

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

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Bidirectional signaling between the sarcolemmal L-type  $\text{Ca}^{2+}$  channel (1,4-dihydropyridine receptor [DHPR]) and the sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  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  $\text{K}^+$  depolarization. The new findings are: (a) For all three genotypes,  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  or the addition of  $\text{Ca}^{2+}$  entry blockers (nifedipine, SKF96365, and  $\text{Ni}^{2+}$ ) enhanced the rate of decay in all genotypes. (d) When  $\text{Ca}^{2+}$  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  $\text{K}^+$  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  $\text{Ca}^{2+}$  channel or 1,4-dihydropyridine receptor (DHPR). The DHPR in turn activates  $\text{Ca}^{2+}$  release from the SR  $\text{Ca}^{2+}$  release channel (RYR type 1 [RYR1]). The communication between the two channels is rapid and does not require  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) 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  $\text{Ca}_v1.1$  (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.

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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  $\text{Ca}^{2+}$  entry (Cherednichenko et al., 2004, 2008; Yang et al., 2007b). However, exactly how MHS mutations potentiate depolarization-induced  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  dependence of the  $\text{Ca}^{2+}$  transient during prolonged depolarization induced by high extracellular  $\text{K}^+$ . 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  $\text{Ca}^{2+}$  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.

### $\text{Ca}^{2+}$ imaging

Differentiated WT and R163C myotubes were loaded with 5  $\mu\text{M}$  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 $\times$ , 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  $\text{Ca}^{2+}$  transient (defined by the average area under the  $\text{Ca}^{2+}$  transient during a 3-min depolarization elicited by elevating  $[\text{K}^+]_e$  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  $\sim 15\text{ M}\Omega$ ) 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  $\text{K}^+$  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  $-60\text{ mV}$ .

### Experimental protocols for $\text{Ca}^{2+}$ transients

WT, R163C Het, or Hom R163C myotubes were exposed to high external potassium ( $[\text{K}^+]_e$ ) 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  $\text{K}^+$  was chosen for the remaining experiments because we have found that this concentration of  $\text{K}^+$  elicits a maximum  $\text{Ca}^{2+}$  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  $[\text{K}^+]_e$  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  $[\text{K}^+]_e$  application. WT and R163C myotubes were exposed to 60 mM  $[\text{K}^+]_e$  in the presence of  $1.8 \times 10^{-5}\text{ M}$   $\text{Ca}^{2+}$  (normal  $\text{Ca}^{2+}$ ) or  $8.7 \times 10^{-6}\text{ M}$   $\text{Ca}^{2+}/5\text{ mM}$   $\text{Mg}^{2+}$  (low  $\text{Ca}^{2+}$ ) solutions. The exposure to the low  $\text{Ca}^{2+}$  solution was done for 10 s before depolarization with 60 mM  $[\text{K}^+]_e$ . WT and Hom R163C myotubes were exposed either to 10  $\mu\text{M}$  nifedipine or 20  $\mu\text{M}$  SKF-96365 for 10 s before depolarization with 60 mM  $[\text{K}^+]_e$ . This time was chosen to minimize any effect on the amplitude of the  $\text{Ca}^{2+}$  transient that we observed with longer incubation times. WT and R163C Het and Hom myotubes were exposed to 60 mM  $[\text{K}^+]_e$  first with normal  $\text{Ca}^{2+}$  solution, allowed to recover for 10 min, then with low  $\text{Ca}^{2+}$  solution, allowed to recover for 10 min, and then finally with low  $\text{Ca}^{2+}$  solution supplemented with 2 mM  $[\text{Ni}^{2+}]_e$ ; the responses were compared. WT and R163C Het and Hom myotubes were exposed to 60 mM  $[\text{K}^+]_e$  for 10 s, and then the  $\text{Ca}^{2+}$  transient was interrupted by replacing the high  $[\text{K}^+]_e$  solution with normal mammalian Ringer's solution, followed by a second application of 60 mM  $[\text{K}^+]_e$  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  $\text{Ca}^{2+}$ -free buffer containing a final concentration of 500  $\mu\text{M}$   $\text{Mn}^{2+}$  (in the presence of 5 mM external  $\text{Mg}^{2+}$ ) with or without the addition of 2 mM  $\text{Ni}^{2+}$ , and then stimulated with 60 mM KCl. The cells were continuously imaged before and after KCl stimulation at an excitation wavelength of  $360 \pm 3\text{ nm}$  (the isosbestic point of Fura-2), and fluorescence intensity was monitored with a 510-nm long-pass filter through a 40 $\times$  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  $\text{Ca}^{2+}$  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  $\text{CaCl}_2$ , 1.2  $\text{MgSO}_4$ , 5 glucose, and 25 HEPES, pH 7.4. Pilot experiments were conducted with Het and Hom R163C and WT myotubes exposed to  $[\text{K}^+]_e$  solutions in which the  $[\text{K}^+] \times [\text{Cl}^-]$  was either maintained constant or not maintained constant. No significant difference was observed in the amplitude and kinetics of the  $\text{Ca}^{2+}$  transients elicited by the high  $\text{K}^+$  solutions under either condition; therefore, we did not maintain the  $[\text{K}^+] \times [\text{Cl}^-]$  in the solutions used experimentally (Hodgkin and Horowicz, 1959). The NaCl concentrations were adjusted to maintain a total ionic strength  $[\text{Na}^+] + [\text{K}^+]$  constant at 130 mM. Caffeine was prepared by adding the desired amount directly into the Ringer's solution or the corresponding high  $[\text{K}^+]_e$  solutions.

