The Journal of General Physiology

A malignant hyperthermia–inducing mutation in RYR1 (R163C): alterations in Ca^{2+} entry, release, and retrograde signaling to the DHPR

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Bidirectional signaling between the sarcolemmal L-type Ca^{2+} channel (1,4-dihydropyridine receptor [DHPR]) and the sarcoplasmic reticulum (SR) Ca^{2+} release channel (type 1 ryanodine receptor [RYR1]) of skeletal muscle is essential for excitation–contraction coupling (ECC) and is a well-understood prototype of conformational coupling. Mutations in either channel alter coupling fidelity and with an added pharmacologic stimulus or stress can trigger malignant hyperthermia (MH). In this study, we measured the response of wild-type (WT), heterozygous (Het), or homozygous (Hom) RYR1-R163C knock-in mouse myotubes to maintained K^+ depolarization. The new findings are: (a) For all three genotypes, Ca^{2+} transients decay during prolonged depolarization, and this decay is not a consequence of SR depletion or RYR1 inactivation. (b) The R163C mutation retards the decay rate with a rank order WT > Het > Hom. (c) The removal of external Ca^{2+} or the addition of Ca^{2+} entry blockers (nifedipine, SKF96365, and Ni^{2+}) enhanced the rate of decay in all genotypes. (d) When Ca^{2+} entry is blocked, the decay rates are slower for Hom and Het than WT, indicating that the rate of inactivation of ECC is affected by the R163C mutation and is genotype dependent (WT > Het > Hom). (e) Reduced ECC inactivation in Het and Hom myotubes was shown directly using two identical K^+ depolarizations separated by varying time intervals. These data suggest that conformational changes induced by the R163C MH mutation alter the retrograde signal that is sent from RYR1 to the DHPR, delaying the inactivation of the DHPR voltage sensor.

INTRODUCTION

In skeletal muscle, excitation–contraction coupling (ECC) is initiated by the activation of the L-type Ca^{2+} channel or 1,4-dihydropyridine receptor (DHPR). The DHPR in turn activates Ca^{2+} release from the SR Ca^{2+} release channel (RYR type 1 [RYR1]). The communication between the two channels is rapid and does not require Ca^{2+} entry through the DHPR, and it is believed that there is a physical interaction between the two proteins (Armstrong et al., 1972; Tanabe et al., 1990; Dirksen and Beam, 1999). In addition to the "orthograde" signal that triggers gating of RYR1, a "retrograde" signal from RYR1 to the DHPR was revealed by the observation that L-type currents of dyspedic (RYR1-null) myotubes were substantially smaller than L-type currents of wild-type (WT) myotubes, despite similar surface membrane expression of the L-type channel (Nakai et al., 1996).

Malignant hyperthermia (MH) is a fulminant pharmacogenetic life-threatening syndrome, which occurs when susceptible individuals are exposed to triggering factors, which include halogenated inhalation anesthetics and/or depolarizing muscle relaxants like succinylcholine (López et al., 1985, 1988; Nelson, 2001, 2002; Treves et al., 2005). The syndrome is associated with massive increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in response to exposure to the triggering agent (López et al., 1988). ECC dysfunction is believed to be the underlying cause of MH susceptibility because of its linkage in humans to more than 122 mutations within 19q13, the gene that codes for RYR1 (Robinson et al., 2006), or two characterized mutations within 1q31-32, the gene that codes $Ca_V1.1$ (Monnier et al., 1997; Jurkat-Rott et al., 2000). MH-susceptible (MHS) pigs and mice possessing a missense mutation (R614C [pigs] and R163C or Y522S [knock-in mice]) in

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Abbreviations used in this paper: DHPR, 1,4-dihydropyridine receptor; ECC, excitation–contraction coupling; Het, heterozygous; Hom, homozygous; MH, malignant hyperthermia; MHS, MH-susceptible WT, wild-type.

The Rockefeller University Press $30.00
J. Gen. Physiol. Vol. 135 No. 6 619–628
www.jgp.org/cgi/doi/10.1085/jgp.200910328

RYR1 develop fulminant episodes when exposed to triggering agents and heat stress (López et al., 1988; Chelu et al., 2006; Yang et al., 2006). In addition, there is evidence that the R163C mutation not only influences RYR1 channel properties (Yang et al., 2003, 2006), but also potentiates depolarization-induced Ca^{2+} entry (Cherednichenko et al., 2004, 2008; Yang et al., 2007b). However, exactly how MHS mutations potentiate depolarization-induced Ca^{2+} entry and modify important aspects of orthograde and retrograde signaling between DHPR and RYR1 is poorly understood.

In the present study, we have examined how an MH mutation in RYR1 (R163C) affects the voltage dependence, time course, and extracellular Ca^{2+} dependence of the Ca^{2+} transient during prolonged depolarization induced by high extracellular K^+. In doing so, we demonstrate that the R163C mutation alters retrograde signaling between RYR1 and the DHPR, as manifested by delayed inactivation of the DHPR voltage sensor, and at this level of depolarization enhances sarcolemmal Ca^{2+} entry.

MATERIALS AND METHODS

Generation of primary myotubes

Primary myoblast cell lines were generated from the hind limb and forelimb muscles of neonatal heterozygous (Het) and homozygous (Hom) F2 R163C C57Bl6/129svJ mice and their WT littermates (Rando and Blau, 1994; Yang et al., 2006). Myoblasts were differentiated into myotubes by withdrawal of growth factors as described previously (Yang et al., 2003). Animals were housed, fed, and sacrificed according to the standards set by the Harvard Medical School Institutional Animal Care and Use Committee.

Ca^{2+} imaging

Differentiated WT and R163C myotubes were loaded with 5 µM Fluo-4-AM (Invitrogen) at 37°C for 30 min in mammalian Ringer's solution as described previously (Yang et al., 2006). The myotubes were then washed several times with mammalian Ringer's solution and transferred to a microscope (Nikon). Fluorescence emission was measured at 516 nm with a long-pass filter using a 40×, 1.3 NA objective lens. Data were collected with a 12-bit digital-intensified CCD at 23 fps (Mega12; Stanford Photonics) from regions consisting of 8–12 individual cells per well and analyzed using QED software (QED Imaging). The magnitude of integrated Ca^{2+} transient (defined by the average area under the Ca^{2+} transient during a 3-min depolarization elicited by elevating $[K^+]$ in the extracellular medium) was compared among genotypes.

