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Ryanodine modification of RyR1 retrogradely affects L-type Ca^{2+} channel gating in skeletal muscle

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Abstract

In skeletal muscle, there is bidirectional signaling between the L-type Ca^{2+} channel (1,4-dihydropyridine receptor; DHPR) and the type 1 ryanodine-sensitive Ca^{2+} release channel (RyR1) of the sarcoplasmic reticulum (SR). In the case of “orthograde signalling” (i.e., excitation-contraction coupling), the conformation of RyR1 is controlled by depolarization-induced conformational changes of the DHPR resulting in Ca^{2+} release from the SR. “Retrograde coupling” is manifested as enhanced L-type current. The nature of this retrograde signal, and its dependence on RyR1 conformation, are poorly understood. Here, we have examined L-type currents in normal myotubes after an exposure to ryanodine (200 μM , 1 hour at 37°C) sufficient to lock RyR1 in a non-conducting, inactivated, conformational state. This treatment caused an increase in L-type current at less depolarized test potentials in comparison to myotubes similarly exposed to vehicle as a result of a ~5 mV hyperpolarizing shift in the voltage-dependence of activation. Charge movements of ryanodine-treated myotubes were also shifted to more hyperpolarizing potentials (~13 mV) relative to vehicle-treated myotubes. Enhancement of the L-type current by ryanodine was absent in *dyspedic* (RyR1 null) myotubes, indicating that ryanodine does not act directly on the DHPR. Our findings indicate that in retrograde signaling, the functional state of RyR1 influences conformational changes of the DHPR involved in activation of L-type current. This raises the possibility that physiological regulators of the conformational state of RyR1 (e.g., Ca^{2+} , CaM, CaMK, redox potential) may also affect DHPR gating.

Keywords

DHPR; α_{1S} ; $\text{Ca}_v1.1$; L-type; RyR1; EC coupling; skeletal muscle; ryanodine

Introduction

Conformational coupling between plasma membrane channels and ER proteins is emerging as an important mechanism by which diverse cell types regulate intracellular Ca^{2+} levels. “Orthograde” coupling, in which the plasma membrane channel controls ER Ca^{2+} release independent of the entry of extracellular Ca^{2+} , has been described in both skeletal muscle (Beam and Horowicz 2004) and neurons (Ouardouz *et al.* 2003; De Crescenzo *et al.* 2006; Kim *et al.* 2007). “Retrograde” coupling whereby a resident ER protein modulates the activity of a plasma membrane ion channel was first described between ryanodine receptors and L-type Ca^{2+} channels in skeletal muscle and not long after in neurons (Nakai *et al.* 1996; Nakai *et al.* 1997; Nakai *et al.* 1998a; Grabner *et al.* 1999; Avila and Dirksen 2000; Chavis *et al.* 1996). The nature of retrograde coupling in each of these cell types remains poorly understood.

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Here, we have examined how conformational state of an ER protein (type 1 ryanodine-sensitive intracellular Ca^{2+} release channel; RyR1) influences gating transitions of a plasma membrane protein (L-type Ca^{2+} channel or 1,4-dihydropyridine receptor; DHPR) in skeletal muscle.

In skeletal muscle, the L-type Ca^{2+} channel serves as the voltage sensor for excitation-contraction (EC) coupling by triggering gating of RyR1 in response to depolarization of the transverse tubular membrane (Beam and Horowicz 2004). The resultant Ca^{2+} efflux into the myoplasm from the sarcoplasmic reticulum (SR) engages the contractile filaments. Since communication between the DHPR and RyR1 is rapid and does not require Ca^{2+} entry via the L-type channel itself, it is believed that there is a physical interaction between the two proteins (Armstrong *et al.* 1973; Tanabe *et al.* 1990; Dirksen and Beam 1999). This view is supported by ultrastructural evidence demonstrating that intra-membranous particles in the plasma membrane, which appear to represent DHPRs, are arranged into groups of four (“tetrads”) with a spacing that places them in register with the four subunits of every other RyR1 (Block *et al.* 1988; Takekura *et al.* 1994; Protasi *et al.* 2002; Takekura *et al.* 2004; Sheridan *et al.* 2006).

In addition to the orthograde signal that is transmitted from the DHPR to RyR1, a retrograde signal was revealed by the observation that L-type Ca^{2+} currents of *dyspedic* (RyR1 null) myotubes were substantially smaller than L-type currents of myotubes harvested from phenotypically normal littermates despite similar membrane expression of the DHPR (Nakai *et al.* 1996). Since retrograde coupling persists in the presence of the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) and depends on the integrity of some of the same structural elements of the DHPR $\text{Ca}_v1.1$ (α_{1S}) subunit that support orthograde coupling (Grabner *et al.* 1999; Nakai *et al.* 1998b; Wilkens *et al.* 2001; Kugler *et al.* 2004; Takekura *et al.* 2004; Bannister *et al.* 2009), it has been postulated that retrograde coupling is supported by protein-protein interactions between RyR1 and the DHPR.