## Statistical analysis

All values are expressed as mean  $\pm$  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 $\text{Ca}^{2+}$ transients during prolonged depolarization

Fig. 1 shows representative  $\text{Ca}^{2+}$  transients elicited from WT and R163C Het and Hom myotubes by a random order titration of  $[\text{K}^+]_e$  (10, 20, 40, and 60 mM). The  $\text{K}^+$ -evoked  $\text{Ca}^{2+}$  transients from the myotubes of all three genotypes had a magnitude and time course that was dependent on  $[\text{K}^+]_e$ . The threshold  $[\text{K}^+]_e$  needed to elicit  $\text{Ca}^{2+}$  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  $[\text{K}^+]_e$  concentration, the integrated magnitude of the  $\text{Ca}^{2+}$  transients showed the rank order as Hom  $\gg$  Het  $>$  WT. WT and R163C myotubes respond to prolonged  $[\text{K}^+]_e$  exposures with an initial brief  $\text{Ca}^{2+}$  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  $[\text{K}^+]_e$  concentration tested (60 mM), the average integrals of the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  transient decay were  $0.107 \pm 0.004 \text{ s}^{-1}$  ( $n = 11$ ),  $0.068 \pm 0.004 \text{ s}^{-1}$  ( $n = 13$ ), and  $0.037 \pm 0.003 \text{ s}^{-1}$  ( $n = 17$ ) in WT and R163C Het and Hom myotubes, respectively ( $P < 0.001$ ).

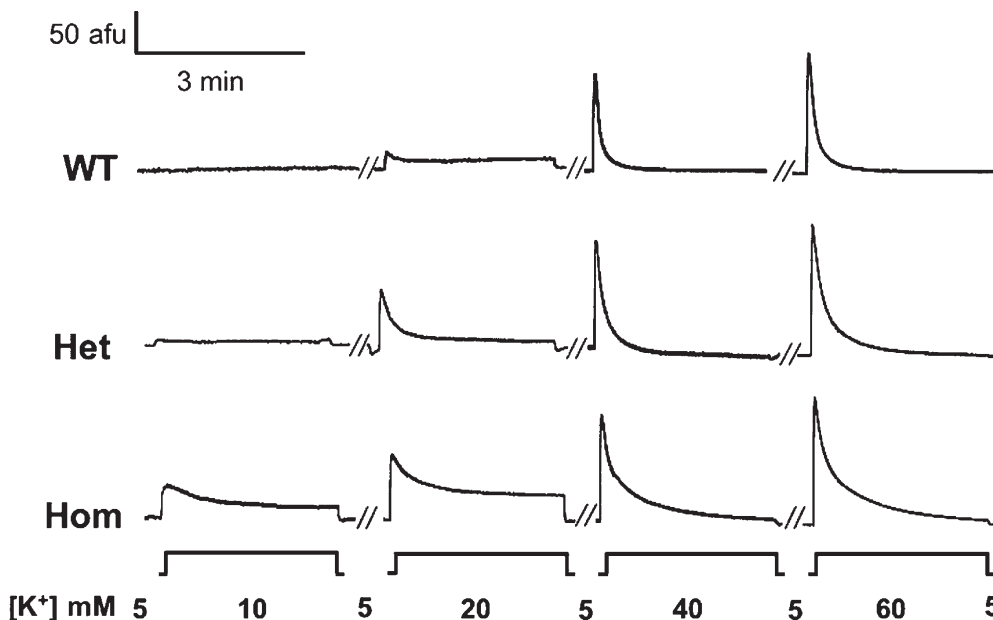
To make certain that membrane depolarization evoked by 60 mM  $[\text{K}^+]_e$  was maintained at similar potentials among the myotubes used in the study, the membrane potential was recorded from each genotype before and during the 180-s depolarization. There was no significant difference ( $P > 0.05$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

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

To test the hypothesis that the spontaneous decay of the  $\text{K}^+$ -evoked  $\text{Ca}^{2+}$  transients was the result of SR  $\text{Ca}^{2+}$  depletion or inactivation of RYR1 by high myoplasmic  $\text{Ca}^{2+}$  concentrations, we elicited SR  $\text{Ca}^{2+}$  release with a 10-s application of 20 mM caffeine before, during, and after exposure to 60 mM  $[\text{K}^+]_e$ . 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  $[\text{K}^+]_e$ . At each interval, caffeine produced a robust release of  $\text{Ca}^{2+}$  whose amplitude was not significantly different ( $P > 0.1$ ) than that measured before the  $[\text{K}^+]_e$  challenge, regardless of genotype. Thus, decay of the  $[\text{K}^+]_e$ -triggered  $\text{Ca}^{2+}$  transient in WT and Hom R163C myotubes does not appear to be related to either depletion of SR  $\text{Ca}^{2+}$  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 $\text{Ca}^{2+}$ accelerates $\text{Ca}^{2+}$ transient decay

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



**Figure 1.**  $\text{K}^+$  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  $[\text{K}^+]_e$  for 180 s to observe the entire time course of the  $\text{Ca}^{2+}$  transients. The peak of the  $\text{Ca}^{2+}$  transients increased and the duration shortened as  $[\text{K}^+]_e$  was elevated from 10 to 60 mM in all three genotypes. Afu, arbitrary fluorescence units.