Intracellular recording

Single-barrel capillary tubing (1.5 mm OD; TW150F-4; WPI) containing an internal filament was used in the construction of microelectrodes for measuring the resting membrane potential. The pipettes were backfilled with 3 M KCl (resistance of ~15 MΩ) and used to impale individual WT and R163C myotubes to measure membrane potential (at room temperature) before and during the time that myotubes were incubated in 60 mM K^+ for 180 s. Results were discarded if tip resistance was altered by cell membrane penetration or from myotubes with resting potential values less negative than −60 mV.

Experimental protocols for Ca^{2+} transients

WT, R163C Het, or Hom R163C myotubes were exposed to high external potassium ($[K^+]_e$) solutions in ascending or descending concentrations (10, 20, 40, and 60 mM KCl) for 180 s for each concentration separated by 180 s of rest. The concentration of 60 mM K^+ was chosen for the remaining experiments because we have found that this concentration of K^+ elicits a maximum Ca^{2+} response in skeletal muscle myotubes (Yang et al., 2007a). WT and Hom R163C myotubes were exposed to 20 mM caffeine for 10 s, and 180 s later to 60 mM $[K^+]_e$ for 180 s. During the 180-s period that the cell was depolarized, a second 10-s application of 20 mM caffeine was applied at intervals ranging from 20 to 200 s after the beginning of the 60-mM $[K^+]_e$ application. WT and R163C myotubes were exposed to 60 mM $[K^+]_e$ in the presence of 1.8×10^{-3} M Ca^{2+} (normal Ca^{2+}) or 8.7×10^{-6} M Ca^{2+}/5 mM Mg^{2+} (low Ca^{2+}) solutions. The exposure to the low Ca^{2+} solution was done for 10 s before depolarization with 60 mM $[K^+]_e$. WT and Hom R163C myotubes were exposed either to 10 µM nifedipine or 20 µM SKF-96365 for 10 s before depolarization with 60 mM $[K^+]_e$. This time was chosen to minimize any effect on the amplitude of the Ca^{2+} transient that we observed with longer incubation times. WT and R163C Het and Hom myotubes were exposed to 60 mM $[K^+]_e$ first with normal Ca^{2+} solution, allowed to recover for 10 min, then with low Ca^{2+} solution, allowed to recover for 10 min, and then finally with low Ca^{2+} solution supplemented with 2 mM $[Ni^{2+}]_e$; the responses were compared. WT and R163C Het and Hom myotubes were exposed to 60 mM $[K^+]_e$ for 10 s, and then the Ca^{2+} transient was interrupted by replacing the high $[K^+]_e$ solution with normal mammalian Ringer's solution, followed by a second application of 60 mM $[K^+]_e$ 3, 5, or 10 s later.

Manganese quench

Differentiated primary WT or R163C Hom myotubes were loaded with 5 mM Fura-2 AM (Invitrogen) for 30 min at 37°C and washed four times for 10 min, each time in imaging buffer. After a baseline was established, the external solution was switched to a Ca^{2+}-free buffer containing a final concentration of 500 µM Mn^{2+} (in the presence of 5 mM external Mg^{2+}) with or without the addition of 2 mM Ni^{2+}, and then stimulated with 60 mM KCl. The cells were continuously imaged before and after KCl stimulation at an excitation wavelength of 360 ± 3 nm (the isosbestic point of Fura-2), and fluorescence intensity was monitored with a 510-nm long-pass filter through a 40× quartz objective using an intensified CCD camera (see above). Data were collected from 3–10 individual cells simultaneously at 15 frames per second. The upward deflection that is seen in all raw traces shown indicates the application of KCl and is an artifact caused by intracellular Ca^{2+} release because it is not possible to be precisely at the isosbestic point of Fura-2 using any filter system.

Solutions

The composition of standard Ringer's solution was (in mM): 125 NaCl, 5 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 5 glucose, and 25 HEPES, pH 7.4. Pilot experiments were conducted with Het and Hom R163C and WT myotubes exposed to $[K^+]_e$ solutions in which the $[K^+] \times [Cl^-]$ was either maintained constant or not maintained constant. No significant difference was observed in the amplitude and kinetics of the Ca^{2+} transients elicited by the high K^+ solutions under either condition; therefore, we did not maintain the $[K^+] \times [Cl^-]$ in the solutions used experimentally (Hodgkin and Horowicz, 1959). The NaCl concentrations were adjusted to maintain a total ionic strength $[Na^+] + [K^+]$ constant at 130 mM. Caffeine was prepared by adding the desired amount directly into the Ringer's solution or the corresponding high $[K^+]_e$ solutions.

Statistical analysis

All values are expressed as mean ± SEM, with the numbers in parentheses indicating the number of myotubes tested. Statistical analysis was performed using paired *t* test, unpaired *t* test, or one-way analysis of variance coupled with Tukey's *t* test for multiple measurements to determine significance ($P < 0.05$). Rates of decay were calculated using the formula $y(t) = ae^{-kt}$.

RESULTS

The MH-causing R163C RyR1 mutation slows the decay of Ca^{2+} transients during prolonged depolarization

Fig. 1 shows representative Ca^{2+} transients elicited from WT and R163C Het and Hom myotubes by a random order titration of $[K^+]_e$ (10, 20, 40, and 60 mM). The K^+-evoked Ca^{2+} transients from the myotubes of all three genotypes had a magnitude and time course that was dependent on $[K^+]_e$. The threshold $[K^+]_e$ needed to elicit Ca^{2+} transients was 10 mM for Het and Hom R163C myotubes, whereas it was 20 mM in WT cells ($P < 0.05$, Tukey's multiple tests; Fig. 1). Independent of the $[K^+]_e$ concentration, the integrated magnitude of the Ca^{2+} transients showed the rank order as Hom >> Het > WT. WT and R163C myotubes respond to prolonged $[K^+]_e$ exposures with an initial brief Ca^{2+} peak followed by a spontaneous exponential decay. This spontaneous decay was seen in all myotubes tested, despite the fact that depolarization of the plasma membrane was maintained throughout the exposure. At the maximum $[K^+]_e$ concentration tested (60 mM), the average integrals of the Ca^{2+} transients were 1.8 and 3.6 times greater in Het and Hom R163C myotubes in relation to WT myotubes, and the peak rates of Ca^{2+} transient decay were $0.107 \pm 0.004\ s^{-1}$ ($n = 11$), $0.068 \pm 0.004\ s^{-1}$ ($n = 13$), and $0.037 \pm 0.003\ s^{-1}$ ($n = 17$) in WT and R163C Het and Hom myotubes, respectively ($P < 0.001$).

