Ca^{2+} Activation of RyR1 Is Not Necessary for the Initiation of Skeletal-Type Excitation-Contraction Coupling

Jennifer J. O'Brien,* Wei Feng,† Paul D. Allen,‡ S. R. Wayne Chen,§ Isaac N. Pessah,† and Kurt G. Beam*

*Department of Anatomy and Neurobiology, Colorado State University, Fort Collins, Colorado 80523 USA; †Department of Molecular Biosciences, University of California at Davis, Davis, California 95616 USA; ‡Department of Anesthesia, Brigham & Women's Hospital, Boston, Massachusetts 02115 USA; and §Department of Physiology and Biophysics, University of Calgary, Calgary, Alberta T2N 4N1, Canada

ABSTRACT   Although an elevation in myoplasmic Ca^{2+} can activate the skeletal muscle ryanodine receptor (RyR1), the function of this Ca^{2+} activation is unclear because extracellular Ca^{2+} influx is unnecessary for skeletal-type EC coupling. To determine whether Ca^{2+} activation of RyR1 is necessary for the initiation of skeletal-type EC coupling, we examined the behavior of RyR1 with glutamate 4032 mutated to alanine (E4032A-RyR1) because this mutation had been shown to dramatically reduce activation by Ca^{2+}. *Proc. Natl. Acad. Sci. USA.* 98:2865–2870). Analysis after reconstitution into planar lipid bilayers revealed that E4032A-RyR1 was negligibly activated by 100 μM Ca^{2+} (P_o too low to be measured). Even in the presence of both 2 mM caffeine and 2 mM ATP, P_o remained low for E4032A-RyR1 (ranging from <0.0001 in 100 μM free Ca^{2+} to 0.005 in 2 mM free Ca^{2+}). Thus, the E4032A mutation caused a nearly complete suppression of activation of RyR1 by Ca^{2+}. Depolarization of E4032A-RyR1-expressing myotubes elicited L-type Ca^{2+} currents of approximately normal size and myoplasmic Ca^{2+} transients that were skeletal-type, but about fivefold smaller than those for wild-type RyR1. The reduced amplitude of the Ca^{2+} transient is consistent either with the possibility that Ca^{2+} activation amplifies Ca^{2+} release during EC coupling, or that the E4032A mutation generally inhibits activation of RyR1. In either case, Ca^{2+} activation of RyR1 does not appear to be necessary for the initiation of Ca^{2+} release during EC coupling in skeletal muscle.

INTRODUCTION

Ryanodine receptors are intracellular Ca^{2+} release channels, currently known to be encoded in mammals by three distinct genes. RyR1 and RyR2 are the predominant forms in skeletal muscle and cardiac muscle, respectively (Takeshima et al., 1989; Zorzato et al., 1990; Nakai et al., 1990), and RyR3 is ubiquitously expressed (Hakamata et al., 1992; Giannini et al., 1992). All three isoforms can be activated by an elevation in cytoplasmic Ca^{2+}. In cardiac muscle, activation of RyR2 by cytoplasmic Ca^{2+} plays a major role in excitation-contraction (EC) coupling. Specifically, depolarization of cardiac muscle cells activates a voltage-gated L-type Ca^{2+} channel, which contains α_{1C} as its principal subunit; the resulting influx of Ca^{2+} provides an increase in cytoplasmic Ca^{2+} sufficient for activating RyR2 to release Ca^{2+} from the sarcoplasmic reticulum (Fabiato, 1985; Nabauer et al., 1989). An elevation of intracellular Ca^{2+} can also serve to activate RyR1, but the role of this pathway is uncertain. Thus, even though skeletal muscle expresses large numbers of L-type Ca^{2+} channels (containing α_{1S} as the principle subunit), EC coupling in skeletal muscle persists after Ca^{2+} influx through α_{1S} is blocked by removal of extracellular Ca^{2+} (Armstrong et al., 1972) or by channel blockers (Gonzalez-Serratos et al., 1982). Therefore, a mechanical interaction with α_{1S} may be

responsible for the initial activation of RyR1 during "skeletal-type" EC coupling (Rios and Brum, 1987; Tanabe et al., 1990) and the role of Ca^{2+}-activation during this initial triggering of RyR1 remains unclear (e.g., Lamb et al., 2001).

Recently, it has been reported that a glutamate conserved in all three RyR isoforms has an important role in Ca^{2+} activation. Specifically, mutation of this glutamate to alanine in RyR2 (E3987A) and in RyR3 (E3885A) caused a 1,000–10,000-fold increase in the EC50 for activation by Ca^{2+}, as measured by recordings of channels reconstituted into bilayers (Chen et al., 1998; Li and Chen, 2001). The corresponding mutation (E4032A) in RyR1 also inhibits activation by Ca^{2+} (Fessenden et al., 2001). Here, we have used expression in dyspedic mouse myotubes, which lack endogenous RyR1 (Takeshima et al., 1994; Buck et al., 1997), to compare the effects of the E4032A mutation on the activation by Ca^{2+} of single RyR channels reconstituted into planar lipid bilayers and the activation of intracellular Ca^{2+} release by depolarization of myotubes via the whole-cell technique. We found that the E4032A mutation causes >100-fold suppression of RyR1 activity in bilayers in response to Ca^{2+}, but only an ~5-fold reduction in depolarization-induced Ca^{2+} release in myotubes. This residual Ca^{2+} release occurred via skeletal-type coupling because it showed a sigmoidal dependence on test potential and persisted after the block of Ca^{2+} influx via L-type Ca^{2+} current. Thus, Ca^{2+} activation of RyR1 does not appear to be essential for the initiation of skeletal-type EC coupling, although it might be important for the overall functioning of EC coupling. In agreement with Avila et al. (2001), retrograde signaling, whereby RyR1 increases the L-type Ca^{2+}

current carried by α_{1S} (Nakai et al., 1996), was little-affected by the E4032A mutation. Therefore, Ca^{2+} release from RyR1 does not appear to play a major role in the retrograde signal from RyR1 to α_{1S}.