TABLE 1  
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	
<b>Normal Ringer's solution</b>		
WT	$-63.6 \pm 0.39$	13
Het R163C	$-63.5 \pm 0.42$	10
Hom R163C	$-63.8 \pm 0.5$	10
<b>60 mM KCl</b>		
WT	$-13.8 \pm 1.4$	13
Het R163C	$-14.6 \pm 1.3$	13
Hom R163C	$-12.6 \pm 1.2$	11

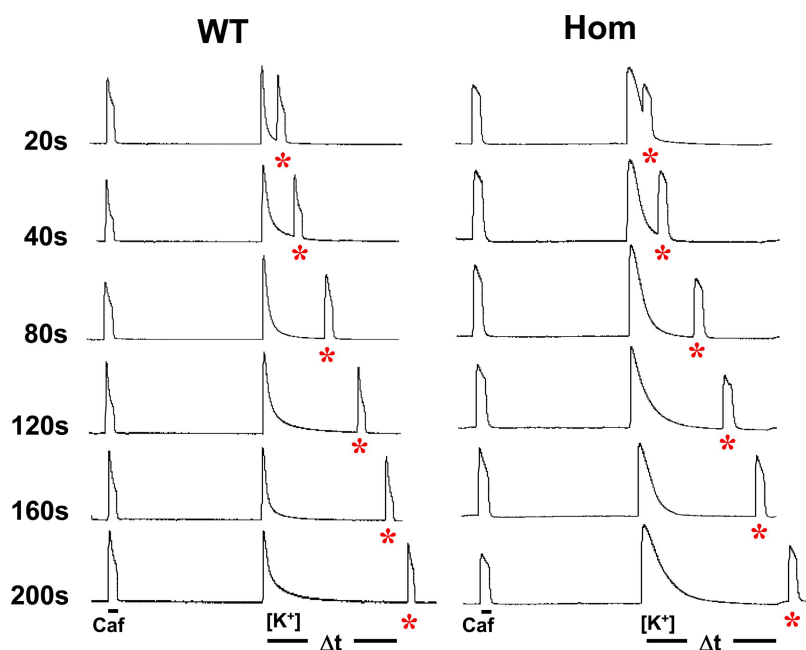
Values are expressed as mean  $\pm$  SE.

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 ( $8.7 \times 10^{-6}$  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  $62 \pm 5.8\%$  ( $n = 9$ ) in

WT,  $74 \pm 7\%$  ( $n = 14$ ) in Het R163C, and by  $80 \pm 9.3\%$  ( $n = 12$ ) in Hom R163C in low  $[Ca^{2+}]_e$  solution, and the rate of  $Ca^{2+}$  decay was increased from  $0.107 \pm 0.004 s^{-1}$  ( $n = 11$ ) to  $0.233 \pm 0.006 s^{-1}$  ( $n = 9$ ), from  $0.068 \pm 0.004 s^{-1}$  ( $n = 13$ ) to  $0.144 \pm 0.004 s^{-1}$  ( $n = 14$ ), and from  $0.037 \pm 0.003 s^{-1}$  ( $n = 17$ ) to  $0.137 \pm 0.005 s^{-1}$  ( $n = 12$ ) 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).

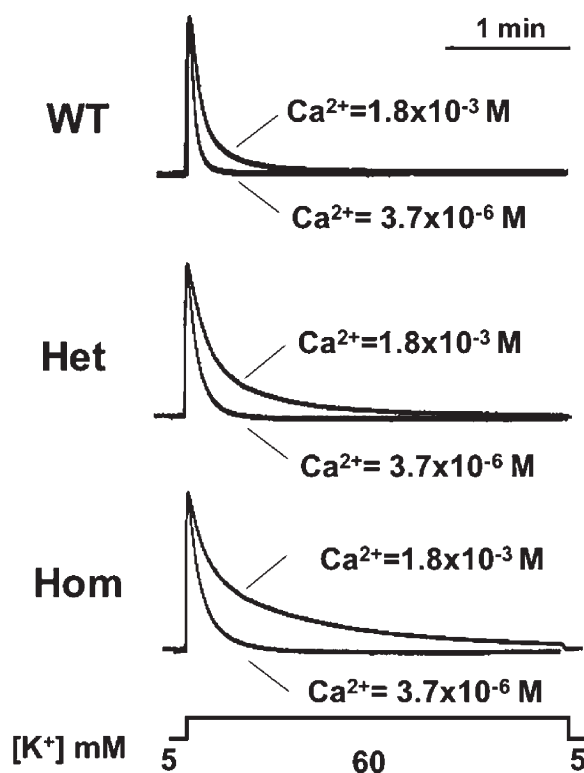
**Effect of  $Ca^{2+}$  entry blockers on  $K^+$ -induced  $Ca^{2+}$  transients**  
Because removal of external  $Ca^{2+}$  would be expected not only to eliminate  $Ca^{2+}$  entry, but also to accelerate inactivation of the voltage sensor for ECC (Rios and Brum, 1987), we also examined the effect 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  $\mu$ M 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  $0.107 \pm 0.004 s^{-1}$  ( $n = 11$ ) to  $0.135 \pm 0.005 s^{-1}$  ( $n = 11$ ) in WT ( $\sim 30\%$ ), and from  $0.037 \pm 0.003 s^{-1}$  ( $n = 17$ ) to  $0.117 \pm 0.002 s^{-1}$  ( $n = 16$ ) in Hom R163C ( $\sim 70\%$ ) myotubes ( $P < 0.001$ ; Fig. 4 A). Exposure of WT and Hom R163C myotubes to 20  $\mu$ M SKF-96365 did not lower  $Ca^{2+}$  transient amplitude, but it accelerated the mean rate of decay from  $0.107 \pm 0.004 s^{-1}$  ( $n = 11$ ) to  $0.146 \pm 0.005 s^{-1}$  ( $n = 9$ ) in WT myotubes ( $\sim 27\%$ ), and from  $0.037 \pm 0.003 s^{-1}$  ( $n = 17$ ) to  $0.072 \pm 0.004 s^{-1}$  ( $n = 13$ ) in Hom R163C ( $\sim 49\%$ ) 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 (\*).