To make certain that membrane depolarization evoked by 60 mM $[K^+]_e$ was maintained at similar potentials among the myotubes used in the study, the membrane potential was recorded from each genotype before and during the 180-s depolarization. There was no significant difference ($P > 0.05$, nonpaired *t* test) in resting membrane potentials or potentials obtained during the 60-mM $[K^+]_e$ challenge, and the level of depolarization was sustained for the duration of the $[K^+]_e$ test, regardless of the myotube's genotype (Table I).

Decay of the Ca^{2+} transient during prolonged depolarization is not due to SR Ca^{2+} depletion or RYR1 inactivation

To test the hypothesis that the spontaneous decay of the K^+-evoked Ca^{2+} transients was the result of SR Ca^{2+} depletion or inactivation of RYR1 by high myoplasmic Ca^{2+} concentrations, we elicited SR Ca^{2+} release with a 10-s application of 20 mM caffeine before, during, and after exposure to 60 mM $[K^+]_e$. Fig. 2 shows the response to caffeine at different intervals (20, 40, 80, 120, 160, and 200 s) after initiating a 180-s challenge to 60 mM $[K^+]_e$. At each interval, caffeine produced a robust release of Ca^{2+} whose amplitude was not significantly different ($P > 0.1$) than that measured before the $[K^+]_e$ challenge, regardless of genotype. Thus, decay of the $[K^+]_e$-triggered Ca^{2+} transient in WT and Hom R163C myotubes does not appear to be related to either depletion of SR Ca^{2+} stores (Hodgkin and Horowicz, 1960b) or inactivation of RYR1 (Smith et al., 1985; Suarez-Isla et al., 1986; Simon et al., 1991; Jong et al., 1995).

Removal of external Ca^{2+} accelerates Ca^{2+} transient decay

It is well established that a relatively short exposure to a low $[Ca^{2+}]_e$ medium has no effect on the peak K^+

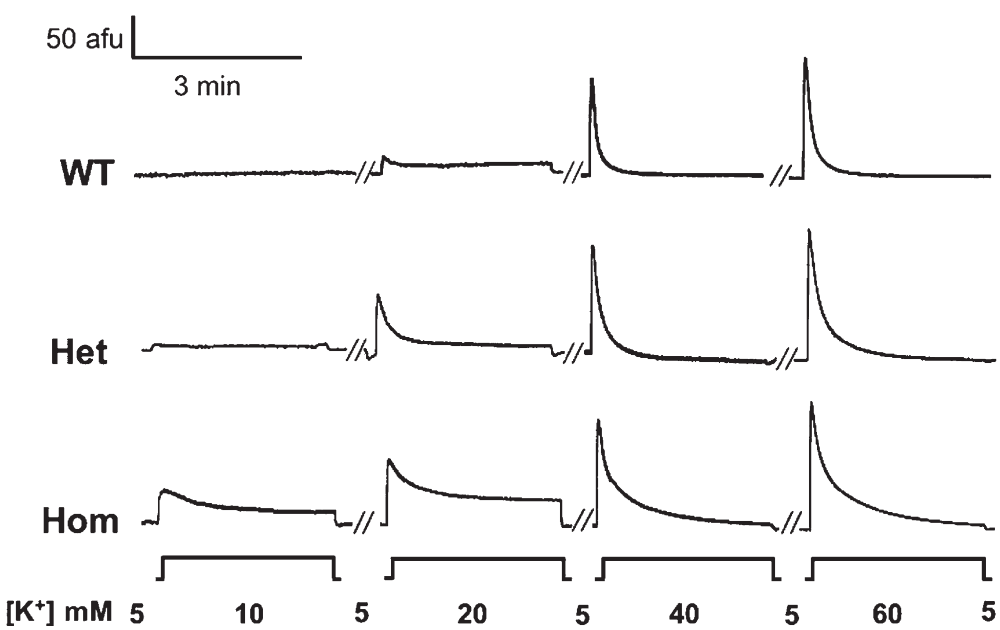

Figure 1. K^+ dose response for WT and R163C Het and Hom myotubes. Representative fluorescence imaging records of WT and R163C (Het and Hom) myotubes loaded with Fluo-4 AM exposed to 10, 20, 40, and 60 mM $[K^+]_e$ for 180 s to observe the entire time course of the Ca^{2+} transients. The peak of the Ca^{2+} transients increased and the duration shortened as $[K^+]_e$ was elevated from 10 to 60 mM in all three genotypes. Afu, arbitrary fluorescence units.

TABLE I

Membrane potentials of WT and R163C Het and Hom mouse myotubes at rest and during depolarization with 60 mM $[K^+]_e$

Treatment and genotype	Membrane potential	*n*
	mV	
Normal Ringer's solution		
WT	−63.6 ± 0.39	13
Het R163C	−63.5 ± 0.42	10
Hom R163C	−63.8 ± 0.5	10
60 mM KCl		
WT	−13.8 ± 1.4	13
Het R163C	−14.6 ± 1.3	13
Hom R163C	−12.6 ± 1.2	11

Values are expressed as mean ± SE.

contracture tension; however, it greatly shortens the contracture time course, reducing the duration of the plateau and increasing the relaxation rate (Bianchi and Shanes, 1959; Frank, 1960; Caputo and Gimenez, 1967). Recently, the L-type current has been identified as the major means of Ca^{2+} entry during prolonged depolarizations or repetitive activity (Bannister and Beam, 2009; Bannister et al., 2009). Such Ca^{2+} entry is enhanced in both Het and Hom R163C myotubes in response to $[K^+]_e$ depolarization and electrical pulse trains (Yang et al., 2007a; Cherednichenko et al., 2008). Here, we have examined the impact of the R163C mutation on external Ca^{2+} effects in more detail. Exposure of WT and Het and Hom R163C myotubes to low Ca^{2+} solution (8.7×10^{-6} M/5 mM Mg^{2+}) did not modify initial Ca^{2+} peak, but it dramatically altered the time course of the Ca^{2+} transients, making their duration shorter and their rate of decay faster in all three genotypes (Fig. 3). The integral of the Ca^{2+} transient was reduced by 62 ± 5.8% (n = 9) in WT, 74 ± 7% (n = 14) in Het R163C, and by 80 ± 9.3% (n = 12) in Hom R163C in low $[Ca^{2+}]_e$ solution, and the rate of Ca^{2+} decay was increased from 0.107 ± 0.004 s^{-1} (n = 11) to 0.233 ± 0.006 s^{-1} (n = 9), from 0.068 ± 0.004 s^{-1} (n = 13) to 0.144 ± 0.004 s^{-1} (n = 14), and from 0.037 ± 0.003 s^{-1} (n = 17) to 0.137 ± 0.005 s^{-1} (n = 12) in WT, Het, and Hom, respectively. The changes in the transient integral and rate of decay were fully reversible in a third depolarization for all genotypes, when $[Ca^{2+}]_e$ was restored in the bath solution (unpublished data).

