ENHANCED EXCITATION-COUPLED CALCIUM ENTRY IN MYOTUBES IS ASSOCIATED WITH EXPRESSION OF RYRI MH MUTATIONS

Tianzhong Yang¹, Paul D Allen¹,*, Isaac N Pessah², Jose R Lopez²,³
¹Department of Anesthesiology Perioperative and Pain Medicine, Brigham and Women’s Hospital, Boston, MA, USA
²Department of Molecular Biosciences, School of Veterinary Medicine, UC Davis, Davis, California, USA
³Centro de Biofisica y Bioquimica, Instituto Venezolano de Investigaciones Cientificas (IVIC), Caracas, Venezuela

Running Title: Enhanced Ca²⁺ entry in Malignant Hyperthermia

*Address Correspondence to: PD Allen, Department of Anesthesiology Perioperative and Pain Medicine, Brigham and Women’s Hospital, 75 Francis St, Boston MA, 02115 E-mail pdalllen@partners.org

Summary

Myotubes expressing wild type RyR1 (WT) or RyR1 with one of three MH mutations R615C, R2163C, and T4826I (MH) were exposed sequentially to 60mM KCl in Ca²⁺-replete and Ca²⁺-free external buffers (Ca⁺ and Ca⁻, respectively) with 3 minutes rest between exposures. While the maximal peak amplitude of the Ca⁺ transient during K⁺ depolarization was similar for WT and MH in both external buffers, the rate of decay of the sustained phase of the transient during K⁺ depolarization (decay rate) in Ca⁺ was 50% slower for MH. This difference was eliminated in Ca⁻, and the relative decay rates were faster for both genotypes than in Ca⁺. The integrated Ca²⁺ transient in Ca⁻ compared to Ca⁺ was reduced by 50-60% for MH and 20% for WT. The decay rate was not affected by [K⁺][Cl⁻] product or NiCl₂ (2 mM) supplementation of Ca⁺. Addition of La³⁺ (0.1 mM), or SKF 96365 (20 μM) to Ca⁺ significantly accelerated decay rates for both WT and MH, but their effect was significantly greater in MH. Nifedipine (1 μM) had no effect suggesting that the mechanism for this difference was not a reduction in L-type Ca²⁺ channel Ca²⁺ current. These data strongly suggest: (1) decay rate in skeletal myotubes is related in part to Ca²⁺ entry through the ECCE channel; (2) MH mutations enhance ECCE compared to wild type; (3) Increased Ca²⁺ entry might play a significant role in the pathophysiology of MH.

INTRODUCTION

Excitation-contraction (EC) coupling in skeletal muscle is a cascade of events which is initiated by the depolarization of T-tubule membrane (dihydropyridine receptor-DHPR), which is followed by activation of the intracellular Ca²⁺ release channels known as ryanodine receptors (RyR1) located at the terminal cisternae of the sarcoplasmic reticulum (SR). These two proteins align during development at the junction of the t-tubule with the terminal cisternae of the sarcoplasmic reticulum (SR) (1)-a collection of structures termed the Calcium Release Unit (CRU) (2). The DHPR may serve a dual function as slow-activated voltage dependent Ca²⁺ channel (3) and as voltage sensor for EC-coupling (4). The DHPR is responsible for activating RyR1 causing Ca²⁺ release from the SR (5-7) into the cytoplasm that in turn triggers muscle contraction. Although the exact signal transduction mechanisms between DHPR and RyR1 are still unknown it is generally accepted that intramembrane charge movements and conformational changes in the DHPR II-III loop couple the T tubule depolarization and Ca²⁺ release from the SR (orthograde conformational coupling) (5,9). It is this massive release of Ca²⁺ from the SR into the cytosol, and not a Ca²⁺ influx from the extracellular space, that is conventionally thought to initiate a series of Ca²⁺ dependant events that results in force generation (10,11).

Experiments in which external Ca²⁺ was removed (12,13) or calcium channel blockers are added to (14) the bathing medium around skeletal muscle fibers show that EC coupling and twitch contraction persists in skeletal muscle cells under these conditions. However, the role of extracellular Ca²⁺ on EC coupling in mammalian skeletal muscle has been reexamined (15). Several groups have demonstrated that stimuli that deplete Ca²⁺ in the SR enhance Ca²⁺ entry through the plasma membrane by a mechanism referred as
store-operated Ca\(^{2+}\) entry (SOCE) (16,17). Although Ca\(^{2+}\) entry through SOCE is easily seen, identification of the channel(s) responsible still awaits discovery. It is highly likely that the mechanism for this entry is at least partially if not completely derived from current passing though Orai 1, 2 and or 3 which is activated by translocation of Stim 1 or Stim 2 from the SR (18-23). In addition to SOCE a new mechanism for Ca\(^{2+}\) entry has recently been described in skeletal myotubes (24,25) that is not linked to Ca\(^{2+}\) depletion of the SR, and is elicited by brief or sustained membrane depolarization that would block conventional SOCE current(s). Activation of this Ca\(^{2+}\) entry pathway depends on interaction among three different Ca\(^{2+}\) channels: the DHPR, RyR1 and an unidentified Ca\(^{2+}\) influx pathway through the plasma membrane that was termed excitation-coupled Ca\(^{2+}\) entry (ECCE) (24).