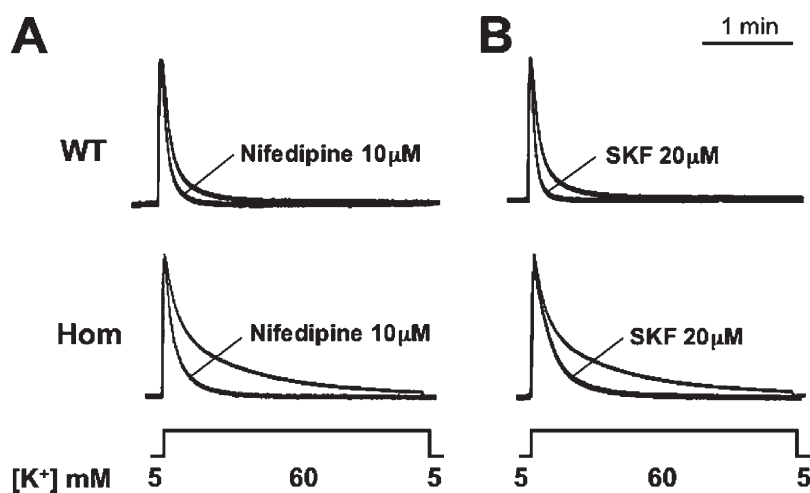


**Figure 3.** Low  $[Ca^{2+}]_e$  increases the rate of decay of the  $Ca^{2+}$  transient in response to 60 mM  $[K^+]_e$ . The average transients obtained from WT and R163C Het and Hom myotubes exposed to 60 mM  $[K^+]_e$  for 3 min in normal  $[Ca^{2+}]_e$  and in low  $[Ca^{2+}]_e$  solution (no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

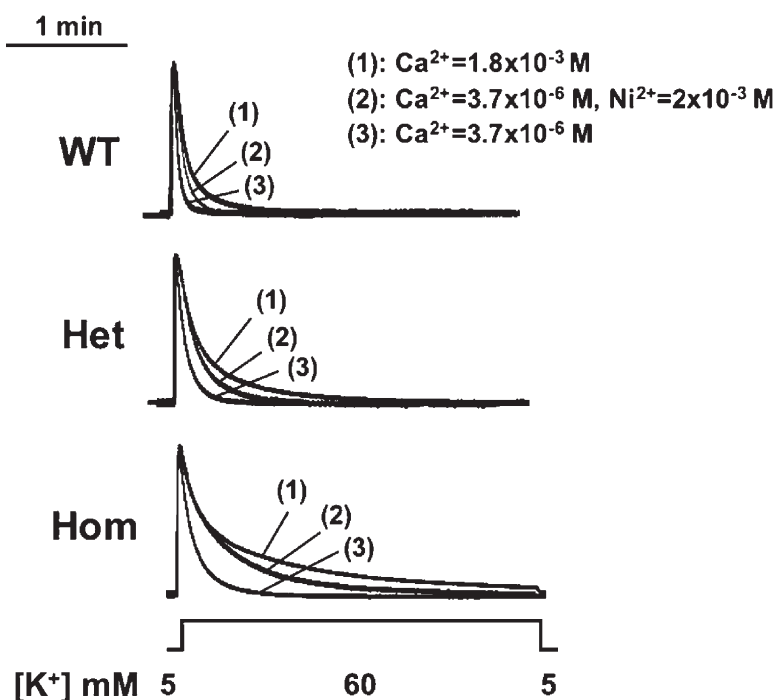
by their ability to partially depolarize the myotubes' membrane at rest, or affect a change in the magnitude of the depolarization induced by 60 mM  $[K^+]_e$ , membrane potentials were measured directly under these experimental conditions. Neither nifedipine nor SKF-96365 modified the resting membrane 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  $K^+$  contractions 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)  $1.8 \times 10^{-3}$  M  $Ca^{2+}$ , (2)  $8.7 \times 10^{-6}$  M  $Ca^{2+}$ , and (3)  $8.7 \times 10^{-6}$  M  $Ca^{2+}$  supplemented with  $2 \times 10^{-3}$  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  $\mu$ M nifedipine (A) or 20  $\mu$ M SKF96365 (B). The cells were exposed to nifedipine or SKF96365 for 10 s before and throughout the  $K^+$  depolarization.



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

suggest that the shortening of the  $\text{Ca}^{2+}$  transient in low  $\text{Ca}^{2+}$  solution was a combined consequence of accelerated inactivation of the DHPR voltage sensor and decreased depolarization-induced  $\text{Ca}^{2+}$  entry (see Table II for decay rates). Furthermore, these data show that the lack of extracellular  $\text{Ca}^{2+}$  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 $\text{Ni}^{2+}$

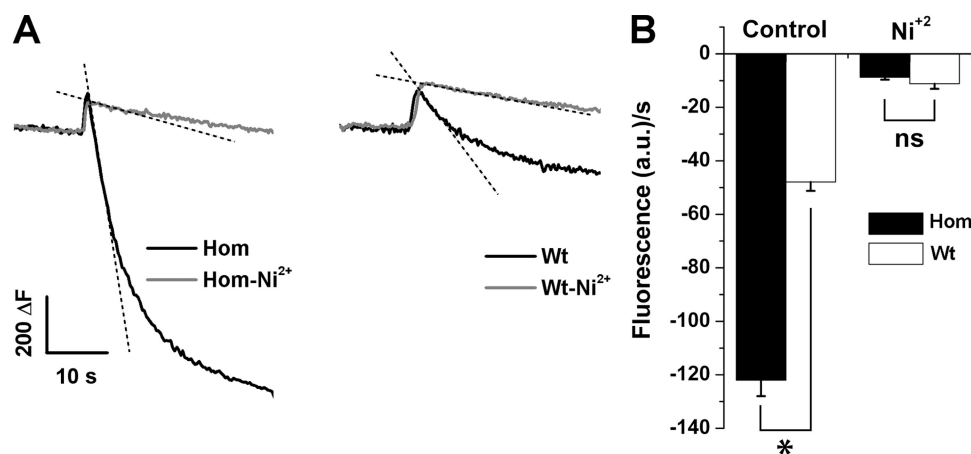
We directly measured the differences in cation entry in response to membrane depolarization using the  $\text{Mn}^{2+}$  quench technique in Hom R163C and WT myotubes. Fig. 6 A shows representative Fura-2 emission traces from Hom R163C and WT myotubes before and after stimulation with 60 mM KCl in the presence and absence of  $\text{Ni}^{2+}$ . As summarized in Fig. 6 B, these data show the average rate of  $\text{Mn}^{2+}$  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  $\text{Ni}^{2+}$ .