MATERIALS AND METHODS

Mutagenesis

The mammalian expression plasmid (E4032A-RyR1-pCDNA3), in which the point mutation E4032A was introduced into RyR1, was constructed by the overlap extension method (Ho et al., 1989) using polymerase chain reaction (PCR). The "outer" two oligonucleotides used were forward, 5′-GTGTTCAACAGCCTCACCGA-3′; and reverse, 5′-GAACTGCT-TCTGGCTGTCCA-3′. The oligonucleotides for the E4032A mutation were forward, 5′-GTCCCTACTGGCAGGGAACGTGGT-3′; and reverse, 5′-CCACGTTCCCTGCCAGTAGGGACA-3′. The sequence of the PCR product was confirmed by DNA sequencing. The *Xho*I (12018)-*Stu*I (12224) fragment was removed from the PCR product and was used to replace the corresponding wild-type region in the *Xho*I (12018)-*Xba*I (3′ end) fragment of the RyR1 cDNA in pBluescript. The *Xho*I (12018)-*Xba*I (3′ end) fragment containing the E4032A mutation was subcloned into the RyR1 cDNA in pHSVprPUC vector lacking the *Xho*I (6466)-*Xho*I (12018) fragment, which was subsequently added back to form the full-length E4032A-RyR1 cDNA. The full-length E4032A-RyR1 cDNA (*Hind*III-*Xba*I) was transferred from pHSVprPUC to pCDNA3 to form the expression plasmid E4032A-RyR1-pCDNA3 .

Single channel analysis

For the measurements of single RyR channels, RyR cDNAs were expressed in 1B5 myotubes. The 1B5 cells were cultured in DMEM (Dulbecco's modified Eagle's medium, GIBCO/BRL, Germantown, MD) containing 20% fetal bovine serum in collagen-coated 100 mm polystyrene plates (Protasi et al., 1998; Moore et al., 1998). When the cells attained 30–50% confluence, they were differentiated within a 20% CO$_2$ incubator at 37°C for 5 days in 5% heat-inactivated-horse serum in DMEM before infection with RyR-cDNA containing viruses (Wang et al., 2000). Briefly, cDNA encoding either wild-type RyR1 or E4032A-RyR1 was added to differentiated 1B5 myotubes at a concentration of 3–5 × 10^5 infectious units (IU)/ml. Cells were harvested and sarcoplasmic reticulum (SR) prepared 48 h after infection.

Crude membrane homogenates from 1B5 myotubes expressing E4032A-RyR1 were prepared as described previously (Moore et al., 1998) and subsequently loaded onto a sucrose gradient consisting of layers of 10%, 27%, and 45% w/w sucrose, 10 mM HEPES pH 7.4. After sedimentation of the crude membranes on this gradient at 40,000 × *g* for 1 h at 4°C, the 27–45% interface containing heavy SR membranes was isolated and diluted in 10 mM HEPES, pH 7.4 and subsequently pelleted at 110,000 × *g* for 1 h. The pellet was resuspended in 10% sucrose, 10 mM HEPES, pH 7.4, divided into small aliquots and either used immediately or stored at −80°C for no more than two weeks.

Heavy SR membrane vesicles were fused with an artificial bilayer lipid membrane (BLM) composed of a 5:2 mixture of natural phosphatidylethanolamine and phosphatidylcholine (PE/PC 5:2 mixture) at 50 mg/ml in decane. The BLM was formed across a 200- to 250-μm hole in a polystyrene cup separating two chambers (*cis* and *trans*), each 0.7 ml. Vesicles (0.1–5 μg protein) were added to the *cis* chamber in the presence of 200 μM Ca^{2+}. The *cis* and *trans* chambers contained 500 mM CsCl, 20 mM HEPES (pH 7.4), and 100 mM CsCl, 20 mM HEPES (pH 7.4), respectively. After fusion, 300 μM EGTA was added to the *cis* chamber to prevent additional fusion events. The *cis* chamber was then perfused with a solution composed of 500 mM CsCl, 20 mM HEPES, pH 7.4 (asymmetrical CsCl 5:1 *cis/trans*). A patch-clamp amplifier (Dagan, model 3900)
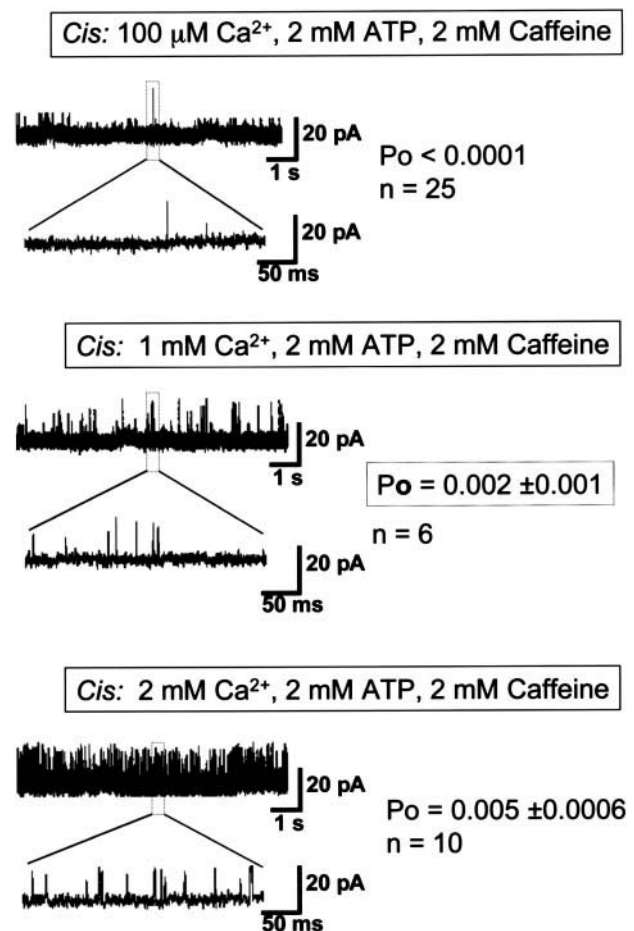

FIGURE 1 E4032A-RyR1 reconstituted into bilayer lipid membranes after expression in 1B5 myotubes is negligibly activated by physiological and pharmacological agonists of RyR1. *Top panel*: of ~100 fusion events for E4032A-RyR1, 25 contained rare and very brief transitions to the open state that prevented calculation of P_o in the presence of *cis* 100 μM Ca^{2+}, 2 mM ATP, and 2 mM caffeine ($P_o <$ 0.0001, $n =$ 25). The remainder produced an offset current (indicative of SR membrane fusion) but no gating events attributable to RyR. *Middle panel*: increasing *cis* Ca^{2+} to 1 mM resulted in a measurable elevation in P_o ($P_o =$ 0.002 ± 0.001, $n =$ 6). *Bottom panel*: in the presence of 2 mM Ca^{2+}, the average P_o determined for E4032A-RyR1 was 0.005 ± 0.0006 ($n =$ 10).

was used to measure currents through a single channel. The data were filtered at 1 kHz (low-pass, 8-pole Bessel filter, Warner Instrument Corp., Hamden, CT) before acquisition at 10 kHz by a DigiData 1200A (Axon Instruments, Foster City, CA). Experimental reagents were added to the *cis* chamber and stirred for 30 s. Subsequent channel-gating behavior was recorded for 1–20 min using Axoscope 8.0 (Axon Instruments). Single-channel data from BLM experiments were analyzed using pCLAMP6 (Axon Instruments), and Fig. 1 was prepared using Origin 4.0 (Microcal, Northampton, MA).