Malignant hyperthermia (MH) is a potentially fatal pharmacogenetic syndrome in which exposure to volatile anesthetics or depolarizing neuromuscular blockers triggers a robust intracellular Ca\(^{2+}\) release through RyR1 from the SR, inducing a cascade of biochemical events that if untreated results in muscle rigidity, rhabdomyolysis, cardiac arrhythmia, and lethal hyperthermia (26,27). This syndrome has been associated with a dysfunction of resting intracellular Ca\(^{2+}\) regulation. To date, about 112 mutations within the gene that codes for type 1 ryanodine receptor (RyR1) have been found on chromosome 19 (28) and two mutations in the \(\alpha_\text{s}\) subunit of the DHPR found on chromosome 1 have been associated with MH (29).

To examine the possible role of abnormal sarcolemmal Ca\(^{2+}\) entry during MH, myotubes expressing mutations R615C, R2163C and T48261, (collectively termed \(\text{MH}\)RyR1s) were exposed to super-maximal concentrations of KCl (60mM K\(^{+}\)) in the presence (Ca\(^{+}\)) and absence (Ca\(^{-}\)) of extracellular Ca\(^{2+}\). In addition, we also conducted experiments in the presence or absence of Ca\(^{2+}\) after the addition of NiCl\(_2\), nifedipine, La\(^{3+}\), or SKF 96365 in an attempt to identify, or if not to identify at least characterize the entry pathway. The results strongly suggest that the accentuated Ca\(^{2+}\) transients seen in myotubes expressing \(\text{MH}\)RyR1s after KCl depolarization that we previously reported (30) were the result of a more robust sarcolemmal Ca\(^{2+}\) entry and not an increased SR Ca\(^{2+}\) release in response to depolarization. This difference potentially plays a role in the pathophysiology of the MH syndrome.

**EXPERIMENTAL PROCEDURES**

**Construction and expression of \(\text{MH}\)RyR1 cDNAs**

A detailed explanation of construction and expression of the \(\text{MH}\)RyR1s (R615C, R2163C and T48261) used in the present study has been previously described (30). All mutated and \(\text{WT}\)RyR1 cDNAs were packaged into HSV1 virions, using a helper virus-free packaging system (31,32) which were then used to transduce dyspedic 1B5 myotubes (32) for 2 hr at an MOI of 0.5, and then cultured for 48 hr prior to imaging. 1B5 cells (RyR-1, RyR-2 and RyR-3 null) were cultured on Matrigel (BD BIOSCIENCE, San Jose, CA) coated 96 well plates (OptiClear® COSTAR 3614) as described previously (30).

**Calcium Imaging of Myotubes**

Differentiated myotubes were loaded with 5 µM Fluo-4AM (Molecular Probes Inc., Eugene, OR) at 37 °C, for 20 min in imaging buffer. The cells were then washed 3 times with imaging buffer (IB), transferred to a Nikon TE2000 microscope. Fluo-4 was excited at 494nm with an argon light source and fluorescence emission was measured at 516 nm using a 40x 1.3na objective. Data were collected with an intensified 12-bit digital intensified CCD at 30fps (Stanford Photonics, Stanford, CA) from regions consisting of 2-12 individual cells, and analyzed using QED software (QED, Pittsburgh Pa). We used the average fluorescence of the calcium transient (area under the curve) to compare responses. Individual response amplitudes were calculated in the following way: the cumulative fluorescence during the 30 s challenge (Ar) minus the average baseline fluorescence for the 10 s immediately prior to the challenge (Ab), was then divided by Ab, and multiplied by 100. In order to compare different experiments individual response amplitudes were normalized to the peak amplitude of the transient obtained in the same cell. The peak amplitude of a transient refers to the highest fluorescence value during the stimulation time minus the average baseline fluorescence for the 10 seconds immediately prior to the challenge.

**Solutions**

Ionic compositions of Ca\(^{2+}\)-replete imaging buffer (Ca\(^{+}\)) with and without K\(^{+}\) (40 or
60mM) are shown in table 1. In KCl solutions, NaCl concentrations were adjusted to maintain a total ionic strength (Na\(^+\) concentration ([Na\(^+\)]) + K\(^+\) concentration ([K\(^+\)]) at 130 mM, but the product of [K\(^+\)] \times [Cl\(^-\)] changes with different concentrations of KCl. In K\(_2\)SO\(_4\) solutions, K\(_2\)SO\(_4\) was used to increase [K\(^+\)] and Na\(_2\)SO\(_4\) was used to replace NaCl in order to maintain the [K\(^+\)] \times [Cl\(^-\)] product at 670 and [Na\(^+\)] + [K\(^+\)] at 130 (the same values as Ca\(^+\)). All nominally Ca\(^{2+}\) free buffers (Ca-)) were prepared using the same protocol as the corresponding regular solutions but without CaCl\(_2\) added. Caffeine, NiCl\(_2\), nifedipine, LaCl\(_3\), and SKF96365 solutions were prepared by adding these compounds directly into either of the external buffers as well as the corresponding KCl solutions. Due to frequent trace Ca\(^{2+}\) contamination in other chemicals, residual free Ca\(^{2+}\) concentrations in Ca- (same composition as Ca+, but no Ca\(^{2+}\) added) and 60 mM Ca\(^{2+}\)-free KCl solution (60KCl-Ca) were measured using Ca\(^{2+}\)-selective microelectrodes and were ~0.8 \mu M and ~1.0 \mu M, respectively.