**TABLE II**  
Effect of  $\text{Ni}^{2+}$  on the rate of  $\text{Ca}^{2+}$  transient decay ( $\text{s}^{-1}$ )

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

#### Double $\text{K}^+$ 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  $\text{Ca}^{2+}$  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  $[\text{K}^+]_e$  for 10 s (Fig. 7,  $\text{Int}_1$ ), and then returned to normal mammalian Ringer's solution for 3, 5, or 10 s (Fig. 7,  $\Delta t$ ), and then reexposed to 60 mM  $[\text{K}^+]_e$  for 10 s (Fig. 7,  $\text{Int}_2$ ). We observed that washout of the elevated  $\text{K}^+$  solution with normal Ringer's solution immediately stopped the  $\text{Ca}^{2+}$  transient in all three genotypes, an effect that can be attributed to the repolarization of the plasma membrane. However, the amplitude of the  $\text{Ca}^{2+}$  transient triggered by reexposure to high  $[\text{K}^+]_e$  was highly genotype dependent (Fig. 7,  $\text{Int}_2$ ). The integral of the  $\text{Ca}^{2+}$  transient during  $\text{Int}_2$  in Hom R163C myotubes was not significantly different from the integral during  $\text{Int}_1$  for all interpulse intervals (Fig. 7;  $P > 0.05$ , Tukey's multiple tests). In Het R163C myotubes,  $\text{Int}_2$  was significantly smaller than  $\text{Int}_1$  at 3 s, but not significantly different from  $\text{Int}_1$  at 5 and 10 s (Fig. 7). In WT myotubes, on the other hand,  $\text{Int}_2$  was significantly lower than  $\text{Int}_1$  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.**  $\text{Mn}^{2+}$  quench in WT and Hom R163C myotubes in the absence and presence of 2 mM  $\text{Ni}^{2+}$ . (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  $\text{Ni}^{2+}$ . (B) The average rates  $\pm$  SEM of  $\text{Mn}^{2+}$  quench during the linear phase of quench after KCl exposure with (left) and without (right) the addition of  $\text{Ni}^{2+}$  to the bath solution. \*,  $P < 0.01$ .

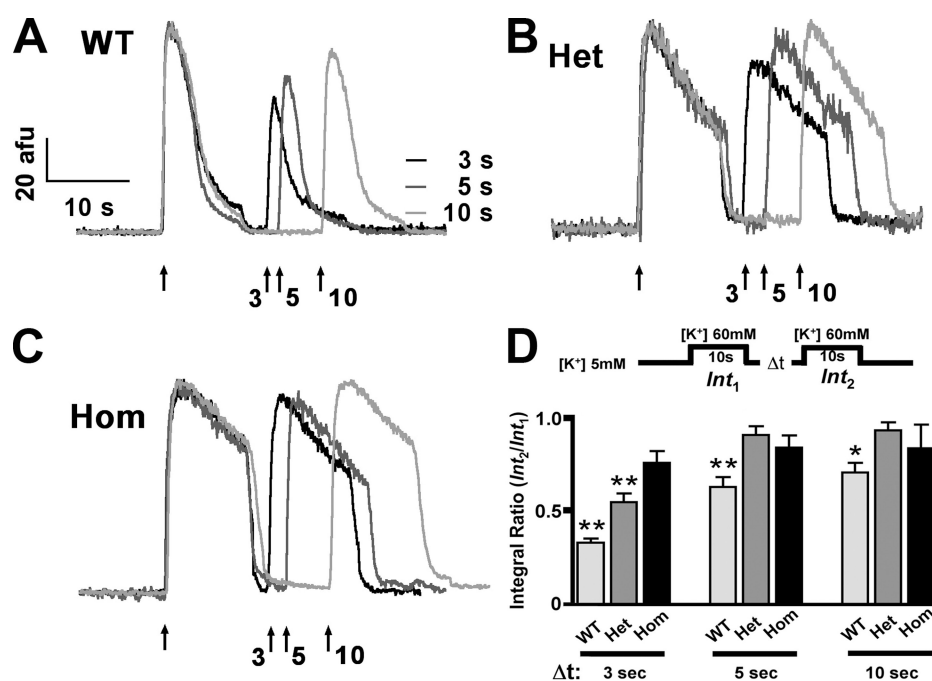
## DISCUSSION

There is general agreement that membrane depolarization elicited by high  $[\text{K}^+]_e$  triggers intracellular  $\text{Ca}^{2+}$  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  $[\text{K}^+]_e$  triggered  $\text{Ca}^{2+}$  transients characterized by a peak  $\text{Ca}^{2+}$  transient that varied with  $[\text{K}^+]_e$  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  $\text{K}^+$  contracture (Hodgkin and Horowitz, 1960b) or the corresponding  $\text{Ca}^{2+}$  transient (Caputo and Bolaños, 1994) depend on the  $[\text{K}^+]_e$  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  $\text{Ca}^{2+}$  transient was smaller and the duration of