Expression of cDNA in primary myotubes

Primary cultures of dyspedic mouse myotubes were obtained as previously described (Nakai et al., 1996; Rando and Blau, 1994). At 6–7 days after initial plating, a single nucleus of each cell was microinjected with 0.3 μg/μl of either E4032A-RyR1-pCDNA3 or RyR1-pCI-neo (Nakai et al.,

1998) and 0.2 μg/μl of an expression plasmid (Jurman et al., 1994) for the surface antigen CD8. Approximately 48 h later, the medium was removed from injected myotubes and replaced with external recording solution (see below) containing beads coated with CD8 antibody (Dynabeads M-450, Dynal AS, Oslo, Norway), which allowed identification of cells that were expressing CD8, and thus candidates to express the RyR construct of interest. Alternatively, the RyR cDNA was injected together with 0.04 μg/μl cDNA for green fluorescent protein (Grabner et al., 1998). After 48 h, the GFP-positive myotubes, while bathed in DMEM, were tested for the ability to contract in response to stimulation (100 V, 10 ms) via an extracellular pipette that was filled 150 mM NaCl and positioned with its tip near (~30 μm) the cell.

Measurement of Ca^{2+} currents and Ca^{2+} transients

Ca^{2+} currents and Ca^{2+} transients were recorded simultaneously (García et al., 1994) using a combination of the whole-cell patch technique and fluorometric monitoring of myoplasmic Ca^{2+} by means of Fluo-3, which was included in the "internal solution" (composition given below). Patch pipettes were pulled from borosilicate glass and had resistances of 1.6 to 2.0 MΩ when filled with internal solution. After a seal was obtained between the patch pipette and a myotube, the cell was washed to remove any Fluo-3 that might have leaked into the bath from the pipette. A period of >5 min was allowed after breaking into whole-cell mode so that Fluo-3 could diffuse throughout the myotube. Cells were held at −80 mV and control (linear capacitive and leak) currents were measured by steps to −110 mV. Cell capacitance was determined by integration of the control current and used to normalize Ca^{2+} currents (pA/pF). Additionally, the average of 10 control currents was digitally scaled and subtracted from test currents to correct for linear components of leakage and capacitive current. The voltage protocol for test currents consisted of a 1-s prepulse to −30 or −20 mV to inactivate T-type current, followed by a 50-ms repolarization to −50 mV, followed by a 200-ms step to the test potential, a 125-ms step to −50 mV, and finally a return to the holding potential.

For the measurement of fluorescence, a rectangular slit and iris diaphragm were adjusted so that the illumination from a 75 W xenon bulb was restricted to a segment of the myotube that avoided both surrounding cells and the patch pipette. To prevent prolonged exposure of cells to this illumination, a digitally controlled shutter was opened 1 s before the test step and closed immediately after the return to the holding potential. Fluorescence emission was recorded with standard filters for fluorescein (excitation centered at 470 nm with half-height width of 20 nm, dichroic 510 nm long pass, emission 520 nm long pass) and a photometer system (Biomedical Instrumentation Group, University of Pennsylvania). Data were acquired and analyzed with a PDP-11/73 computer system (INDEC, Sunnyvale, CA).

For the measurement of changes of myoplasmic calcium in response to application of caffeine, intact myotubes were rinsed with serum-free DMEM and then incubated for 1 h at 37°C in serum-free DMEM containing 5.5 μM Fluo-3 AM (Molecular Probes, Eugene, OR). After loading, the cells were returned to normal culture medium (DMEM with serum) and placed in the incubator for a minimum of 30 min before experimentation. Fluorescence responses were recorded with pClamp 8.0 (Axon Instruments).

Solutions

The internal solution contained (in mM) 145 cesium glutamate, 8 MgATP (1 mM free Mg^{2+}), 2 CsCl, 10 HEPES, 10 EGTA, and 0.5 K$_5$ Fluo-3 (Molecular Probes). The external solution used for measuring Ca^{2+} currents and Ca^{2+} transients contained (in mM) 145 TEACl, 10 HEPES, 10 CaCl$_2$, and 0.003 TTX. To test the effects of Cd^{2+} plus La^{3+} (0.5 and 0.1 mM, respectively) or of caffeine (1 or 10 mM), individual, patch-clamped

myotubes were analyzed before and after the external solution was replaced with external solution containing the indicated substances. The effects of caffeine were tested on only a single myotube per culture dish.

Analysis

Because intracellular Ca^{2+} release was reduced severalfold for E4032A-RyR1, we characterized release in terms of ΔF, the change in fluorescence from baseline (F), rather than as $\Delta F/F$, because ΔF was quite stable during the course of an experiment, whereas the baseline fluorescence gradually increased, apparently due to incomplete removal of myoplasmic Ca^{2+}. Additionally, ΔF was measured 40 ms after the onset of depolarization to help reduce the contribution of the entry of extracellular Ca^{2+} due to Ca^{2+} current. Values of ΔF were plotted versus test potential (V) and fitted according to the equation:

$$\Delta F = \Delta F_{max}/[1 + \exp - (V - V_{1/2})/k_F] \qquad (1)$$

where ΔF_{max} is the maximum fluorescence change, $V_{1/2}$ is the potential eliciting a half-maximal ΔF, and k_F is a parameter related to the steepness of voltage-dependence. In addition to measuring amplitude, we visually fitted a straight line to the onset of the transient to determine the initial slope (in ΔF/ms).

Data are reported as mean ± SEM and exclude cells that had peak Ca^{2+} currents <1 pA/pF and transients indistinguishable from baseline noise, on the assumption that these cells were likely expressing CD8, but not the RyR construct. Statistical comparisons were made with Student's t-test.