**Determination of threshold for Maximum Ca\(^{2+}\) transient in response to KCl**

From our previous studies we had determined that 50 mM [K\(^+\)]\(_o\) was sufficient to produce the maximum peak Ca\(^{2+}\) transient in myotubes (30). However, to make certain that our previous observations did not diverge from the measurements performed in the present study, we measured the magnitude of Ca\(^{2+}\) transients in myotubes expressing WT RyR1 produced by challenges with 40, 60, 80 and 100 mM [K\(^+\)]\(_o\), applied in a random order.

**Statistics**

All values are expressed as mean±SE. Prism software (version 4.0b) was used for statistical analysis (GraphPad Software, San Diego California USA, www.graphpad.com). Student’s t tests, One-way ANOVA and Tukey’s multiple comparison tests were used to compare the response sizes and peak amplitudes.

**RESULTS**

**Effect of extracellular Ca\(^{2+}\) on KCl-induced Ca\(^{2+}\) transients**

A significant observation in our previous study (30) was that dyspedic 1B5 myotubes expressing MH RyR1s consistently exhibited Ca\(^{2+}\) transients (area under the curve) in response to 60mM KCl (60KCl) that were greater than those in myotubes expressing WT RyR1. The selection of 60 mM [K\(^+\)]\(_o\) to induce Ca\(^{2+}\) transients in WT RyR1 and MH RyR1 myotubes for this study was based both on our results obtained from the dose-response relationship measured in WT myotubes (see figure 1) and previously published data (33, 34). The collective results from these studies show that in muscle fibers the amount of membrane depolarization caused by >50 mM [K\(^+\)]\(_o\) will elicit an elevation of myoplasmic [Ca\(^{2+}\)] above the level required to saturate the contractile apparatus.

Closer examination of data from our previous study revealed differences in the shapes of the Ca\(^{2+}\) transients induced by K\(^+\) between WT RyR1 and each of the seven MH RyR1s. Although there was no difference in the peak amplitude between the two groups, all MH RyR1 Ca\(^{2+}\) transients had a slower rate of decay than WT RyR1 Ca\(^{2+}\) transients (see Figure. 1A, inset), and accounted for the greater areas under the Ca\(^{2+}\) transients elicited by 60KCl in myotubes expressing MH RyR1s.

To determine if Ca\(^{2+}\) entry contributed to the slower decline of the Ca\(^{2+}\) transient in myotubes expressing MH RyR1s during KCl depolarization, we increased the duration of depolarization from 10 sec as it was in the previous study (30) to 30 sec to record their rates of decay of the sustained phase of the transient and increased the interval between two 60KCl challenges from 50 sec (30) to 3 min to permit the myotubes to return to their original resting intracellular free Ca\(^{2+}\) concentration between responses. As shown in figure 2A, the 1B5 myotubes expressing WT RyR1 or each of three common MH RyR1s (R615C, R2163C, and T4826I, from RyR1 gene “hot spots” I, II and III respectively) were sequentially exposed to 60KCl, 60KCl-Ca, and 60KCl using the protocol described above. Consistent with our previous observation (2), all three types of MH myotubes demonstrated transients with much slower decay rates of the sustained phase of the transient during 60KCl depolarization compared to those expressing WT RyR1. Prior to exposure to 60KCl the Ca\(^{2+}\) buffer was exchanged with nominally Ca\(^{2+}\)-free (Ca-) buffer for 5 sec. The decay rate of the sustained phase of the Ca\(^{2+}\) transient during exposure to 60KCl-Ca was accelerated for all four types (WT and 3 MH) of myotubes and was more
rapid in all groups than the rate obtained from

\( \text{WT RyR1} \) depolarized in Ca\(^{2+}\) buffer. On the other hand, the peak amplitude of the response was not significantly different for all three KCl exposures. In addition the ratio of the peak amplitude to the amplitude at 30 sec in Ca\(^{2+}\) free media (“pure RyR1 Ca\(^{2+}\) release”) was not different between cells expressing \( \text{WT RyR1} \) compared to those expressing any of the three \( \text{MH RyR1s} \). After 3 min rest, the rate of decay of the sustained phase of the Ca\(^{2+}\) transient after the third 60 KCl exposure was essentially the same as the first 60 KCl response in both \( \text{WT RyR1} \) and \( \text{MH RyR1} \) myotubes. Therefore the increased rate of decay seen during the 60 KCl Ca\(^{2+}\) response was not the result of progressive store depletion in either group. Superimposing the first 60 KCl response with the 60 KCl Ca\(^{2+}\) response in the same myotube clearly showed the pronounced difference in their decay rate in the presence and absence of external Ca\(^{2+}\). Importantly the relative increase in the decay rate in Ca- was much greater for all \( \text{MH RyR1s} \) than for \( \text{WT RyR1} \). Comparison of the response sizes (figure 2B) demonstrated that (1) all three groups of MH myotubes had significantly larger total Ca\(^{2+}\) responses (p<0.01) to 60 KCl compared to \( \text{WT RyR1} \), (2) the response sizes to 60 KCl Ca\(^{2+}\) were significantly smaller than those to 60 KCl in both WT and MH myotubes, and (3) the peak and total response sizes to 60 KCl Ca\(^{2+}\) for \( \text{WT RyR1} \) and \( \text{MH RyR1} \) myotubes was the same. The ratio of the 60 KCl Ca\(^{2+}\) response size to the 60 KCl (figure 2C) response size for each of the three \( \text{MH RyR1s} \) was significantly (p<0.01) less than that for \( \text{WT RyR1} \) (42.6±3.9%, 48.6±1.6%, and 52.0±3.3% for \( \text{R615C RyR1} \), \( \text{R2163C RyR1} \), and \( \text{T4826I RyR1} \), respectively vs. 80.5±4.6% for \( \text{WT RyR1} \)), reflecting the difference in the rate of decay during depolarization for \( \text{MH RyR1s} \) (for superimposed normalized responses see figure 2A). Due to the similarity in the ratios of these three MH mutations in their slower rate of decay compared to \( \text{WT RyR1} \), we randomly picked T4826I for further investigation for the possible mechanisms for this difference.