the  $\text{Ca}^{2+}$  transient was greatly prolonged at low  $[\text{K}^+]_e$ . Although the peak amplitude of the  $\text{Ca}^{2+}$  transient was enhanced with increasing  $[\text{K}^+]_e$ , the duration of the transient decreases. The inverse relationship between  $[\text{K}^+]_e$  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  $\text{K}^+$  produced  $\text{Ca}^{2+}$  transients in primary R163C myotubes, which activated at lower membrane potentials and decayed more slowly than in WT myotubes. Thus, these alterations of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  transients for WT (A), Het (B), and Hom (C) myotubes induced by the following protocol: 60 mM  $[\text{K}^+]_e$  ( $\text{Int}_1$ ) for 10 s; 2.5 mM  $[\text{K}^+]_e$  for 3, 5, or 10 s; 60 mM  $[\text{K}^+]_e$  ( $\text{Int}_2$ ). (D) The integrals of the second  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  release. Furthermore, it is known that the time course of  $\text{K}^{+}$ -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  $\text{Ni}^{2+}$  was substituted for  $\text{Ca}^{2+}$ , it was shown that depolarization-induced  $\text{Ca}^{2+}$  entry is enhanced in Het and Hom R163C, but the majority of the difference in the rate of decay of the  $\text{Ca}^{2+}$  transient in Het and Hom R163C myotubes is due to prolonged SR  $\text{Ca}^{2+}$  release caused by either a delay in the onset or a reduction in the rate of DHPR voltage sensor inactivation.

The double  $\text{K}^{+}$  application protocol (Fig. 7) confirmed this idea by demonstrating that the second  $\text{Ca}^{2+}$  transient of the pair was significantly smaller in WT myotubes than in R163C myotubes with all delays. Furthermore, the rate of decay of the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channel.

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## REFERENCES

Almers, W., and P.T. Palade. 1981. Slow calcium and potassium currents across frog muscle membrane: measurements with a vaseline-gap technique. *J. Physiol.* 312:159–176.

Araya, R., J.L. Liberona, J.C. Cárdenas, N. Riveros, M. Estrada, J.A. Powell, M.A. Carrasco, and E. Jaimovich. 2003. Dihydropyridine receptors as voltage sensors for a depolarization-evoked, IP3R-mediated, slow calcium signal in skeletal muscle cells. *J. Gen. Physiol.* 121:3–16. doi:10.1085/jgp.20028671

Armstrong, C.M., F.M. Bezanilla, and P. Horowicz. 1972. Twitches in the presence of ethylene glycol bis-(aminoethyl ether)-N,N'-tetracetic acid. *Biochim. Biophys. Acta.* 267:605–608. doi:10.1016/0005-2728(72)90194-6

Bannister, R.A., and K.G. Beam. 2009. The cardiac  $\alpha(1\text{C})$  subunit can support excitation-triggered  $\text{Ca}^{2+}$  entry in dysgenic and dyspedic myotubes. *Channels (Austin)*. 3:268–273.

Bannister, R.A., I.N. Pessah, and K.G. Beam. 2009. The skeletal L-type  $\text{Ca}^{2+}$  current is a major contributor to excitation-coupled  $\text{Ca}^{2+}$  entry. *J. Gen. Physiol.* 133:79–91. doi:10.1085/jgp.200810105

Bannister, R.A., E. Estève, J.M. Eltit, I.N. Pessah, P.D. Allen, J.R. López, and K.G. Beam. 2010. A malignant hyperthermia-inducing

mutation in RYR1 (R163C): consequent alterations in the functional properties of DHPR channels. *J. Gen. Physiol.* 135:629–640.

Bianchi, C.P., and A.M. Shanes. 1959. Calcium influx in skeletal muscle at rest, during activity, and during potassium contracture. *J. Gen. Physiol.* 42:803–815. doi:10.1085/jgp.42.4.803

Blinks, J.R., R. Rüdel, and S.R. Taylor. 1978. Calcium transients in isolated amphibian skeletal muscle fibres: detection with aequorin. *J. Physiol.* 277:291–323.

Bolaños, P., C. Caputo, and L. Velaz. 1986. Effects of calcium, barium and lanthanum on depolarization-contraction coupling in skeletal muscle fibres of *Rana pipiens*. *J. Physiol.* 370:39–60.

Brum, G., E. Ríos, and E. Stefani. 1988. Effects of extracellular calcium on calcium movements of excitation-contraction coupling in frog skeletal muscle fibres. *J. Physiol.* 398:441–473.

Caputo, C. 1972a. The effect of low temperature on the excitation-contraction coupling phenomena of frog single muscle fibres. *J. Physiol.* 223:461–482.

Caputo, C. 1972b. The time course of potassium contractures of single muscle fibres. *J. Physiol.* 223:483–505.

Caputo, C. 1976. The effect of caffeine and tetracaine on the time course of potassium contractures of single muscle fibres. *J. Physiol.* 255:191–207.

Caputo, C. 1981. Nickel substitution for calcium and the time course of potassium contractures of single muscle fibres. *J. Muscle Res. Cell Motil.* 2:167–182. doi:10.1007/BF00711867

Caputo, C., and P. Bolaños. 1994. Fluo-3 signals associated with potassium contractures in single amphibian muscle fibres. *J. Physiol.* 481:119–128.

Caputo, C., and M. Gimenez. 1967. Effects of external calcium deprivation on single muscle fibers. *J. Gen. Physiol.* 50:2177–2195. doi:10.1085/jgp.50.9.2177

Chelu, M.G., S.A. Goonasekera, W.J. Durham, W. Tang, J.D. Lueck, J. Riehl, I.N. Pessah, P. Zhang, M.B. Bhattacharjee, R.T. Dirksen, and S.L. Hamilton. 2006. Heat- and anesthesia-induced malignant hyperthermia in an RyR1 knock-in mouse. *FASEB J.* 20:329–330.