RESULTS

To analyze the effects of the E4032A mutation at the single channel-level, wild-type and mutant RyR1 were produced by transduction of myotubes obtained by differentiation of 1B5 cells, an immortal myogenic cell line lacking a functional copy of the native RyR1 gene. As observed after reconstitution of native RyR1 from rabbit skeletal muscle junctional SR, wild-type RyR1 channels isolated from transduced 1B5 myotubes have been shown to exhibit large-conductance events (>400 pS for 100 mM Cs$^+$) with rapid, full-conductance gating transitions that are sensitive to modulation by Ca^{2+}, Mg^{2+}, caffeine, adenine nucleotides, ryanodine, and ruthenium red (Moore et al., 1998; Wang et al., 2000). Consistent with this previous work, we found that reconstituted wild-type RyR1 was strongly activated by exposure on the *cis* side to 100 μM Ca^{2+} (P_o ranging from 0.64 to 0.95, averaging 0.88 ± 0.08, n = 9), whereas the reconstituted E4032A-RyR1 was not (P_o too low to be measured, data not shown). In fact, several attempts to activate E4032A-RyR1 by *cis* exposure to a combination of activators (100 μM free Ca^{2+}, 2 mM ATP, and 2 mM caffeine) yielded negligible gating activity (Fig. 1, *top panel*, P_o < 0.0001). Thus, the E4032A mutation severely impairs activation by Ca^{2+} compared to wild-type RyR1 isolated from 1B5 myotubes and measured under similar conditions (Wang et al., 2000). We also examined the behavior of E4032A-RyR1 in the presence of mM Ca^{2+} in the *cis* solution since mM Ca^{2+} was found to cause measurable activation (P_o of 0.016) of RyR3 bearing the mutation (E3885A) homologous to that of E4032A-RyR1 (Chen

et al., 1998). When Ca^{2+} was raised to 1–5 mM, channel activity of E4032A-RyR1 exhibited only a modest enhancement (not shown), but reached a discernible level when a combination of strong activators was included in the *cis* chamber (Fig. 1, *middle* and *bottom panels*). However, unlike wild-type RyR1 and E3885A-RyR3, the gating activity of E4032A-RyR1 remained extremely low (typically $P_o < 0.005$) under all the conditions tested. The inability to cause an appreciable P_o for E4032A-RyR1 makes it difficult to quantify the effects of the mutation on Ca^{2+} activation. However, comparison of P_o for wild-type RyR1 in 100 μM Ca^{2+} (0.88) with the maximal P_o that we observed for E4032A-RyR1 under any condition (<0.005 in 2 mM Ca^{2+}, 2 mM ATP and 2 mM caffeine), suggests a >100-fold decrement in the maximal ability of Ca^{2+} to cause activation, alone or together with other strongly activating ligands.

To determine whether the E4032A mutation affects the ability of RyR1 to mediate EC coupling, the mutant protein was expressed in dyspedic myotubes, which lack endogenous RyR1 (Takeshima et al., 1994), but do express other key proteins of the triad junction, including α_{1S} (Buck et al., 1997). When cells expressing E4032A-RyR1 were focally stimulated (100 V, 10 ms), no evoked contractions were observed in the 28 cells tested, whereas all 13 cells expressing wild-type RyR1 contracted when stimulated. Thus, the E4032A mutation impairs the ability of RyR1 to mediate EC coupling. To obtain a more quantitative estimate of this impairment, we used the whole-cell variant of the patch clamp technique together with Fluo-3 to measure Ca^{2+} transients. As shown in Fig. 2 a, depolarization caused Ca^{2+} release via E4032A-RyR1, but this was about fivefold smaller than for RyR1. Specifically, at $+40$ mV, ΔF values for E4032A-RyR1-expressing cells was 35.31 ± 4.47 ($n = 28$) compared with 181.67 ± 20.36 ($n = 27$) for RyR1 (significantly different, $p \ll 0.001$). The rate of fluorescence release (ΔF/ms) at $+40$ mV showed a similar reduction from 7.84 ± 1.18 ($n = 27$) for RyR1 to 1.54 ± 0.20 for E4032A-RyR1 ($n = 28$). No transients were observed in dyspedic myotubes ($n = 10$). The amplitude of Ca^{2+} transients for E4032A-RyR1 showed a sigmoidal dependence on voltage similar to that of RyR1 (Fig. 2 b). Under the assumption that SR Ca^{2+} content was similar in myotubes expressing wild-type and mutant RyR1, the reduced amplitude of Ca^{2+} transients for E4032A-RyR1 implies that the mutant RyRs are activated to a lesser extent during EC coupling.

The results above show that although E4032A-RyR1 does not release enough Ca^{2+} to mediate electrically evoked contractions, it does support depolarization-evoked Ca^{2+} transients of small amplitude. To determine whether Ca^{2+} release via E4032A-RyR1 reflects skeletal-type EC coupling, Ca^{2+} transients were measured in individual cells for a test pulse to $+40$ mV immediately before and after addition of 0.5 mM Cd^{2+} and 0.1 mM La^{3+} to the bathing
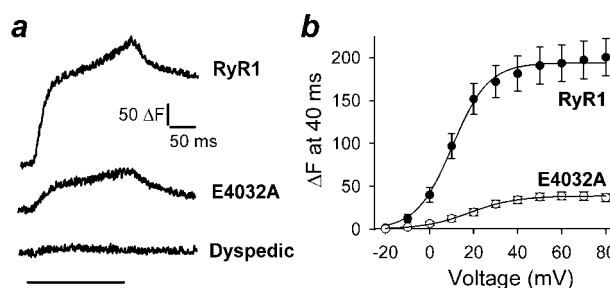

FIGURE 2 The mutation E4032A in RyR1 reduces depolarization-induced Ca^{2+} release. (*a*) Representative Ca^{2+} transients (ΔF) from control dyspedic myotubes and dyspedic myotubes expressing E4032A-RyR1 or RyR1. The transients were elicited by a 200-ms depolarization to $+40$ mV (*black bar*). (*b*) Voltage dependence of the Ca^{2+}-induced increase of fluorescence measured 40 ms after the onset of depolarization for E4032A-RyR1 ($n = 28$) and RyR1 ($n = 27$). Ca^{2+} transients were absent in dyspedic myotubes ($n = 10$; not shown). The smooth curves represent best-fits of the Boltzmann expression (Eq. 1) with values of $k = 10.66$ and 8.13 mV, and $V_{1/2} = 18.34$ and 10.36 mV, for E4032A-RyR1 and wild-type RyR1, respectively.

medium. This addition effectively blocked Ca^{2+} current (Fig. 3 a, lower sets of traces), without altering the amplitude of the Ca^{2+} transients for either RyR1 or E4032A-RyR1 (Fig. 3 a, upper set of traces; Fig. 3 b). These results, together with the sigmoidal voltage-dependence of the transients (Fig. 2 b) imply that E4032A-RyR1 is able to mediate skeletal-type EC coupling.