It is well known that extracellular [Cl\(^{-}\)] also contributes to the membrane potential of frog muscle fibers when extracellular [K\(^{+}\)] and [Cl\(^{-}\)] were changed reciprocally to keep a constant product (35). In our 60 mM KCl solution both [K\(^{+}\)] and [Cl\(^{-}\)] were increased, substantially increasing the product of [K\(^{+}\)]\times[Cl\(^{-}\)], compared to Ca\(^{2+}\) buffers without KCl. (Table 1). Thus it was theoretically possible that the responses to 60 KCl we observed could have been affected by a Cl\(^{-}\) overload. To rule this in or out, we exposed \( \text{WT RyR1} \) and \( \text{T4826I RyR1} \) myotubes to 60 mM KCl whose [K\(^{+}\)]\times[Cl\(^{-}\)] product in the buffer was the same as Ca\(^{2+}\) buffer. Comparison of the shape and response sizes after depolarization with these two K\(^{+}\) solutions ([K\(^{+}\)]\times[Cl\(^{-}\)] not constant versus [K\(^{+}\)]\times[Cl\(^{-}\)] constant) showed that they were not significantly different from one another (figure 3A).

It has also been demonstrated in single frog muscle fibers that removing extracellular Ca\(^{2+}\) to levels identical with that used for our Ca-experiments resulted in a slight membrane depolarization (9±2 mV) and potentiation of twitch responses (12). Substitution of Ni\(^{2+}\) for missing Ca\(^{2+}\) in extracellular solutions resolved this problem (36). To examine this as a possible mechanism for the differences we observed between \( \text{WT RyR1} \) and \( \text{MH RyR1} \), we prepared 60 KCl\(_{\text{Ni}}\) solution by adding 2 mM NiCl\(_{2}\) to the 60 KCl Ca\(^{2+}\) solution. Myotubes expressing \( \text{T4826I RyR1} \) were exposed to 60 KCl, 60 KCl\(_{\text{Ca}}\), 60 KCl\(_{\text{Ni}}\) and 60 KCl sequentially (figure 3B). As observed before, the rate of decay of the Ca\(^{2+}\) transient in response to 60 KCl Ca\(^{2+}\) depolarization was accelerated compared to the 60 KCl response. Addition of 2 mM NiCl\(_{2}\) (60 KCl Ca\(^{2+}\)) did not slow the accelerated rate of decay. However the slow rate of decay of the sustained phase of KCl responses in \( \text{T4826I RyR1} \) myotubes was restored in Ca\(^{2+}\) buffer. (figure 3B).

**Effects of Ca\(^{2+}\) entry blockers on depolarization-induced Ca\(^{2+}\) entry**

To better define the mechanism for the enhanced Ca\(^{2+}\) entry caused by depolarization of cells with \( \text{MH RyR1} \) mutations we used a pharmacologic library to attempt to block the response. To do this we chose (0.1 mM) lanthanum chloride (La\(^{3+}\); a non specific Ca\(^{2+}\) entry blocker (37)), 20 μM SKF 96365 (a SOCE and TRP channel blocker (38) that has also been shown to block cation influx attributable to ECCE (24),) and 1 μM nifedipine (the selective L-type Ca\(^{2+}\) channel blocker (39). As shown in figure 4A, La\(^{3+}\) (0.1 mM) added 10 sec after 60 KCl exposure immediately accelerated the decay rate of the sustained phase of the Ca\(^{2+}\) transients for both
WT RyR1 and T4826I RyR1 myotubes (responses labeled '2' for WT RyR1 and T4826I RyR1). The 60 KCl responses ('3') with La$^{3+}$ (0.1 mM) added 5 sec before KCl exposure demonstrated a more significantly accelerated the decay rate of the sustained phase for both WT and T4826I RyR1 myotubes, which is better illustrated by superimposed normalized traces on the right side of figure 4. The last ('4) 60 KCl responses in the absence of extracellular La$^{3+}$ partially restored the slower decay rate of the sustained phase in both WT RyR1 and T4826I RyR1 myotubes, although the recovery was not complete compared to the '1 responses. The ratio of response sizes for 60 KCl + La$^{3+}$ ('3) to 60 KCl ('1) (figure 4B) is significantly smaller (p < 0.01) for T4826I (64.7 ± 3.1%) compared to WT (85.3 ± 3.6%), indicating a significantly larger portion of the 60 KCl response in T4826I RyR1 myotubes was diminished by La$^{3+}$ compared to WT RyR1. Of note, no change in the peak amplitude of the KCl responses was observed with the sequential KCl stimulations.

The decay rate of the sustained phase of the Ca$^{2+}$ transient during KCl depolarization was drastically accelerated in both WT and T4826I myotubes by 20 µM SKF 96365 ('2 responses in figure 5A). Removing SKF 96365 prior to the next 60 KCl stimulation restored the slower decay of sustained phase to pre-exposure rates in T4826I myotubes. 20 µM of SKF 96365 appears to accelerate the decay rate instantaneously (figure 5, inset) and the ratios of response '2 to response '1 sizes for WT and T4826I are 72.8 ± 3.2 % and 49.0 ± 3.9% respectively, which suggests a more significant effect (p < 0.01) of SKF 96365 on T4826I myotubes compared to WT myotubes (figure 5). Interestingly, the response '2 to response '1 ratios for the SKF 96365 effect tended to be smaller than even those for 60 KCl CS shown in figure 2 (80.5 ± 4.6 % and 52.0 ± 3.3% for WT and T4826I, respectively).