Effect of Ca^{2+} entry blockers on K^+-induced Ca^{2+} transients

Because removal of external Ca^{2+} would be expected not only to eliminate Ca^{2+} entry, but also to accelerate inactivation of the voltage sensor for ECC (Rios and Brum, 1987), we also examined the effect of two organic Ca^{2+} entry blockers, nifedipine and SKF-96365 (Almers and Palade, 1981; Rivet et al., 1989; Leung and Kwan, 1999; Araya et al., 2003; Bannister et al., 2009), in the presence of Ca^{2+} replete external solutions.

Exposure to 10 µM nifedipine in the presence of normal external Ca^{2+} concentrations did not affect Ca^{2+} transient amplitude, but it accelerated the mean rate of decay from 0.107 ± 0.004 s^{-1} (n = 11) to 0.135 ± 0.005 s^{-1} (n = 11) in WT (~30%), and from 0.037 ± 0.003 s^{-1} (n = 17) to 0.117 ± 0.002 s^{-1} (n = 16) in Hom R163C (~70%) myotubes ($P < 0.001$; Fig. 4 A). Exposure of WT and Hom R163C myotubes to 20 µM SKF-96365 did not lower Ca^{2+} transient amplitude, but it accelerated the mean rate of decay from 0.107 ± 0.004 s^{-1} (n = 11) to 0.146 ± 0.005 s^{-1} (n = 9) in WT myotubes (~27%), and from 0.037 ± 0.003 s^{-1} (n = 17) to 0.072 ± 0.004 s^{-1} (n = 13) in Hom R163C (~49%) myotubes ($P < 0.001$; Fig. 4 B). To test whether the effects of nifedipine or SKF96365 on the decay rate of the Ca^{2+} transients could be explained

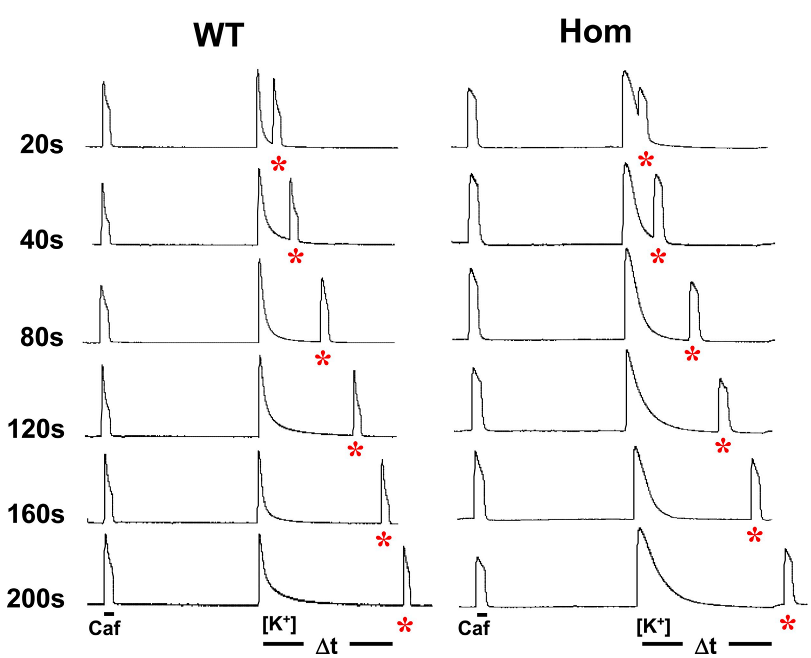

Figure 2. Decay of the Ca^{2+} transient is not due to store depletion or to RYR1 inactivation. Representative fluorescence records of WT and Hom R163C myotubes loaded with Fluo-4 AM in response to a 10-s exposure to 20 mM caffeine (Caf) and a 180-s exposure to 60 mM $[K^+]_e$, followed by a second 10-s application of 20 mM caffeine (*) applied at different intervals (20–200 s) after the Ca^{2+} transient was elicited by 60 mM $[K^+]_e$. The decay of the Ca^{2+} transient during K^+ depolarization does not appear to be related to SR Ca^{2+} depletion, nor is it due to RYR1 inactivation based on the amplitude of the second caffeine response (*).

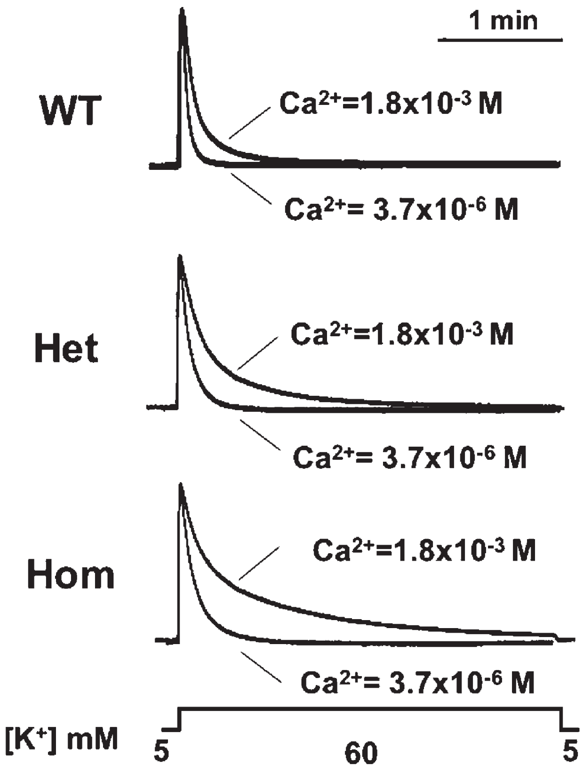

Figure 3. Low $[Ca^{2+}]_e$ increases the rate of decay of the Ca^{2+} transient in response to 60 mM $[K^+]_e$. The average transients obtained from WT and R163C Het and Hom myotubes exposed to 60 mM $[K^+]_e$ for 3 min in normal $[Ca^{2+}]_e$ and in low $[Ca^{2+}]_e$ solution (no added Ca^{2+} and no EGTA). Data were normalized by making the peak transient of each response equal to 1.