In addition to receiving the EC coupling signal from α_{1S}, RyR1 transmits a retrograde signal that acts to increase the magnitude of the L-type Ca^{2+} current produced by α_{1S} (Nakai et al., 1996). Fig. 4 illustrates representative L-type Ca^{2+} currents (*a*) and average peak current versus voltage relationships (*b*) for dyspedic myotubes and myotubes expressing RyR1 or E4032A-RyR1. Although having similar voltage dependence, the peak currents differed considerably in magnitude for the three groups. At $+30$ mV, peak current density for E4032A-RyR1 (-6.53 ± 0.54 pA/pF, $n = 28$) was much larger ($p \ll 0.001$) than for dyspedic myotubes (-1.35 ± 0.30 pA/pF, $n = 10$), but not quite as large ($p < 0.005$) as for wild-type RyR1 (-10.11 ± 0.79 pA/pF, $n = 35$).

Because caffeine is a potentiator of Ca^{2+}-induced Ca^{2+} release (Meissner et al., 1997), it was of interest to determine whether caffeine affected depolarization-evoked Ca^{2+} release via E4032A-RyR1. The application of 10 mM caffeine to myotubes at the holding potential caused a large release of Ca^{2+} in RyR1-expressing myotubes (113.36 $\pm$ 28.76 ΔF; $n = 4$), but caused no release in E4032A-RyR1-expressing myotubes ($n = 7$; Fig. 5 a). This difference provides additional support for the idea that the E4032A mutation impairs Ca^{2+} activation. Despite having little effect on E4032A-RyR1 at the holding potential, 10 mM caffeine did cause a large increase in the amplitude of the depolarization-induced Ca^{2+} tran-
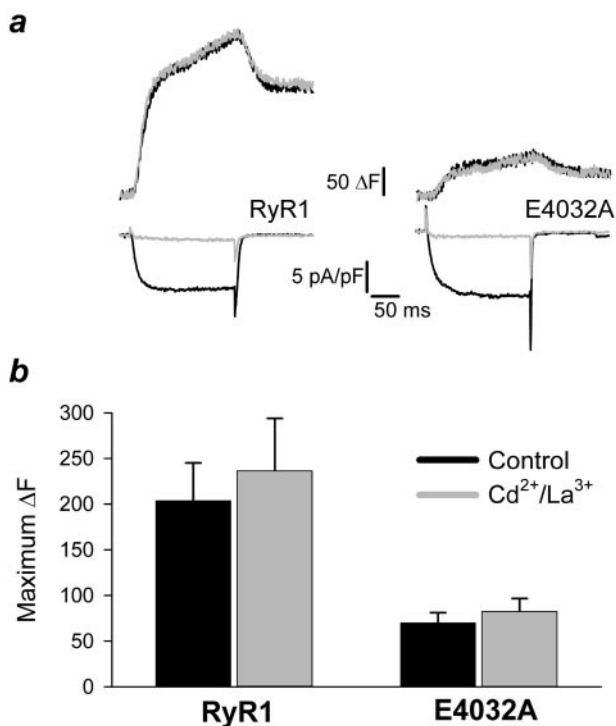
FIGURE 3 E4032A-RyR1 supports skeletal-type EC coupling. (*a*) Representative Ca^{2+} transients and Ca^{2+} currents recorded at +40 mV before (*black*) and after (*gray*) application of Cd^{2+} and La^{3+} to block Ca^{2+} entry. The transients before and after the block of Ca^{2+} current almost completely overlapped, indicating that, like RyR1, E4032A-RyR1 supports skeletal-type EC coupling. (*b*) Average Ca^{2+} transient amplitude at +40 mV was not significantly different before (*black*) and after (*gray*) application of Cd^{2+} and La^{3+} for either RyR1 (*n* = 7; *p* > 0.5) or E4032A-RyR1 (*n* = 7; *p* > 0.5).

sient (Fig. 5 *b*), which on average was about fourfold (ΔF increased from 20.6 $\pm$ 3.0 to 78.2 $\pm$ 12.39, *n* = 10, *p* < 0.001). In the presence of 10 mM caffeine, the ΔF versus voltage relationship for E4032A-RyR1 remained sigmoidal, although steeper and somewhat left-shifted compared to control (Fig. 5 *c*). Additionally, caffeine increased the initial rate of rise of the Ca^{2+} transient in E4032A-RyR1-expressing cells from 0.81 $\pm$ 0.13 to 3.25 $\pm$ 0.58 $\Delta F/ms$ (*n* = 10; *p* $\ll$ 0.001).

In addition to increasing the amplitude of the Ca^{2+} transient in myotubes expressing E4032A-RyR1, the application of 10 mM caffeine also caused the half-rise time for activation of Ca^{2+} current to decrease from 23.4 $\pm$ 2.2 ms to 11.6 $\pm$ 0.6 ms (*n* = 10, *p* < 0.001). The effect of caffeine on the rate of activation of Ca^{2+} current appeared to be unrelated to its effect on Ca^{2+} release. First, the half-rise time for activation of Ca^{2+} current in the absence of caffeine was similar (*p* $\geq$ 0.15) in cells expressing wild-type RyR1 (18.1 $\pm$ 1.3 ms, *n* = 35) to that in cells expressing E4032A-RyR1 (21.2 $\pm$ 1.7 ms, *n* = 28), despite the fact that Ca^{2+} release flux was severalfold lower for E4032A-RyR1 (see above). Second, 10 mM caffeine caused half-rise times
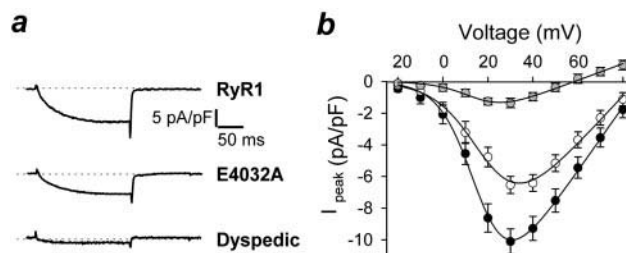