Nifedipine administered 5 sec before 60 KCl_Nif (60 KCl containing nifedipine (Nif) 1 µM) stimulation ('2 responses in figure 6A) had no significant effect on the sustained phase in either WT or MH myotubes compared to the corresponding control '1 responses.

Caffeine-induced Ca$^{2+}$ transient is not mediated by sarcolemmal Ca$^{2+}$ entry

To investigate if the presence of extracellular Ca$^{2+}$ has any effect on the Ca$^{2+}$ transient elicited by direct RyR1 activators, 20mM caffeine was used to stimulate Ca$^{2+}$ release from SR from myotubes expressing WT RyR1 and T4826I RyR1, using the same experimental protocol (figure 7A). In contrast to the KCl responses shown before, the responses to regular 20mM caffeine (20Caff) and Ca$^{2+}$-free 20mM caffeine (20Caff_Ca) were identical in both WT RyR1 and T4826I RyR1 myotubes. These similarities were clearly illustrated when the normalized data were superimposed. Interestingly, the peak amplitude of the caffeine responses decreased significantly with sequential caffeine successive caffeine challenges, and was independent of external Ca$^{2+}$ (figure 7B).

As a result, the response sizes showed a similar decrease in both WT RyR1 and T4826I RyR1 myotubes (figure 7C). When the peak amplitudes and response sizes for the first 20Caff response and the 20Caff_Ca response were superimposed (figure 7D), the decreases in peak amplitude were coincident with the decrease in response size (area under the curve) for both genotypes, suggesting that the decreased response size to 20Caff_Ca resulted from decreased peak amplitude rather than a change in the sustained phase. These observations are also born out when the cells are stimulated with caffeine in the presence of SKF 96365. (figure 7E). Neither WT RyR1 nor T4826I RyR1 expressing cells showed any decrease in the amplitude or area under the curve of their 20Caff_Ca transient in the presence of 20µM SKF 96365 compared to the control 20Caff response.

DISCUSSION

The purpose of this study was to determine whether sarcolemmal Ca$^{2+}$ entry was responsible for maintaining and prolonging the Ca$^{2+}$ transient in MH skeletal muscle myotubes in response to depolarization and if so, attempt to use pharmacological means to better define the pathway for this entry. Our study demonstrates that in myotubes expressing WT RyR1 or MH RyR1 there is a significant amount of Ca$^{2+}$ entry in response to K$^{+}$-induced depolarization. This Ca$^{2+}$ entry is most easily detectable during the sustained phase of a long depolarization that follows the peak Ca$^{2+}$ transient and is enhanced in cells expressing any of three widely spaced MH RyR1s. Removing extracellular Ca$^{2+}$ significantly reduced the area under the Ca$^{2+}$ transient in both WT RyR1 and MH RyR1 expressing myotubes and was attributed to an accelerated decay of the sustained
phase without affecting the peak amplitude. Interestingly the rate of decay of the Ca\(^{2+}\) signal during the sustained phase was significantly greater in myotubes expressing any of the MT-RyR1’s tested. Sarcolemmal channel blockers La\(^{3+}\) or SKF 96365 both instantaneously accelerated the rate of decay in both WT-RyR1 and MT-RyR1 myotubes, and similar to the responses seen with low extracellular Ca\(^{2+}\) their effect is larger in myotubes expressing MT-RyR1s than in WT-RyR1. Although these two Ca\(^{2+}\) entry blockers like almost all pharmacologic agents have off site actions and thus are never specific for a single channel type, we can be certain their ability to reduce Ca\(^{2+}\) entry was not due to their ability to block L-type Ca\(^{2+}\) current as nifedipine had no significant effect on the Ca\(^{2+}\) entry in either WT-RyR1 or MT-RyR1s myotubes. The importance of extracellular Ca\(^{2+}\) during K\(^{+}\)-induced contracture has been previously suggested in early studies of frog muscle fibers (12,40,41). In these studies it was found that Ca\(^{2+}\) influx, measured by Ca\(^{45}\) entry, was almost proportional to the extracellular Ca\(^{2+}\) concentration during the K\(^{+}\) contracture (41). The same study also showed that the difference between the height of contractures evoked by high K\(^{+}\) solutions (95mM) with or without Ca\(^{2+}\) was very small or absent, whereas the duration time of the contracture is directly proportional to the extracellular Ca\(^{2+}\) concentration. Similar results were obtained in a later study on effects of external calcium deprivation on single frog muscle fibers (12), which demonstrated that Ca\(^{2+}\) removal immediately increased the rate of relaxation of K\(^{+}\) contractures, whereas a longer time (3 min.) of Ca\(^{2+}\) withdrawal was needed to decrease the action potential amplitude. On the other hand, experiments in which muscle fibers were subject to brief electrical stimulation, removal of extracellular Ca\(^{2+}\) did not appear to impair EC coupling and force production in amphibian skeletal muscle cells (12,13). More recently a Ca\(^{2+}\) entry that is not linked to Ca\(^{2+}\) depletion of the SR, named excitation coupled calcium entry (ECCE) has been shown to be elicited by K\(^{+}\) depolarization and trains of electrical pulses (24). Activation of ECCE depends on interaction among three different Ca\(^{2+}\) channels: the DHPR, RyR1 and an as of yet unidentified Ca\(^{2+}\) channel at the plasma membrane (24). Its distinctive properties are: (1) it is initiated by depolarization; (2) It is dependent on the presence of both RyR1 and DHPR (3) it is independent of the DHPR L-type Ca\(^{2+}\) current, (4) It does not depend on SR Ca\(^{2+}\) release and (5) It can be effectively inhibited by La\(^{3+}\) or SKF96365 (24).