by their ability to partially depolarize the myotubes' membrane at rest, or affect a change in the magnitude of the depolarization induced by 60 mM $[K^+]_e$, membrane potentials were measured directly under these experimental conditions. Neither nifedipine nor SKF-96365 modified the resting membrane potential or the ability of 60 mM $[K^+]_e$ to depolarize the myotubes to the expected membrane potential (unpublished data).

The reduced rate of ECC voltage sensor inactivation that results from the R163C mutation is apparent when Ni^{2+} is substituted for Ca^{2+}

Although both nifedipine and SKF-96365 accelerated Ca^{2+} transient decay, the decay rates remained different between WT and Hom myotubes, providing further support for the hypothesis that the R163C mutation affects the rate at which the DHPR transitions into the ECC-inactivated state. To further test this hypothesis, we examined the effect of replacement of external Ca^{2+} by Ni^{2+}. Previous investigations in amphibian muscle have shown that when Ni^{2+} is used to replace Ca^{2+} in the extracellular medium, the normal time course of K^+ contractures is maintained (Caputo, 1981; Lorković and Rüdel, 1983), despite the fact that Ni^{2+} blocks Ca^{2+} current conducted by amphibian skeletal L-type channels (Almers and Palade, 1981; Obejero-Paz et al., 2008). Fig. 5 shows superimposed averaged records from experiments in which WT and R163C Het and Hom myotubes were exposed to 60 mM $[K^+]_e$ under three different experimental conditions: in the presence of (1) 1.8×10^{-3} M Ca^{2+}, (2) 8.7×10^{-6} M Ca^{2+}, and (3) 8.7×10^{-6} M Ca^{2+} supplemented with 2×10^{-3} M Ni^{2+} (see Materials and methods for details). As shown above, the removal of Ca^{2+} significantly accelerated the rate of decay compared with the response in normal Ca^{2+} solutions. Importantly, the addition of Ni^{2+} to low $[Ca^{2+}]_e$ solutions also resulted in prolongation of the K^+-evoked Ca^{2+} transients in WT, Het, and Hom myotubes, although it did not restore the rate of decay to control levels. Under all three conditions, the decay rate was significantly slower in Hom and Het myotubes compared with WT. If the decay rates of the Ca^{2+} transients in the presence of Ni^{2+} are taken to indicate the normal course of SR Ca^{2+} release without any influence from Ca^{2+} entry, the fraction of the total Ca^{2+} release that is presumably due to the inactivation of the DHPR caused by the lack of extracellular Ca^{2+} was 35.7% of the area under the curve in WT, 44.1% in Het R163C, and 64.3% in Hom R163C myotubes. These results

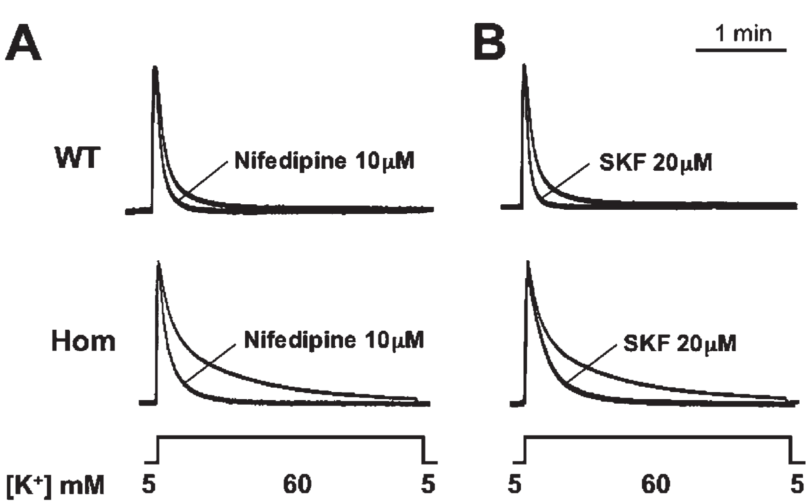

Figure 4. The effect of nifedipine and SKF-96365 on the decay of the K^+-induced Ca^{2+} transients. The average normalized traces (n = 8–10) of Ca^{2+} transients from WT and Hom R163C myotubes in response to 60 mM $[K^+]_e$ for 180 s before and after exposure to 10 µM nifedipine (A) or 20 µM SKF96365 (B). The cells were exposed to nifedipine or SKF96365 for 10 s before and throughout the K^+ depolarization.