FIGURE 4 The E4032A mutation of RyR1 slightly impairs retrograde signaling. (*a*) Representative peak Ca^{2+} currents (at +20 mV) from control, E4032A-RyR1-expressing and RyR1-expressing dyspedic myotubes. (*b*) Average peak current versus voltage relationships for dyspedic myotubes (*gray circles*; *n* = 10) and dyspedic myotubes expressing E4032A-RyR1 (*white circles*; *n* = 28) or RyR1 (*black circles*; *n* = 35).

for Ca^{2+} current in four cells expressing wild-type RyR1 to decrease from 18.0 $\pm$ 2.0 ms to 10.3 $\pm$ 0.3 ms, despite causing a reduction in the amount of calcium released (maximum ΔF fell from 355.3 $\pm$ 60.5 to 139.3 $\pm$ 52.9, presumably because the caffeine caused a loss of Ca^{2+} from the SR).

DISCUSSION

In this paper we have characterized the consequences of the mutation E4032A on the function of RyR1. Expression in 1B5 dyspedic myotubes followed by reconstitution into planar lipid bilayers demonstrated that E4032A-RyR1 is not appreciably activated by Ca^{2+}, even when the additional strong activators caffeine and ATP are also present. Expression in primary dyspedic myotubes followed by whole-cell measurements showed that the E4032A mutation reduced depolarization-induced Ca^{2+} release about fivefold as compared with wild-type RyR1, and as a result impaired the ability of RyR1 to mediate EC coupling. This reduction implies that the E4032A mutation impairs activation of Ca^{2+} release in response to depolarization (under the assumption that the mutation did not cause a reduction in SR Ca^{2+} content). The Ca^{2+} release that did occur for E4032A-RyR1 appeared to reflect skeletal-type EC coupling because its amplitude showed a sigmoidal voltage-dependence and because it persisted after the addition of extracellular Cd^{2+} and La^{3+} to block Ca^{2+} influx. In addition to being able to mediate EC coupling (at a reduced level), E4032A-RyR1 was also able to cause retrograde enhancement of L-type Ca^{2+} current via α_{1S}. Compared to control dyspedic cells, the L-current density was about fivefold larger for E4032A-RyR1-expressing myotubes, a retrograde enhancement somewhat smaller than that found for wild-type RyR1 (7.5-fold).

The results reported here raise the question of whether there is a direct relationship between activation of RyR1 by α_{1S} (in response to depolarization) and the activation of RyR1 by Ca^{2+}. Fig. 6 illustrates two extreme possibilities
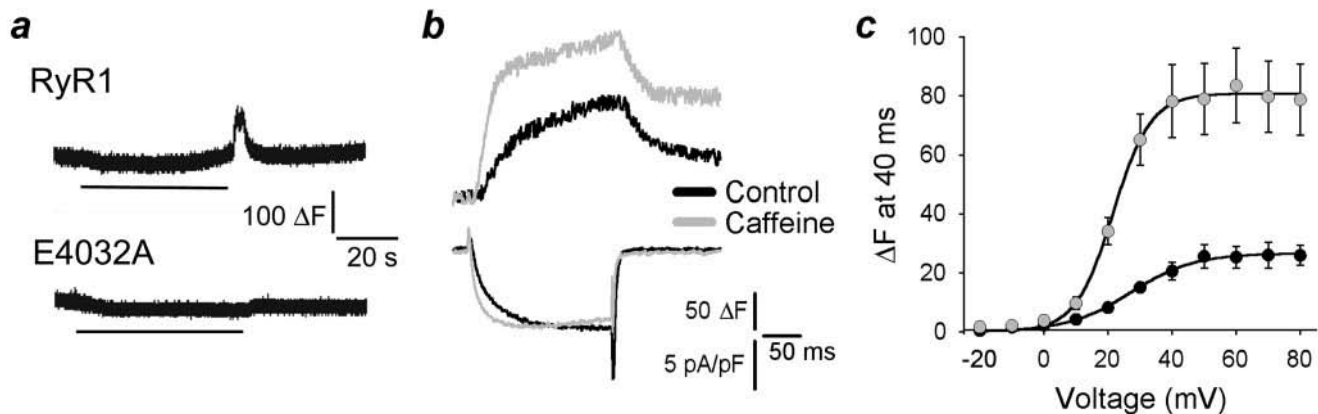
FIGURE 5 Caffeine enhances depolarization-induced Ca^{2+} release for E4032A-RyR1. (a) Caffeine (10 mM) causes Ca^{2+} release from RyR1 expressing myotubes at the holding potential (-80 mV), but not from myotubes expressing E4032A-RyR1. Similar responses were obtained from a total of eight cells expressing E4032A-RyR1 ($\Delta F = 0$) and four cells expressing RyR1 ($\Delta F = 113.36 \pm 28.76$). Bath perfusion (horizontal bar) was used to introduce 10 mM caffeine, which then remained for the duration of the experiment. (b) Representative Ca^{2+} transients (top) and Ca^{2+} currents (bottom) at +40 mV before (black) and after (gray) the application of 10 mM caffeine. (c) Voltage dependence of the Ca^{2+} transients measured in E4032A-RyR1-expressing myotubes before and after application of 10 mM caffeine ($n = 10$). The data have been fitted with Eq. 1, as shown by the smooth curve. The values for k are 9.87 and 5.89 mV and for $V_{1/2}$ are 27.13 and 21.69 mV before and after caffeine was applied.

for the relationship between the two modes of activation. In scheme 1 the two modes of activation are completely independent, whereas in scheme 2 the only function of α_{1S} is to lower the threshold for activation by Ca^{2+} such that resting Ca^{2+} is sufficient to cause activation. Scheme 2 can be viewed as the converse of the proposal that α_{1S} functions to raise the threshold for the inhibition of RyR1 by Mg^{2+} (Lamb and Stephenson, 1991, 1994). If scheme 1 were correct, it might be possible to abolish activation by Ca^{2+} without any effect on depolarization-induced Ca^{2+} release. If scheme 2 were correct, then eliminating activation by Ca^{2+} would also abolish activation by α_{1S}. An argument against scheme 2 is that the E4032A mutation does not abolish depolarization-induced Ca^{2+} release, although one
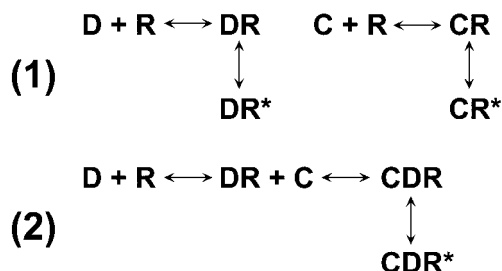

