28, P < 0.001) in MHS myoballs and from 113 ± 11 (n = 23) to 91 ± 12 nM (n = 23, P < 0.001) in MHN myoballs.

The effect of caffeine and 4-CmC on \([Ca^{2+}]_i\) in BAPTA-loaded MHN and MHS myoballs is shown in Figs. 3 and 4. Figure 3 shows that in a BAPTA-treated MHS myoball, subsequent challenges with caffeine elicited responses that were similar to those observed in untreated MHS myoballs. All groups required the same concentration (2 mM) to induce intracellular \(Ca^{2+}\) release and had a similar increase in \([Ca^{2+}]_i\), induced by 2 mM caffeine (207 ± 21 nM in MHN, n = 10; and 218 ± 40 nM in MHS, n = 9). Figure 4 shows that a similar effect was observed in BAPTA-treated MHS myoballs challenged with 4-CmC. In BAPTA-treated MHS myoballs, the concentration of 4-CmC required to induce elevation of \([Ca^{2+}]_i\) in MHS cells was shifted from 0.5 to 10 μM, and the increase in \([Ca^{2+}]_i\) in response to 10 μM 4-CmC was reduced to 256 ± 37 nM (n = 10), a level similar to that observed in MHN myoballs (268 ± 63 nM, n = 15) (Fig. 4). It is important to note that loading with 10 μM BAPTA-AM had no significant effect on caffeine- or 4-CmC-mediated elevation of \([Ca^{2+}]_i\), in MHN myoballs (Figs. 3 and 4). Thus it appears that the increase in intracellular buffering capacity induced by BAPTA at this particular concentration did not alter the caffeine- and 4-CmC-induced \(Ca^{2+}\) releases in MHS myoballs.

In summary, these results suggest that the MHS myoball phenotype of an increased response to caffeine and 4-CmC at submaximal concentrations is at least in part the result of the high \([Ca^{2+}]_i\), observed in MHS myoballs.
DISCUSSION

We have found that the resting \([\text{Ca}^{2+}]_i\) in quiescent myoballs from MHS muscle with a homozygous Arg615Cys RyR1 mutation is higher than that in MHN myoballs. These results provide independent verification of a chronically elevated skeletal muscle resting \([\text{Ca}^{2+}]_i\), previously reported in adult MHS skeletal muscle fibers (26, 27, 29) and dyspedic myotubes expressing MH mutations (50). In addition, MHS myoballs have a larger response than MHN myoballs to caffeine and 4-CmC at submaximal agonist concentrations. Furthermore, we were able to abolish the increased responsiveness to caffeine and 4-CmC in MHS myoballs by enhancing the intracellular buffering capacity with BAPTA, which reduced resting \([\text{Ca}^{2+}]_i\) to MHN levels.

The high \([\text{Ca}^{2+}]_i\) that we found in quiescent MHS skeletal muscle myoballs is not due to the microelectrode impalement causing plasma membrane injury and/or leakage of \(\text{Ca}^{2+}\) ions into the myoplasm from the extracellular space. This conclusion is based on the fact that it) there was no acute or additional increase in observed resting \([\text{Ca}^{2+}]_i\), in polarized myoballs when the \([\text{Ca}^{2+}]_i\) measurements were obtained in the presence of high-\(\text{Ca}^{2+}\) solution, and similar evidence for good \(\text{Ca}^{2+}\) microelectrode sealing into the membrane during intracellular \(\text{Ca}^{2+}\) measurements has been reported in neurons from Helix aspersa (2) and heart muscle cells (31); and 2) there was no sustained depolarization in myoballs during the time that intracellular \(\text{Ca}^{2+}\) measurements were obtained, which is a finding that almost always is coincident with sarcolemmal membrane damage. We did observe sustained depolarization when occasional cell membrane damage did occur during microelectrode impalements in MHN and MHS myoballs. This depolarization was always associated with sustained and irreversible elevation of resting \([\text{Ca}^{2+}]_i\), in the millimolar range. When this did occur, data obtained for these cells were discarded. Another possibility to be considered is that the signals recorded by our \(\text{Ca}^{2+}\)-selective microelectrodes from MHS myoballs were influenced in some systematic way by an unknown component of the myoplasm present in MHS cells. However, there is no experimental evidence or even a hypothesis that supports or makes such a supposition. The increase in \([\text{Ca}^{2+}]_i\) in myoballs was less (2-fold compared with 3-fold) than the level we have observed in adult swine muscle fibers.