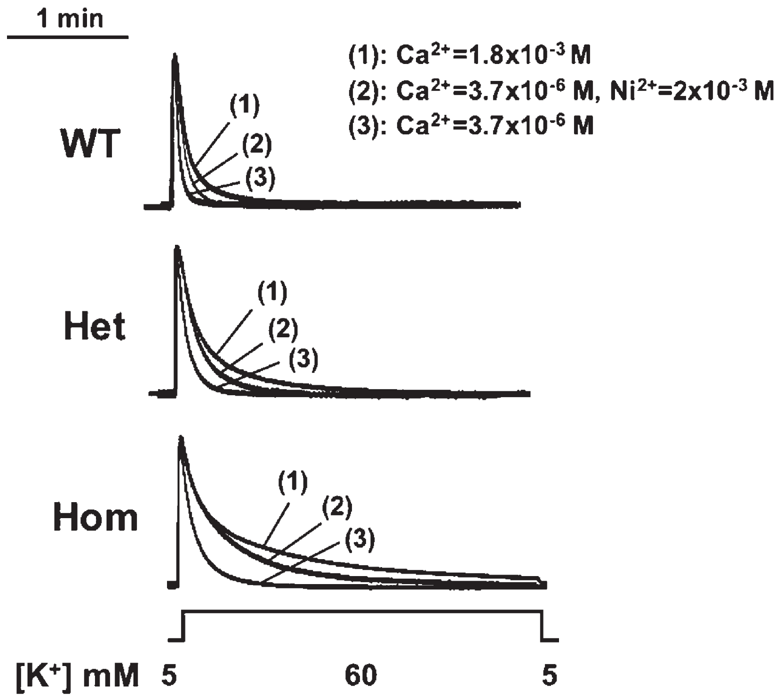

Figure 5. Ni^{2+} partially reverses the effect of low $[Ca^{2+}]_e$ on the Ca^{2+} transient. The average normalized Ca^{2+} transients from WT ($n = 14$), Het ($n = 12$), and Hom ($n = 18$) R163C myotubes, which were depolarized for 180 s by 60 mM $[K^+]_e$ in the presence of 1.8×10^{-3} M Ca^{2+}, followed by a 5-min rest in normal Ringer's solution, depolarized for 180 s again in low Ca^{2+} solution (3.7×10^{-6} M), followed by a second 5-min rest, and then depolarized for a third time in low Ca^{2+} solution supplemented with 2×10^{-3} M Ni^{2+} (see Table II for the rates of Ca^{2+} transient decay for each condition).

suggest that the shortening of the Ca^{2+} transient in low Ca^{2+} solution was a combined consequence of accelerated inactivation of the DHPR voltage sensor and decreased depolarization-induced Ca^{2+} entry (see Table II for decay rates). Furthermore, these data show that the lack of extracellular Ca^{2+} affects the inactivation of the DHPR ECC voltage sensor in the Het and Hom R163C myotubes to a much greater extent than in WT myotubes.

Cation entry is enhanced by R163C and can be blocked by Ni^{2+}

We directly measured the differences in cation entry in response to membrane depolarization using the Mn^{2+} quench technique in Hom R163C and WT myotubes. Fig. 6 A shows representative Fura-2 emission traces from Hom R163C and WT myotubes before and after stimulation with 60 mM KCl in the presence and absence of Ni^{2+}. As summarized in Fig. 6 B, these data show the average rate of Mn^{2+} quench measured during the linear phase of quench after KCl exposure was significantly greater in Hom R163C myotubes than in WT myotubes ($P < 0.01$). Furthermore, cation entry was almost completely abolished in both groups of myotubes by the addition of 5 mM Ni^{2+}.

TABLE II

Effect of Ni^{2+} on the rate of Ca^{2+} transient decay (s^{-1})

Genotype	Low Ca^{2+}	n	Low Ca^{2+} and Ni^{2+}	n
WT	0.223 ± 0.006	10	0.139 ± 0.004	10
Het R163C	0.144 ± 0.004	13	0.085 ± 0.003	13
Hom R163C	0.137 ± 0.004	14	0.049 ± 0.002	13

Double K^+ pulse protocol to assess DHPR voltage sensor inactivation

Depolarization converts resting DHPR voltage sensors into their active state, after which they spontaneously change conformation and transition into their inactive state. The amount of SR Ca^{2+} release in response to depolarization is proportional to the degree that the voltage sensors are in their active state (Dulhunty and Gage, 1988; Dulhunty, 1992), when the T-tubule is depolarized. WT and R163C Het and Hom myotubes were initially exposed to 60 mM $[K^+]_e$ for 10 s (Fig. 7, Int_1), and then returned to normal mammalian Ringer's solution for 3, 5, or 10 s (Fig. 7, Δt), and then reexposed to 60 mM $[K^+]_e$ for 10 s (Fig. 7, Int_2). We observed that washout of the elevated K^+ solution with normal Ringer's solution immediately stopped the Ca^{2+} transient in all three genotypes, an effect that can be attributed to the repolarization of the plasma membrane. However, the amplitude of the Ca^{2+} transient triggered by reexposure to high $[K^+]_e$ was highly genotype dependent (Fig. 7, Int_2). The integral of the Ca^{2+} transient during Int_2 in Hom R163C myotubes was not significantly different from the integral during Int_1 for all interpulse intervals (Fig. 7; $P > 0.05$, Tukey's multiple tests). In Het R163C myotubes, Int_2 was significantly smaller than Int_1 at 3 s, but not significantly different from Int_1 at 5 and 10 s (Fig. 7). In WT myotubes, on the other hand, Int_2 was significantly lower than Int_1 at all three intervals tested (i.e., 3, 5, and 10 s; $P < 0.001$). These results indicate that the DHPR voltage sensor inactivates more slowly in MHS myotubes than in WT.

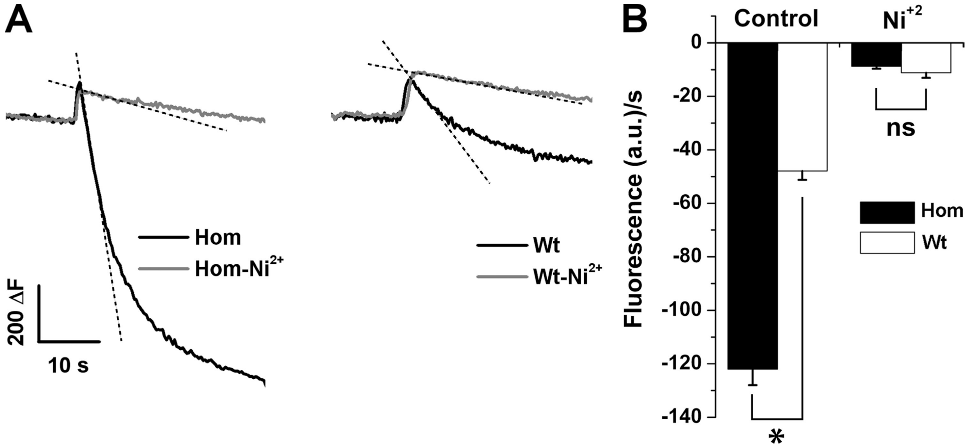

Figure 6. Mn^{2+} quench in WT and Hom R163C myotubes in the absence and presence of 2 mM Ni^{2+}. (A) Representative Fura-2 emission traces from WT and R163C Hom myotubes before and after stimulation with 60 mM KCl in the presence and absence of Ni^{2+}. (B) The average rates ± SEM of Mn^{2+} quench during the linear phase of quench after KCl exposure with (left) and without (right) the addition of Ni^{2+} to the bath solution. *, P < 0.01.