Previous studies of KCl responses in MH muscle cells showed significantly larger muscle contractures and lower KCl contracture thresholds in muscle fibers dissected from MH susceptible swine compared to WT (42-44). In addition, it has been reported that the total calcium transient in MH susceptible muscle fibers was larger than control fibers when exposed to sub-contracture and contracture concentrations of KCl (30,43,46).

This study is the first detailed investigation of the effects of external Ca\(^{2+}\) on the kinetics of the Ca\(^{2+}\) transient in response to K\(^{+}\) depolarization in myotubes expressing WT-RyR1 and MT-RyR1s. Our results demonstrate that a significant portion of the observed KCl depolarization induced Ca\(^{2+}\) transient is the result of sarcolemmal Ca\(^{2+}\) entry in both groups of myotubes. This is most easily detectable during the sustained phase that follows the peak of the Ca\(^{2+}\) transient. The data from our previous study on seven common MT-RyR1s expressed in dyspedic myotubes gave us the first clue that the kinetics of MH associated Ca\(^{2+}\) transients, especially the sustained phase, is significantly different compared to WT-RyR1 myotubes (30). The experimental protocol adopted in this study was specifically designed to facilitate a more detailed characterization of the sustained phase of the KCl response, i.e. the 3 min interval between KCl stimulations allowed the myotubes to restore the SR calcium pools so the peak amplitudes of repeated KCl responses remained the same in any individual cell.

We have characterized the class of channels responsible for the increased Ca\(^{2+}\) entry seen in cells expressing MT-RyR1s during KCl induced-Ca\(^{2+}\) transients in myotubes by exposing them to several different experimental conditions:

1. After exposure to nominally Ca\(^{2+}\) free imaging buffer. Cells were exposed to Ca\(^{2+}\) shortly (5 sec) before the exposure to 60K\(_{\text{Ca}}\) to ensure the KCl response occurred in a calcium-free solution while minimizing the possible reduction of SR calcium stores caused by Ca\(^{2+}\) leakage out of the cell. EGTA was not used in the Ca\(^{2+}\)-free solutions because (1) high concentrations of EGTA results
in a blockade of excitation-contraction coupling (46) and (2) adding EGTA (1 or 2 mM) to extracellular solutions can significantly increases the spontaneous Ca\(^{2+}\) release activities in primary myotubes (unpublished observations). We believe that the Ca\(^{2+}\)-free buffers used in this study didn’t have an observable effect on the membrane potential and the voltage sensitivity of the myotubes because (1) the peak amplitudes of KCl-induced Ca\(^{2+}\) transients in the presence or absence of extracellular Ca\(^{2+}\) are the same; and (2) supplementation of the Ca\(^{2+}\)-free KCl solution with 2 mM NiCl\(_2\), which has been shown to prevent the partial depolarization observed in Ca\(^{2+}\) free media (12) did not change the amplitude or rate of decay of the sustained phase of KCl-induced Ca\(^{2+}\) transients in myotubes.

2. In the presence of sarcolemmal Ca\(^{2+}\) entry channel blockers, La\(^{3+}\), a non-specific blocker of membrane Ca\(^{2+}\) permeability and SKF 96365 which has been widely used as a SOC channel blocker (38) accelerated the decay of the sustained phase of the Ca\(^{2+}\) transients in both \(w\_T\)RyR1 and \(T4826\)RyR1 myotubes proving that the sustained phase of the KCl responses is largely due to Ca\(^{2+}\) entry. Their pharmacological effects were more evident in myotubes expressing \(T4826\)RyR1 than \(w\_T\)RyR1 expressing myotubes. Both compounds have been demonstrated to effectively block ECCE (24) which suggests the possibility that ECCC is enhanced in myotubes carrying MH mutations.

3. Any conventional contribution of the slow inward Ca\(^{2+}\) current through the DHPR to the Ca\(^{2+}\) entry observed during sustained depolarization in \(w\_T\)RyR1 and \(M\_T\)RyR1 myotubes was ruled out by the fact that neither group was affected by 1 \(\mu\)M nifedipine.

Another interesting finding in support of a role for Ca\(^{2+}\) entry during KCl depolarization being the cause of the difference between \(w\_T\)RyR1 and \(M\_T\)RyR1 was the result seen when 20mM caffeine was used to stimulate Ca\(^{2+}\) release in myotubes expressing \(w\_T\)RyR1 and \(T4826\)RyR1. The fact that the responses to 20mM caffeine were almost identical in Ca\(^{2+}\) replete, Ca\(^{2+}\)-free, and Ca\(^{2+}\) replete buffer containing SKF 96365 in both \(w\_T\)RyR1 and \(T4826\)RyR1 myotubes rules out the possible contribution of an increased SR Ca\(^{2+}\) release being responsible for the larger KCl induced Ca\(^{2+}\) transients seen in cells expressing \(M\_T\)RyRs. In addition, the fact that both the peak amplitude and response size of the caffeine responses for myotubes expressing \(T4826\)RyR1 were smaller and decreased more significantly with sequential caffeine stimulations compared to those for \(w\_T\)RyR1 suggest that our previous conclusion that the SR Ca\(^{2+}\) load is not diminished in MH myotubes (30) was incorrect and gives support to hypothesis that there are a greater number of \(M\_T\)RyR1 channels in leak state in the SR than are present in cells expressing \(w\_T\)RyR1 (47).