FIGURE 6 Two schemes indicating pathways for activation of RyR1 (R), with horizontal arrows indicating reversible binding steps, and vertical arrows indicating conformational changes that convert RyR1 to the active state, indicated by the asterisk (*). According to scheme 1, depolarization causes a domain of the α_{1S} (D, possibly the II-III loop) to bind to and activate RyR1 independently of the activation occurring in response to Ca^{2+} (C). In scheme 2, the binding of D cannot by itself induce activation, which requires Ca^{2+}. Specifically, the binding of D increases the affinity of RyR1 for Ca^{2+} so that the threshold for activation is below that of the resting myoplasmic concentration.

could argue that E4032A-RyR1 has sufficient residual Ca^{2+} sensitivity to support a reduced level of depolarization-induced release. However, the effect of the mutation on depolarization-induced release is trivial compared to its effect on Ca^{2+} activation. Specifically, the bilayer experiments indicate that the E4032A mutation depressed activation by Ca^{2+} by >100-fold, whereas the reduction in depolarization-induced release was only 5-fold. Thus, it seems unlikely that Ca^{2+} activation plays an obligatory role in depolarization-induced release.

Because the E4032A mutation affects activation of RyR1 both by depolarization (as shown in myotubes) and by Ca^{2+} (as shown in bilayers), it is possible that the two activation pathways interact with one another in intact cells. Such an interaction could take place either in the same RyR molecule or between different RyR molecules. In the latter case, for example, the Ca^{2+} initially released from some RyRs in response to depolarization could induce release from adjacent RyRs via Ca^{2+} activation (Rios and Pizarro, 1991; Schneider, 1994). Put another way, ΣCa^{2+} = VGCR + CICR, where ΣCa^{2+} is the total amount of Ca^{2+} released by depolarization, part of which is from RyRs activated directly by DHPRs (VGCR) and part from RyRs secondarily activated by Ca^{2+} (Rios and Pizarro, 1991; Schneider, 1994). Accordingly, the interpretation of the fivefold reduction in depolarization-induced Ca^{2+} release in myotubes expressing E4032A-RyR1 depends on knowing whether the mutation affects only CICR. If this were the case, one would conclude that CICR is responsible for the bulk of total Ca^{2+} release and that VGCR contributes only a relatively small fraction ($\sim$20%). Alternatively, it is common that synergistic interactions occur between ligands that activate an allosteric protein; that is, activation of a single RyR by depo-

larization would be potentiated by Ca^{2+}, and activation of an RyR by Ca^{2+} would be potentiated by depolarization. Thus, a mutation that depressed CICR might well be expected to depress VGCR. However, even if the two activation pathways were completely independent, there is no reason why the E4032A mutation could not affect both.

Caffeine is widely thought to act on ryanodine receptors by reducing the threshold for activation by Ca^{2+} (e.g., Meissner et al., 1997). Thus, it is of interest that the application of 10 mM caffeine caused an approximately fourfold augmentation in the depolarization-evoked release of Ca^{2+} by E4032A-RyR1 without having any discernible effect at the holding potential. This augmentation appears to be difficult to explain if the only effect of caffeine is on the Ca^{2+}-activation threshold. In bilayers, Ca^{2+} caused minimal activation of E4032A-RyR1, even when 2 mM caffeine was present (Fig. 1). Thus, rather than simply shifting the apparent Ca^{2+}-activation threshold by enhancing the binding affinity of the activator site for Ca^{2+}, caffeine may have the more general effect on ryanodine receptors of reducing the energy of channel activation and shifting the equilibrium toward the open state.

A limitation in our comparison of the properties of wild-type RyR1 and E4032A-RyR1 is that we have no independent measure of the expression level or junctional targeting of the mutant protein. However, it seems unlikely that expression or targeting is significantly altered because E4032A-RyR1 caused a substantial increase in the amplitude of L-type Ca^{2+} current compared to that in non-injected dyspedic cells. This result suggests that E4032A-RyR1 is expressed at a level similar to that of RyR1, and is like RyR1 in being able to increase the magnitude of current normalized by the number of α_{1S} subunits in the plasma membrane (Nakai et al., 1996; Avila et al., 2001). Avila et al. (2001) also found that retrograde signaling by E4032A-RyR1 was similar to that of RyR1, although E4032A-RyR1 lacked the ability to cause an up-regulation of the number of α_{1S} subunits in the plasma membrane that occurs on the time scale of several days. Because E4032A-RyR1 was able to mediate retrograde enhancement of Ca^{2+} current despite a greatly reduced orthograde signaling (EC coupling), it suggests that the two processes are not tightly linked. Further support for this idea is that caffeine caused a large increase in depolarization-induced Ca^{2+} release by E4032A-RyR1 without much effect on the amplitude of L-type current (Fig. 5). Thus, it does not seem likely that Ca^{2+} release is the signal for retrograde enhancement of L-type current.

The depression of Ca^{2+} activation by the E4032A mutation could mean that the mutation directly affects the Ca^{2+} binding site for activation (Chen et al., 1998; Li and Chen, 2001). An alternative possibility is that the E4032A mutation induces a folding error that impairs channel-opening conformational changes (Fessenden et al., 2001). According to this latter interpretation, calcium would be less effective at overcoming the impairment of channel-opening than would orthograde activation via the DHPR, and this orthograde activation would be directly potentiated by caffeine. However, no matter which interpretation is correct, the essential conclusion from our work is that Ca^{2+} activation of RyR1 is not essential for the initiation of skeletal-type EC coupling.

REFERENCES

Armstrong, C. M., F. M. Bezanilla, and P. Horowicz. 1972. Twitches in the presence of ethylene glycol bis(-aminoethyl ether)-*N,N*-9-tetraacetic acid. *Biochim. Biophys. Acta.* 267:605–608.