Cherednichenko, G., A.M. Hurne, J.D. Fessenden, E.H. Lee, P.D. Allen, K.G. Beam, and I.N. Pessah. 2004. Conformational activation of  $\text{Ca}^{2+}$  entry by depolarization of skeletal myotubes. *Proc. Natl. Acad. Sci. USA.* 101:15793–15798. doi:10.1073/pnas.0403485101

Cherednichenko, G., C.W. Ward, W. Feng, E. Cabrales, L. Michaelson, M. Samso, J.R. López, P.D. Allen, and I.N. Pessah. 2008. Enhanced excitation-coupled calcium entry in myotubes expressing malignant hyperthermia mutation R163C is attenuated by dantrolene. *Mol. Pharmacol.* 73:1203–1212. doi:10.1124/mol.107.043299

Cota, G., and E. Stefani. 1981. Effects of external calcium reduction on the kinetics of potassium contractures in frog twitch muscle fibres. *J. Physiol.* 317:303–316.

Dirksen, R.T., and K.G. Beam. 1999. Role of calcium permeation in dihydropyridine receptor function. Insights into channel gating and excitation-contraction coupling. *J. Gen. Physiol.* 114:393–403. doi:10.1085/jgp.114.3.393

Dulhunty, A.F. 1992. The voltage-activation of contraction in skeletal muscle. *Prog. Biophys. Mol. Biol.* 57:181–223. doi:10.1016/0079-6107(92)90024-Z

Dulhunty, A.F., and P.W. Gage. 1988. Effects of extracellular calcium concentration and dihydropyridines on contraction in mammalian skeletal muscle. *J. Physiol.* 399:63–80.

Ebata, H., J.S. Mills, K. Nemcek, and J.D. Johnson. 1990. Calcium binding to extracellular sites of skeletal muscle calcium channels regulates dihydropyridine binding. *J. Biol. Chem.* 265:177–182.

Frank, G.B. 1960. Effects of changes in extracellular calcium concentration on the potassium-induced contracture of frog's skeletal muscle. *J. Physiol.* 151:518–538.