In summary, theses results demonstrate that RyR1 MH mutations are associated with an enhanced Ca\(^{2+}\) entry through the sarcolemma during depolarization. They further suggest that Ca\(^{2+}\) entry may contribute to maintaining Ca\(^{2+}\) homeostasis in mammalian skeletal EC coupling and may play an important role in the pathophysiology of malignant hyperthermia.

References

FIGURE LEGENDS

Figure 1. The effects of increasing KCl concentration on the depolarization induced Ca\textsuperscript{2+} transient. Average calcium fluorescence response in N=7 1B5 cells expressing \( \text{WT RyR1} \) after being stimulated with 40, 60, 80 and 100 mM KCl applied in random order for individual cells. (afu=arbitrary fluorescence units) Note that in 1B5 cells expressing \( \text{WT RyR1} \), as KCl concentration increases inactivation becomes faster, to the point where at 100mM KCl inactivation begins before the peak Ca\textsuperscript{2+} response is attained.

Figure 2. The effects of nominally Ca\textsuperscript{2+} free imaging buffer on the size of the Ca\textsuperscript{2+} transient in WT and MH myotubes. Top inset, comparison of KCl Ca\textsuperscript{2+} transients between myotubes expressing \( \text{WT RyR1} \) and 7 common \( \text{MH RyR1s} \) (from Yang et al 2003 (3)) Panel A. Response to 60mM KCl in Ca\textsuperscript{2+} replete (\( \text{oKCl} \)) and nominally Ca\textsuperscript{2+} free (\( \text{oKCl}_{\text{Ca}} \)) IB. Responses are superimposed on the far right. B. Total response size to 60mM KCl in \( \text{WT} \) and MH myotubes in Ca\textsuperscript{2+} replete and nominally Ca\textsuperscript{2+} free IB. \( \text{WT}=100\% \) C. The ratio of the response to 60mM KCl between Ca\textsuperscript{2+} replete and nominally Ca\textsuperscript{2+} free IB.

Figure 3. The effects of [K\textsuperscript{+}]x[Cl\textsuperscript{-}] product and moderate membrane depolarization on the Ca\textsuperscript{2+} transient in response to KCl depolarization. Panel A. Ca\textsuperscript{2+} transients in response to 40 and 60 mM KCl is unchanged in response to changes in the [K\textsuperscript{+}]x[Cl\textsuperscript{-}] product (responses directly overlaid at right and Bar graph (far right)) Panel B. Relieving the partial depolarization caused by removing Ca\textsuperscript{2+} in nominally free Ca\textsuperscript{2+} IB does not change the rate of decay of the sustained phase of the Ca\textsuperscript{2+} transient in response to 60mM KCl. (responses directly overlaid at right and Bar graph (far right))

Figure 4. The effects Lanthanum (La\textsuperscript{3+}) on the Ca\textsuperscript{2+} transient in response to KCl depolarization. Panel A. La\textsuperscript{3+} immediately increases the decay rate of the sustained phase of the Ca\textsuperscript{2+} transient in response to 60mM KCl. Responses with and without La\textsuperscript{3+} compared at right. Panel B. The increase in the decay rate in response to La\textsuperscript{3+} is greater in MH than in \( \text{WT} \) myotubes.

Figure 5. The effects of SKF96365 on the Ca\textsuperscript{2+} transient in response to KCl depolarization. Figure 3. Panel A. SKF 96365 immediately increases the decay rate of the sustained phase of the Ca\textsuperscript{2+} transient in response to 60mM KCl. Responses with and without SKF 96365 compared at right. Panel B. The increase in the decay rate in response to SKF 96365 is greater in MH than in \( \text{WT} \) myotubes.

Figure 6. The effects of Nifedipine on the Ca\textsuperscript{2+} transient in response to KCl depolarization. 1\( \mu \text{M} \) Nifedipine has no effect on the rate of decay of the sustained phase of the Ca\textsuperscript{2+} transient in response to 60 mM KCl depolarization. With and without nifedipine compared directly at right (Panel A). There is also no difference in the effect of nifedipine between \( \text{WT} \) and MH myotubes (Panel B).

Figure 7. The effects of nominally free Ca\textsuperscript{2+} media and SKF 96563 on 20mM caffeine induced Ca\textsuperscript{2+} transients. Panel A shows responses in Ca\textsuperscript{2+} replete or nominally Ca\textsuperscript{2+} free IB with normalized responses shown at the right. Note that there is a steady decline in the total response on successive caffeine exposure, presumably due to reduction in SR stores. The Peak amplitude (Panel B) and the total response size (area under the curve of the Ca\textsuperscript{2+} transient)(Panel C) are significantly smaller in MH myotubes (p<0.01) than the comparable responses in \( \text{WT} \) myotubes. Both the total response (RS) and Peak (PA) amplitude decline proportionally in MH and \( \text{WT} \) myotubes. (Panel D) and in Panel E it can be seen that SKF 96365 has no effect on the peak amplitude or total response of the Ca\textsuperscript{2+} transient in response to 20mM Caffeine. Responses with and without SKF 96365 compared at the right (Panel E).
### Table 1: Composition of buffer solutions