Avila, G., K. M. S. O'Connell, L. A. Groom, and R. T. Dirksen. 2001. Ca^{2+} release through ryanodine receptors regulates skeletal muscle L-type Ca^{2+} channel expression. *J. Biol. Chem.* 276:17732–17738.

Buck, E. D., H. T. Nguyen, I. N. Pessah, and P. D. Allen. 1997. Dyspedic mouse skeletal muscle expresses major elements of the triadic junction but lacks detectable ryanodine receptor protein and function. *J. Biol. Chem.* 272:7360–7367.

Chen, S. R. W., K. Ebisawa, X. Li, and L. Zhang. 1998. Molecular identification of the ryanodine receptor Ca^{2+} sensor. *J. Biol. Chem.* 273:14675–14678.

Fabiato, A. 1985. Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *J. Gen. Physiol.* 85:247–289.

Fessenden, J. D., L. Chen, Y. Wang, C. Franzini-Armstrong, P. D. Allen, and I. N. Pessah. 2001. Ryanodine receptor point mutant E4032A reveals an allosteric interaction with ryanodine. *Proc. Natl. Acad. Sci. U.S.A.* 98:2865–2870.

García, J., T. Tanabe, and K. G. Beam. 1994. Relationship of calcium transients to calcium currents and charge movements in myotubes expressing skeletal and cardiac DHP receptors. *J. Gen. Physiol.* 103:125–147.

Giannini, G., E. Clementi, R. Ceci, G. Marziali, and V. Sorrentino. 1992. Expression of a ryanodine receptor-Ca^{2+} channel that is regulated by TGF-β. *Science.* 257:91–94.

Gonzalez-Serratos, H., R. Valle-Aguilera, D. A. Lathrop, and M. C. Garcia. 1982. Slow inward calcium currents have no obvious role in muscle excitation-contraction coupling. *Nature.* 298:292–294.

Grabner, M., R. T. Dirksen, and K. G. Beam. 1998. Tagging with green fluorescent protein reveals a distinct subcellular distribution of L-type and non-L-type Ca^{2+} channels expressed in dysgenic myotubes. *Proc. Natl. Acad. Sci. U.S.A.* 95:1903–1908.

Hakamata, Y., J. Nakai, H. Takeshima, and K. Imoto. 1992. Primary structure and distribution of a novel ryanodine receptor/calcium release channel from rabbit brain. *FEBS Lett.* 312:229–235.

Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene.* 77:51–59.

Jurman, M. E., L. M. Boland, Y. Liu, and G. Yellen. 1994. Visual identification of individual transfected cells for electrophysiology using antibody-coated beads. *Biotechniques.* 17:876–881.

Lamb, G. D., M. A. Cellini, and D. G. Stephenson. 2001. Different Ca^{2+} releasing action of caffeine and depolarization in skeletal muscle fibres of the rat. *J. Physiol.* 531:715–728.

Lamb, G. D., and D. G. Stephenson. 1991. Effect of Mg^{2+} on the control of Ca^{2+} release in skeletal muscle fibres of the toad. *J. Physiol.* 434:507–528.

Lamb, G. D., and D. G. Stephenson. 1994. Effect of intracellular pH and [Mg^{2+}] on excitation-contraction coupling in skeletal muscle fibres of the rat. *J. Physiol.* 478:331–339.

Li, P., and S. R. W. Chen. 2001. Molecular basis of Ca^{2+} activation of the mouse cardiac Ca^{2+} release channel (ryanodine receptor). *J. Gen. Physiol.* 118:33–44.

Meissner, G., E. Rios, A. Tripathy, and D. A. Pasek. 1997. Regulation of skeletal muscle Ca^{2+} release channel (ryanodine receptor) by Ca^{2+} and monovalent cations and anions. *J. Biol. Chem.* 272:1628–1638.

Moore, R. A., H. Nguyen, J. Galceran, I. N. Pessah, and P. D. Allen. 1998. A transgenic myogenic cell line lacking ryanodine receptor protein for homologous expression studies: reconstitution of Ry1R protein and function. *J. Cell Biol.* 140:843–851.

Nabauer, M., G. Callewaert, L. Cleeman, and M. Morad. 1989. Regulation of calcium release is gated by calcium current, not gating charge, in cardiac myocytes. *Science.* 244:800–803.

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.

Nakai, J., T. Imagawa, Y. Hakamata, M. Shigekawa, H. Takeshima, and S. Numa. 1990. Primary structure and functional expression from cDNA of the cardiac ryanodine receptor/calcium release channel. *FEBS Lett.* 271:169–177.

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 Ca^{2+} channels. *J. Biol. Chem.* 273:13403–13406.

Protasi, F., C. Franzini-Armstrong, and P. D. Allen. 1998. Role of ryanodine receptors in the assembly of calcium release units in skeletal muscle. *J. Cell Biol.* 140:831–842.

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.

Rios, E., and G. Brum. 1987. Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle. *Nature.* 325:717–720.

Rios, E., and G. Pizarro. 1991. Voltage sensor of excitation-contraction coupling in skeletal muscle. *Physiol. Rev.* 71:849–908.

Schneider, M. F. 1994. Control of calcium release in functioning skeletal muscle fibers. *Annu. Rev. Physiol.* 56:463–484.

Takeshima, H., M. Iino, H. Takekura, M. Nishi, J. Kuno, O. Minowa, H. Takano, and T. Noda. 1994. Excitation-contraction uncoupling and muscular degeneration in mice lacking functional skeletal muscle ryanodine-receptor gene. *Nature.* 369:556–559.

Takeshima, H., S. Nishimura, T. Matsumoto, H. Ishida, K. Kangawa, N. Minamino, H. Matsuo, M. Ueda, M. Hanaoka, T. Hirose, and S. Numa. 1989. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature.* 339:439–445.

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.

Wang, Y., C. Fraefel, F. Protasi, R. A. Moore, J. D. Fessenden, I. N. Pessah, A. DiFrancesco, X. Breakefield, and P. D. Allen. 2000. HSV-1 amplicon vectors are a highly efficient gene delivery system for skeletal muscle myoblasts and myotubes. *Am. J. Physiol. Cell Physiol.* 278:C619–C626.

Zorzato, F., J. Fujii, K. Otsu, M. Phillips, N. M. Green, F. A. Lai, G. Meissner, and D. H. MacLennon. 1990. Molecular cloning of cDNA encoding human and rabbit forms of the Ca^{2+} release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 265:2244–2256.