DISCUSSION

There is general agreement that membrane depolarization elicited by high $[K^+]_e$ triggers intracellular Ca^{2+} transients in amphibian (Blinks et al., 1978; López et al., 1983; Snowdowne, 1985; Caputo and Bolaños, 1994) and mammalian skeletal muscle (López et al., 2005). Exposure of WT and R163C myotubes to prolonged high $[K^+]_e$ triggered Ca^{2+} transients characterized by a peak Ca^{2+} transient that varied with $[K^+]_e$ and subsided over time, despite the fact that the membrane remained depolarized.

As has been shown previously, the duration and the peak amplitude of a K^+ contracture (Hodgkin and Horowicz, 1960b) or the corresponding Ca^{2+} transient (Caputo and Bolaños, 1994) depend on the $[K^+]_e$ and hence on membrane potential. The results of the present study confirm these essential features in all three genotypes studied (Fig. 1). Thus, the peak amplitude of the Ca^{2+} transient was smaller and the duration of the Ca^{2+} transient was greatly prolonged at low $[K^+]_e$. Although the peak amplitude of the Ca^{2+} transient was enhanced with increasing $[K^+]_e$, the duration of the transient decreases. The inverse relationship between $[K^+]_e$ and transient duration is related to the rate of inactivation of the voltage sensor of the DHPR (Caputo and Bolaños, 1994).

In agreement with previous work on dyspedic myotubes expressing RYR1 with any of seven MH-causing mutations (Yang et al., 2003, 2007a), we found in the present work that elevated K^+ produced Ca^{2+} transients in primary R163C myotubes, which activated at lower membrane potentials and decayed more slowly than in WT myotubes. Thus, these alterations of Ca^{2+} handling may be a common consequence of many MH mutations of RYR1. These observations are in agreement with those reported by Gallant and co-workers (Gallant et al., 1982; Gallant and Lentz, 1992), in which the mechanical

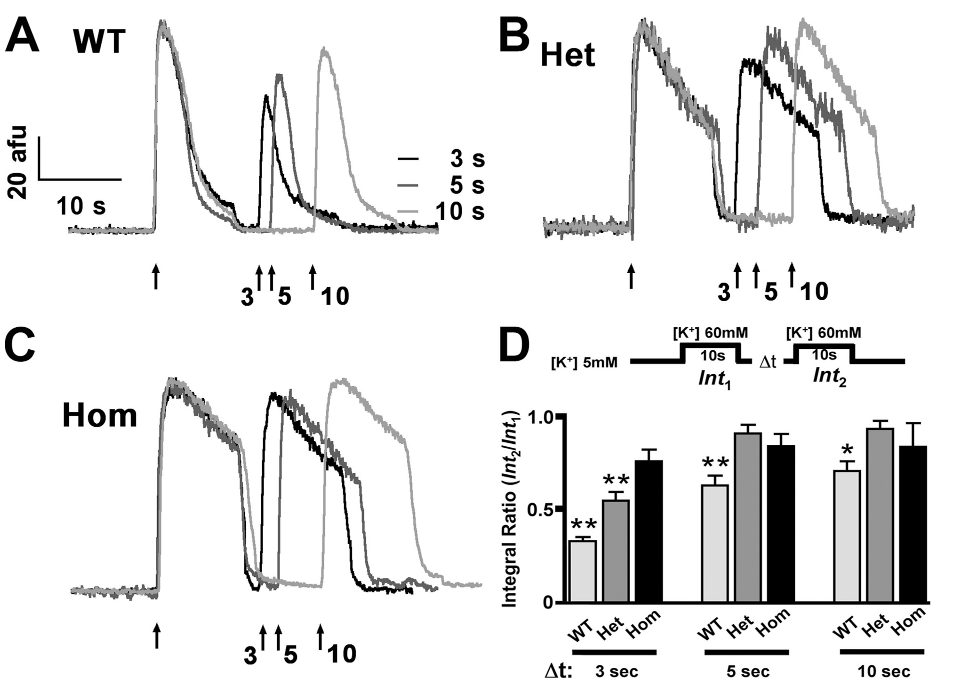

Figure 7. Inactivation of the DHPR voltage sensor is genotype dependent. Representative Ca^{2+} transients for WT (A), Het (B), and Hom (C) myotubes induced by the following protocol: 60 mM $[K^+]_e$ (Int_1) for 10 s; 2.5 mM $[K^+]_e$ for 3, 5, or 10 s; 60 mM $[K^+]_e$ (Int_2). (D) The integrals of the second Ca^{2+} transient were expressed as a percentage of the first fluorescence signal (**, P < 0.01; Tukey's test for multiple comparisons). The amplitude of the second Ca^{2+} transient was used as an index of DHPR inactivation at the time the second stimulus was applied.

threshold for $[K^+]_e$ was lower in MHS than in WT pig skeletal muscle fibers.

Interestingly, the rate of decay of the Ca^{2+} transient elicited by $[K^+]_e$ was slower in Hom and Het R163C compared with WT myotubes. Our data show that the decay in the peak of the Ca^{2+} transient during membrane depolarization observed in WT and R163C myotubes is related neither to SR Ca^{2+} depletion (Hodgkin and Horowicz, 1960b) nor to inactivation of RYR1 due to the high Ca^{2+} present in the myoplasm (Smith et al., 1985; Suarez-Isla et al., 1986; Simon et al., 1991; Jong et al., 1995) because the application of 20 mM caffeine was able to induce a robust Ca^{2+} release in all genotypes at all time points during the decay phase of the Ca^{2+} transient during the prolonged K^+ depolarization (Fig. 2). The most probable explanation for this observation is that an alteration in the conformation of the RYR1 tetramer caused by even one MHS allele affects both orthograde and retrograde coupling between the DHPR and RYR1 (Nakai et al., 1996, 1998). Specifically, our data demonstrate at least two retrograde effects of the R163C mutation on DHPRs that alter the properties of ECC. The first is that the threshold for activation of Ca^{2+} release induced by $[K^+]_e$ is lowered by the RYR1 MH mutation (this change is confirmed electrophysiologically in the companion paper [see Bannister et al. in this issue]). Second, the R163C RyR1 mutation slows the rate of inactivation of the DHPR voltage sensor for ECC (Figs. 1 and 7). Additionally, our data suggest that the R163C mutation increases Ca^{2+} entry during prolonged depolarization.