<table>
<thead>
<tr>
<th></th>
<th>Ca(^{2+}) replete buffer (Ca(^{+}))</th>
<th>KCl 40mM (40KCl)</th>
<th>KCl 60mM (60KCl)</th>
<th>KCl 60mM (Ca(^{2+}) free) (60KCl, Ca(^{-}))</th>
<th>K(_2)SO(_4) 20mM (20K(_2)SO(_4))</th>
<th>K(_2)SO(_4) 30mM (30K(_2)SO(_4))</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (mM)</td>
<td>125</td>
<td>90</td>
<td>70</td>
<td>12.75</td>
<td>7.17</td>
<td></td>
</tr>
<tr>
<td>KCl (mM)</td>
<td>5</td>
<td>40</td>
<td>60</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO(_4) (mM)</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
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<tr>
<td>Hepes (pH7.4)</td>
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<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
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</tr>
<tr>
<td>Glucose (mM)</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
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<tr>
<td>CaCl(_2) (mM)</td>
<td>2</td>
<td>2</td>
<td>2</td>
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<tr>
<td>Na(_2)SO(_4) (mM)</td>
<td></td>
<td></td>
<td></td>
<td>38.625</td>
<td>31.415</td>
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</tr>
<tr>
<td>K(_2)SO(_4) (mM)</td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>30</td>
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<tr>
<td>[Na(^{+})] + [K(^{-})]</td>
<td>130</td>
<td>130</td>
<td>130</td>
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<tr>
<td>[K(^{-})] × [Cl(^{-})]</td>
<td>670</td>
<td>5360</td>
<td>8040</td>
<td>8040</td>
<td>670</td>
<td>670</td>
</tr>
</tbody>
</table>
Figure 2

A. 

R164C  G342R  R615C  R2163C  V2168M  R2458H  T4825I

--- WT RYR1  60 KCl (10 sec)

--- 60 KCl  60 KCl -Ca

B. 

Response Size

--- 60 KCl  60 KCl -Ca

C. 

Response Ratio

--- 60 KCl  60 KCl -Ca

--- WT RYR1 (n=54)  R615C (n=21)  R2163C (n=68)  T4825I (n=23)

--- WT RYR1 (n=54)  R615C (n=21)  R2163C (n=68)  T4825I (n=23)
A.

\[ \frac{[K^+] [Cl^-]}{[K^+]} = 8040 \]

\[ \frac{[K^+] [Cl^-]}{[K^+]} = 670 \]

B.

\[ \frac{[K^+] [Cl^-]}{[K^+]} = 5360 \]

\[ \frac{[K^+] [Cl^-]}{[K^+]} = 670 \]
Figure 4

A.  

\[ \text{wtRyR1:} \]

\[ \begin{align*}
&\text{KCl} \\
&\text{La}^{3+} 0.1\text{mM}
\end{align*} \]

\[ \begin{align*}
&\text{30sec} \\
&\text{3 min. interval}
\end{align*} \]

\[ \begin{align*}
&\text{#1} \\
&\text{#2} \\
&\text{#3} \\
&\text{#4}
\end{align*} \]

\[ \text{T4825I:} \]

\[ \begin{align*}
&\text{KCl} \\
&\text{La}^{3+} 0.1\text{mM}
\end{align*} \]

\[ \begin{align*}
&\text{#1} \\
&\text{#2} \\
&\text{#3} \\
&\text{#4}
\end{align*} \]

B.  

\[ \begin{align*}
\text{Response ratio (3/1 %)}
\end{align*} \]

\[ \begin{align*}
&\text{wtRyR1} \\
&\text{T4825I}
\end{align*} \]

\[ \begin{align*}
&n = 28 \\
&n = 31
\end{align*} \]
Figure 5

A. 

**wtRyR1:**

- 25 KCl (30 sec)
- #1
- 3 min. interval

**T4825I:**

- 60 KCl
- #1

B. 

Normalized responses

<table>
<thead>
<tr>
<th>Response ratio (#2 / #1 %)</th>
</tr>
</thead>
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<tr>
<td><strong>wtRyR1</strong></td>
</tr>
<tr>
<td><strong>T4825I</strong></td>
</tr>
</tbody>
</table>

- n = 26
- n = 21

*
Figure 6

A.  

\[ \text{Response ratio (\#2 / \#1 \%)} \]

B.  

Normalized responses

\[ \text{WT RyR1 T4825I} \]

\[ n = 24 \quad n = 25 \]
Figure 7

A.

Normalized responses

B.

C.

D.

Response Size (RS) & Peak Amplitude (PA)

- \( w_{1} \text{RYR1 (RS)} \)
- \( T4825I (RS) \)
- \( w_{1} \text{RYR1 (PA)} \)
- \( T4825I (PA) \)
Figure 7

E.

$w_7$ RyR1:

<table>
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<tr>
<th>25</th>
<th>30sec</th>
<th>3 min. interval</th>
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<tbody>
<tr>
<td>#1</td>
<td>#2</td>
<td>#3</td>
</tr>
</tbody>
</table>

T4825I:

<table>
<thead>
<tr>
<th>20 Caf</th>
<th>20 Caffeine, SKF</th>
<th>20 Caf</th>
</tr>
</thead>
<tbody>
<tr>
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Tianzhong Yang, Paul D. Allen, Isaac N. Pessah and Jose R. Lopez

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