We think that this quantitative difference \([\text{Ca}^{2+}]_i\) may be related to the fact that myoballs have an embryonic phenotype and that the cause of the intracellular \(\text{Ca}^{2+}\) elevation is not fully developed.

\([\text{Ca}^{2+}]_i\) in quiescent muscle cells represents the balance between the \(\text{Ca}^{2+}\) transporting systems in the plasma membrane (\(\text{Na}^+/\text{Ca}^{2+}\) exchange, plasma membrane \(\text{Ca}^{2+}\)-ATPase), in the sarcoplasmic reticulum (SR) (\(\text{Ca}^{2+}\)-ATPase), and the passive \(\text{Ca}^{2+}\) release from intracellular stores through the \(\text{Ca}^{2+}\) leak channels in the SR (7, 24, 33, 37). The precise mechanism for the high resting \([\text{Ca}^{2+}]_i\), found in MHS myoballs, myotubes, and adult myofibers is still not known. However, it must be linked to a dysregulation of intracellular \(\text{Ca}^{2+}\) homeostasis. We think that it is the result of a chronic alteration of intracellular \(\text{Ca}^{2+}\) release or leak combined with a resetting of the set point and/or a deficiency in the \(\text{Ca}^{2+}\) transport by the SR and/or the plasma membrane (9, 11, 12, 29, 34, 38).

The enhanced sensitivity of MHS muscle cells to caffeine and 4-CmC has been observed by numerous groups (16, 22, 45, 48, 51), and thus similar behavior in MHS myoballs was expected. However, the mechanism for this increased sensitivity has been the subject of debate. Our laboratory previously showed that partial depolarization of MHN fibers with KCl increased their \([\text{Ca}^{2+}]_i\), and resulted in increased caffeine response (27). BAPTA is a high-affinity \(\text{Ca}^{2+}\) chelator (46, 47) that has been used in several previous studies to reduce \([\text{Ca}^{2+}]_i\) in adult skeletal muscle fibers (3, 19). As an intracellular \(\text{Ca}^{2+}\) buffer, BAPTA has several important advantages over other \(\text{Ca}^{2+}\) chelators, because it is practically insensitive to intracellular pH changes, has a greater selectivity for \(\text{Ca}^{2+}\) over \(\text{Mg}^{2+}\), is faster than EDTA and EGTA at taking up and releasing \(\text{Ca}^{2+}\), and its dissociation constant is 110 nM (46, 47). In this study, we have shown that loading MHN and MHS myoballs with BAPTA-AM induced a decline in resting \([\text{Ca}^{2+}]_i\), by 25% in MHN myoballs and by 51% MHS myoballs. The BAPTA concentration used was previously shown not to bind to either the DHPR or RyR1 receptors and not to interfere with intramembranous charge movement (42). Thus any effects caused by BAPTA on caffeine and 4-CmC-mediated SR \(\text{Ca}^{2+}\) release in myoballs is likely to be related only to its ability to buffer myoplasmic \([\text{Ca}^{2+}]_i\); if there are other acute effects caused by BAPTA, they are likely to be related to its ability to buffer myoplasmic \([\text{Ca}^{2+}]_i\); if there are other acute effects.

![Graph showing BAPTA modifies caffeine-induced \(\text{Ca}^{2+}\) release in MHS myoballs.](http://ajpcell.physiology.org/)