Previous reports have shown that skeletal muscle fibers can contract after electrical stimulation or under whole cell voltage clamp with short step depolarizations in low extracellular Ca^{2+} concentrations (Armstrong et al., 1972; Bolaños et al., 1986). However, the importance of extracellular Ca^{2+} both in the presence (Lüttgau and Spiecker, 1979; Caputo, 1981; Cota and Stefani, 1981) and absence of Ca^{2+} buffers (Caputo and Gimenez, 1967; Lüttgau and Spiecker, 1979) for maintenance of muscle K^+ contractures has also been well demonstrated. Our data provide evidence that external Ca^{2+} plays a significant physiological role for the maintenance of Ca^{2+} transients induced by elevated $[K^+]_e$ in WT and R163C MHS myotubes (Fig. 3). In fact, the duration of the Ca^{2+} transient was shortened (increased rate of decay of peak Ca^{2+}) in nominally Ca^{2+}-free external solution or in the presence of Ca^{2+} entry blockers (Figs. 3 and 4) regardless of genotype. We have ruled out the possibility that the increased rate of decay in the presence of low $[Ca^{2+}]_e$ might be a consequence of reduced SR Ca^{2+} content resulting from prolonged membrane depolarization (Hodgkin and Horowicz, 1960a,b; Caputo and Gimenez, 1967), as the addition of 5 mM Mg^{2+} to the external solutions prevents this depolarization (unpublished data; see Materials and methods). Furthermore, if resting SR Ca^{2+} content was reduced by the lower $[Ca^{2+}]_e$, the peak amplitude (early phase) of the Ca^{2+} transient during long depolarization would have also been reduced. Because a reduction in peak transient amplitude was not observed in our experiments on intact myotubes, it appears that SR Ca^{2+} stores were not significantly reduced by exposure to lower $[Ca^{2+}]_e$. This also argues against the alternative explanation that the slower rate decay in R163C compared with WT could have been due to changes in electrical properties of the myotubes during high $[K^+]_e$ exposure, as was reported in amphibian muscle fibers (Stefani and Chiarandini, 1973). If this were the case, the peak transient amplitude would also be reduced (Fig. 1). The most likely explanation is that the main alteration caused by low $[Ca^{2+}]_e$ is an inactivation of the voltage sensor and a reduction in SR Ca^{2+} release with no change in the rate of Ca^{2+} removal from the myoplasm (Brum et al., 1988). The suggested mechanism for this is the existence of a high affinity Ca^{2+} binding site or sites on the external side of the DHPR (Ebata et al., 1990), which modulate its inactivation process (Rios and Brum, 1987). In addition, the removal of external Ca^{2+} compromises the Ca^{2+} entry, which sustains the time course of the Ca^{2+} transients in WT and R163C myotubes, induced by $[K^+]_e$ depolarization and long trains of electrical stimuli (Cherednichenko et al., 2004, 2008). This combined explanation is directly supported by our result that when Ni^{2+} was added to the low Ca^{2+} solution, the rate of decay of the Ca^{2+} transient was significantly retarded but not completely restored (Fig. 5). It is important to point out that the sarcolemma is not permeable to Ni^{2+}, and when it is present it blocks the inward Ca^{2+} current during depolarization of amphibian skeletal muscle fibers (Palade and Almers, 1985). In this regard, we have found that 2 mM Ni^{2+} blocks the L-type Ca^{2+} current in both WT and R163C myotubes (unpublished data) and divalent cation entry during depolarization (Fig. 6). Interestingly, we found the contribution of extracellular Ca^{2+} to be more substantial in both Hom and Het R163C myotubes than in WT.

Exposure of WT and R163C myotubes to the L-type Ca^{2+} channel antagonist nifedipine or the nonspecific cation channel blocker SKF-96365 also accelerated the rate of decline of Ca^{2+} transients (Fig. 4) in the presence of extracellular Ca^{2+}. The fact that we did not observe a significant reduction in the peak Ca^{2+} transient amplitude in either WT or R163C myotubes shows that peak Ca^{2+} transient amplitude is largely controlled by SR Ca^{2+} release, and the brief exposure to both drugs prevented any secondary effects of these drugs. Although both drugs are known to block Ca^{2+} entry via the DHPR (Bannister et al., 2009), nifedipine caused a more rapid decline in the Ca^{2+} transient because it also alters the inactivation of the DHPR voltage sensor.

The general model for ECC suggests that Ca^{2+} release in skeletal muscle is controlled by a voltage sensor, which is an integral function of the DHPR that initially activates and subsequently inactivates to tightly control SR Ca^{2+} release. Furthermore, it is known that the time course of K^+-triggered contractures is determined by the onset and time course of the voltage sensor inactivation process (Caputo, 1976; Bolaños et al., 1986), which is initiated immediately after activation begins (Caputo, 1972a,b). From the experiments in which Ni^{2+} was substituted for Ca^{2+}, it was shown that depolarization-induced Ca^{2+} entry is enhanced in Het and Hom R163C, but the majority of the difference in the rate of decay of the Ca^{2+} transient in Het and Hom R163C myotubes is due to prolonged SR Ca^{2+} release caused by either a delay in the onset or a reduction in the rate of DHPR voltage sensor inactivation.

The double K^+ application protocol (Fig. 7) confirmed this idea by demonstrating that the second Ca^{2+} transient of the pair was significantly smaller in WT myotubes than in R163C myotubes with all delays. Furthermore, the rate of decay of the Ca^{2+} transient was slower in Hom than in Het R163C myotubes, suggesting that the conformation of R163C has a significant impact through retrograde signaling from RYR1 to the DHPR on the voltage sensor inactivation process, in addition to the retrograde signals that control L-type Ca^{2+} current. In the companion paper (Bannister et al., 2010), we have examined the effects of the R163C mutation in RYR1 on the biophysical properties of the skeletal muscle L-type Ca^{2+} channel.

This work was supported by a fellowship grant from the Fondation pour la Recherche Médicale (SPE20040901554 to E. Estève), the Muscular Dystrophy Association (MDA4155 to R.A. Bannister and MDA4319 to K.G. Beam), and the National Institutes of Health (NIH P01AR052534 to P.D. Allen and I.N. Pessah, and R01AR44750 to K.G. Beam).

Kenneth C. Holmes served as editor.

Submitted: 18 September 2009
Accepted: 21 April 2010

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