- Gallant, E.M., and L.R. Lentz. 1992. Excitation-contraction coupling in pigs heterozygous for malignant hyperthermia. *Am. J. Physiol.* 262:C422–C426.
- Gallant, E.M., G.A. Gronert, and S.R. Taylor. 1982. Cellular membrane potentials and contractile threshold in mammalian skeletal muscle susceptible to malignant hyperthermia. *Neurosci. Lett.* 28:181–186. doi:10.1016/0304-3940(82)90149-5
- Hodgkin, A.L., and P. Horowicz. 1959. The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* 148:127–160.
- Hodgkin, A.L., and P. Horowicz. 1960a. The effect of sudden changes in ionic concentrations on the membrane potential of single muscle fibres. *J. Physiol.* 153:370–385.
- Hodgkin, A.L., and P. Horowicz. 1960b. Potassium contractures in single muscle fibres. *J. Physiol.* 153:386–403.
- Jong, D.S., P.C. Pape, S.M. Baylor, and W.K. Chandler. 1995. Calcium inactivation of calcium release in frog cut muscle fibers that contain millimolar EGTA or Fura-2. *J. Gen. Physiol.* 106:337–388. doi:10.1085/jgp.106.2.337
- Jurkat-Rott, K., T. McCarthy, and F. Lehmann-Horn. 2000. Genetics and pathogenesis of malignant hyperthermia. *Muscle Nerve*. 23:4–17. doi:10.1002/(SICI)1097-4598(200001)23:1<4::AID-MUS3>3.0.CO;2-D
- Leung, Y.M., and C.Y. Kwan. 1999. Current perspectives in the pharmacological studies of store-operated  $\text{Ca}^{2+}$  entry blockers. *Jpn. J. Pharmacol.* 81:253–258. doi:10.1254/jip.81.253
- López, J.R., L. Alamo, C. Caputo, R. DiPollo, and S. Vergara. 1983. Determination of ionic calcium in frog skeletal muscle fibers. *Biophys. J.* 43:1–4. doi:10.1016/S0006-3495(83)84316-1
- López, J.R., L. Alamo, C. Caputo, J. Wikinski, and D. Ledezma. 1985. Intracellular ionized calcium concentration in muscles from humans with malignant hyperthermia. *Muscle Nerve*. 8:355–358. doi:10.1002/mus.880080502
- López, J.R., P.D. Allen, L. Alamo, D. Jones, and F.A. Sreter. 1988. Myoplasmic free  $[\text{Ca}^{2+}]$  during a malignant hyperthermia episode in swine. *Muscle Nerve*. 11:82–88. doi:10.1002/mus.880110113
- López, J.R., N. Linares, I.N. Pessah, and P.D. Allen. 2005. Enhanced response to caffeine and 4-chloro-m-cresol in malignant hyperthermia-susceptible muscle is related in part to chronically elevated resting  $[\text{Ca}^{2+}]_i$ . *Am. J. Physiol. Cell Physiol.* 288:C606–C612. doi:10.1152/ajpcell.00297.2004
- Lorković, H., and R. Rüdel. 1983. Influence of divalent cations on potassium contracture duration in frog muscle fibres. *Pflügers Arch.* 398:114–119. doi:10.1007/BF00581057
- Lüttgau, H.C., and W. Spiecker. 1979. The effects of calcium deprivation upon mechanical and electrophysiological parameters in skeletal muscle fibres of the frog. *J. Physiol.* 296:411–429.
- Monnier, N., V. Procaccio, P. Stieglitz, and J. Lunardi. 1997. Malignant hyperthermia susceptibility is associated with a mutation of the alpha 1-subunit of the human dihydropyridine-sensitive L-type voltage-dependent calcium-channel receptor in skeletal muscle. *Am. J. Hum. Genet.* 60:1316–1325. doi:10.1086/515454
- Nakai, J., R.T. Dirksen, H.T. Nguyen, I.N. Pessah, K.G. Beam, and P.D. Allen. 1996. Enhanced dihydropyridine receptor channel activity in the presence of ryanodine receptor. *Nature*. 380:72–75. doi:10.1038/380072a0
- Nakai, J., N. Sekiguchi, T.A. Rando, P.D. Allen, and K.G. Beam. 1998. Two regions of the ryanodine receptor involved in coupling with L-type  $\text{Ca}^{2+}$  channels. *J. Biol. Chem.* 273:13403–13406. doi:10.1074/jbc.273.22.13403
- Nelson, T.E. 2001. Heat production during anesthetic-induced malignant hyperthermia. *Biosci. Rep.* 21:169–179. doi:10.1023/A:1013696124358
- Nelson, T.E. 2002. Malignant hyperthermia: a pharmacogenetic disease of  $\text{Ca}^{++}$  regulating proteins. *Curr. Mol. Med.* 2:347–369. doi:10.2174/1566524023362429
- Obejero-Paz, C.A., I.P. Gray, and S.W. Jones. 2008.  $\text{Ni}^{2+}$  block of  $\text{Ca}_v3.1$  ( $\alpha 1\text{G}$ ) T-type calcium channels. *J. Gen. Physiol.* 132:239–250. doi:10.1085/jgp.200809988
- Palade, P.T., and W. Almers. 1985. Slow calcium and potassium currents in frog skeletal muscle: their relationship and pharmacologic properties. *Pflügers Arch.* 405:91–101. doi:10.1007/BF00584528
- Rando, T.A., and H.M. Blau. 1994. Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. *J. Cell Biol.* 125:1275–1287. doi:10.1083/jcb.125.6.1275
- Rios, E., and G. Brum. 1987. Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle. *Nature*. 325:717–720. doi:10.1038/325717a0
- Rivet, M., C. Cognard, and G. Raymond. 1989. The slow inward calcium current is responsible for a part of the contraction of patch-clamped rat myoballs. *Pflügers Arch.* 413:316–318. doi:10.1007/BF00583547
- Robinson, R., D. Carpenter, M.A. Shaw, J. Halsall, and P. Hopkins. 2006. Mutations in RYR1 in malignant hyperthermia and central core disease. *Hum. Mutat.* 27:977–989. doi:10.1002/humu.20356
- Simon, B.J., M.G. Klein, and M.F. Schneider. 1991. Calcium dependence of inactivation of calcium release from the sarcoplasmic reticulum in skeletal muscle fibers. *J. Gen. Physiol.* 97:437–471. doi:10.1085/jgp.97.3.437
- Smith, J.S., R. Coronado, and G. Meissner. 1985. Sarcoplasmic reticulum contains adenine nucleotide-activated calcium channels. *Nature*. 316:446–449. doi:10.1038/316446a0
- Snowdowne, K.W. 1985. Subcontracture depolarizations increase sarcoplasmic ionized calcium in frog skeletal muscle. *Am. J. Physiol.* 248:C520–C526.
- Stefani, E., and D.J. Chiarandini. 1973. Skeletal muscle: dependence of potassium contractures on extracellular calcium. *Pflügers Arch.* 343:143–150. doi:10.1007/BF00585709
- Suarez-Isla, B.A., C. Orozco, P.F. Heller, and J.P. Froehlich. 1986. Single calcium channels in native sarcoplasmic reticulum membranes from skeletal muscle. *Proc. Natl. Acad. Sci. USA*. 83:7741–7745. doi:10.1073/pnas.83.20.7741
- Tanabe, T., K.G. Beam, B.A. Adams, T. Niidome, and S. Numa. 1990. Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling. *Nature*. 346:567–569. doi:10.1038/346567a0
- Treves, S., A.A. Anderson, S. Ducreux, A. Divet, C. Bleunven, C. Grasso, S. Paesante, and F. Zorzato. 2005. Ryanodine receptor 1 mutations, dysregulation of calcium homeostasis and neuromuscular disorders. *Neuromuscul. Disord.* 15:577–587. doi:10.1016/j.nmd.2005.06.008
- Yang, T., T.A. Ta, I.N. Pessah, and P.D. Allen. 2003. Functional defects in six ryanodine receptor isoform-1 (RyR1) mutations associated with malignant hyperthermia and their impact on skeletal excitation-contraction coupling. *J. Biol. Chem.* 278:25722–25730. doi:10.1074/jbc.M302165200
- Yang, T., J. Riehl, E. Esteve, K.I. Matthaie, S. Goth, P.D. Allen, I.N. Pessah, and J.R. Lopez. 2006. Pharmacologic and functional characterization of malignant hyperthermia in the R163C RyR1 knock-in mouse. *Anesthesiology*. 105:1164–1175. doi:10.1097/0000542-200612000-00016
- Yang, T., P.D. Allen, I.N. Pessah, and J.R. Lopez. 2007a. Enhanced excitation-coupled calcium entry in myotubes is associated with expression of RyR1 malignant hyperthermia mutations. *J. Biol. Chem.* 282:37471–37478. doi:10.1074/jbc.M701379200
- Yang, T., E. Esteve, I.N. Pessah, T.F. Molinski, P.D. Allen, and J.R. López. 2007b. Elevated resting  $[\text{Ca}^{2+}]_i$  in myotubes expressing malignant hyperthermia RyR1 cDNAs is partially restored by modulation of passive calcium leak from the SR. *Am. J. Physiol. Cell Physiol.* 292:C1591–C1598. doi:10.1152/ajpcell.00133.2006