Fig. 4. BAPTA changes 4-chloro-m-cresol (4-CmC)-elicited \(\text{Ca}^{2+}\) release in MHS myoballs. \([\text{Ca}^{2+}]_i\), was measured in MHN myoballs (open bars) and MHS myoballs (closed bars) after incubation in BAPTA (10 \(\mu\)M and was then exposed to different caffeine concentrations (0.5–2 mM). Data are presented as means ± SD, and values above each bar represent the number of \([\text{Ca}^{2+}]_i\) measurements. *\(P < 0.001\), significant difference vs. pre-BAPTA measurements.
caused by BAPTA-AM cleavage products in these cells (40), they should be similar in the two genotypes, because the same loading conditions were used for both cell types. It is important to point out that fluorescent Ca^{2+} indicators such as fura-2, indo, fluo-3, and fluo-4 are structurally derived from BAPTA and share many of the physical characteristics of their parent molecule, including similar Ca^{2+} affinities, binding kinetics, and cytoplasmic mobility (43), although their affinities for Ca^{2+} are lower (Kd is 224 nM for fura, 230 nM for indo, and 400 nM for fluo-3) (15, 36, 43). The effect of BAPTA on resting [Ca^{2+}], in MHS myoballs demonstrates clearly that [Ca^{2+}] can be modulated by experimental maneuvers that increase the apparent Ca^{2+}-buffering capacity of the cytoplasm, which could artifically lower the measured [Ca^{2+}]), from the true cytoplasmic [Ca^{2+}] in these cells. It is possible that the increase in Ca^{2+}-buffering capacity of the cytoplasm associated with fluorescent dye loading, the changes in emission spectrum due to binding to soluble and structural proteins, and the lack of accurate calibration (fluorescence ratios measured in vivo are used to calibrate curves determined in vitro), as well as the inability to properly and accurately determine the constants used to calculate final [Ca^{2+}] (Kd for Ca^{2+}), may explain the discrepancy between our results in MHS cells (myoballs, myotubes, and muscle fibers) and the values for [Ca^{2+}], obtained by others in MHS cells in which this value was obtained using fluorescent Ca^{2+} dyes (4, 8, 17, 18, 20, 36, 43, 46, 47), even when an increase in [Ca^{2+}], has been observed in MH cells compared with wild-type cells (10).

It was previously hypothesized that the increased sensitivity of MHS muscle fibers to caffeine is a consequence of high resting [Ca^{2+}], and not an increased sensitivity of the MHS SR Ca^{2+} release channel to caffeine per se (27, 41). Our data in the present study support this hypothesis by showing that a reduction of [Ca^{2+}], in MHS myoballs elicited by increasing the myoplasmic buffering capacity eliminated the enhanced responsiveness of MHS myoballs to caffeine and 4-CmC. Thus, on the basis of these combined data, it appears that the elevation of resting [Ca^{2+}], is an important factor in the enhanced response to these agents observed in MHS myotubes, myoballs, and muscle fibers. Yang et al. (51) showed that expression of recombinant RyR1 bearing of six of the most common MH-RyR1 proteins in RyR-null myotubes (including Arg615Cys) not only exhibited significantly higher sensitivity to caffeine and 4-CmC compared with those expressing wt-RyR1 but also were more sensitive to K+ depolarization. SR isolated from these cells tested by [3H]ryanodine binding analysis revealed that although none of the MH-RyR1 proteins showed altered EC50 for activation by Ca^{2+}, all possessed significantly impaired inhibition by Ca^{2+} and Mg^{2+}. When tested at a [Ca^{2+}] of 100 nM, [3H]ryanodine binding analysis of all MH-RyR1 showed amplified binding in response to caffeine compared with wild-type cells. Similar results have been reported by other researchers who used several different isolated membrane preparations (14, 23, 35, 39). These studies indicated that there is a primary dysfunction of RyR1 under resting conditions and that one consequence is a heightened agonist sensitivity that is characteristic of MHS muscle. On this basis, we conclude that there are three mutually enforcing mechanisms that lead to an enhanced response of MHS muscle to direct agonists and increased release of SR Ca^{2+}, chronically elevated resting [Ca^{2+}], impaired feedback inhibition of MH-RyR1 by Ca^{2+} and Mg^{2+}, and a mutation-induced enhanced sensitivity to agonist activation.

ACKNOWLEDGMENTS

We thank for Dr. Stuart Taylor for advice on the preparation of swine myoballs and Dr. Claudio Perez for suggestions after reading the manuscript.

GRANTS

This work was partially supported by grants from Fondo Nacional de Ciencia, Tecnología e Innovación (FONACIT)-S3 of Venezuela (to J. R. López) and the Muscular Dystrophy Association (to P. D. Allen) and by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-46513 (to P. D. Allen and I. N. Pessah).

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