Studies that have examined the pharmacology of RyR1 in lipid bilayers and/or isolated SR vesicles have demonstrated that lower concentrations of ryanodine ($< 1 \mu\text{M}$) induce high P_o gating (Rosseau *et al.* 1987; Smith *et al.* 1988; Buck *et al.* 1992), while higher concentrations of ryanodine irreversibly lock the channel in a permanently inactivated state (Buck *et al.* 1992; Zimányi *et al.* 1992). Significantly, conformational changes induced by high ryanodine result in a 2 nm decrease in distance between adjacent tetradic particles, supporting the hypothesis that DHPRs are docked to RyR1 (Paolini *et al.* 2004). The observations of Paolini and colleagues (2004) raise the question of whether the conformational state of RyR1 might also regulate gating transitions of the DHPRs. Here, we demonstrate that the high ryanodine-induced conformation of RyR1 enhances skeletal muscle L-type Ca^{2+} current by shifting activation in the hyperpolarizing direction. Thus, our results indicate that retrograde communication between these channels depends on the conformational state of RyR1 and that, in turn, DHPR gating may be retrogradely influenced by physiological modulators of RyR1.

Methods

Myotube culture and ryanodine treatment

All procedures involving mice were approved by the University of Colorado-Denver Institutional Animal Care and Use Committee. Primary cultures of phenotypically normal (+/+ or +/*mdg*) and *dyspedic* (RyR1^{-/-}) myotubes were prepared as described previously (Beam and Franzini-Armstrong 1997). Cultures were grown for 4–5 days in a humidified 37°C incubator with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM; #15-017-CM, Mediatech, Herndon, VA), supplemented with 10% fetal bovine serum/10% horse serum (Hyclone Laboratories, Logan, UT). This medium was then replaced with differentiation medium (DMEM supplemented with 2% horse serum). Ryanodine (#K6017, Sigma, St. Louis,

MO) was reconstituted in 40 or 100% EtOH and diluted to either 200 or 10 μ M in differentiation medium.

Measurement of L-type Ca^{2+} currents and charge movements

Electrophysiological experiments were performed at room temperature ($\sim 25^\circ\text{C}$) 3–5 days following differentiation. Pipettes were fabricated from borosilicate glass and had resistances of $\sim 2.0 \text{ M}\Omega$ when filled with internal solution, which consisted of (mM): 140 Cs-aspartate, 10 $\text{Cs}_2\text{-EGTA}$, 5 MgCl_2 , and 10 HEPES, pH 7.4 with CsOH. The external solution contained (mM): 145 TEA-Cl, 10 CaCl_2 , 0.003 tetrodotoxin (TTX), and 10 HEPES, pH 7.4 with TEA-OH. For measurement of intramembrane charge movements, ionic currents were blocked by the addition of 0.5 mM CdCl_2 + 0.1 mM LaCl_3 to the external solution. Electronic compensation was used to reduce the effective series resistance (usually to $< 1 \text{ M}\Omega$) and the time constant for charging the linear cell capacitance (usually to $< 0.5 \text{ ms}$). L-type currents were corrected for linear components of leak and capacitive current by digital scaling and subtraction of the average of eleven, 30-mV hyperpolarizing pulses from a holding potential of -80 mV . All charge movements were corrected for linear cell capacitance and leakage currents using a -P/6 subtraction protocol. Filtering was at 2 kHz (eight pole Bessel filter; Frequency Devices, Inc.) and digitization was either at 10 kHz (L-type currents) or 20 kHz (charge movements). Voltage clamp command pulses were exponentially rounded with a time constant of 50–500 μs and a 1-s prepulse to -20 mV followed by a 50-ms repolarization to -50 mV was administered before the test pulse (prepulse protocol; see Adams *et al.* 1990) to inactivate low-voltage activated Ca^{2+} channels and voltage-gated Na^+ channels. Cell capacitance was determined by integration of a transient from -80 mV to -70 mV using Clampex 8.0 and was used to normalize current amplitudes (pA/pF).

L-type current-voltage (I-V) curves were fitted according to:

$$I = G_{\max} * (V - V_{\text{rev}}) / \{1 + \exp[-(V - V_{1/2})/k_G]\}, \quad (1)$$

where I is the current for the test potential V, V_{rev} is the reversal potential, $G_{\max}$ is the maximum Ca^{2+} channel conductance, $V_{1/2}$ is the half-maximal activation potential and k_G is the slope factor.

Plots of the integral of the ON charge movement (Q_{on}) as a function of test potential (V) were fitted according to:

$$Q_{\text{on}} = Q_{\max} / \{1 + \exp[(V_Q - V)/k_Q]\}, \quad (2)$$

where $Q_{\max}$ is the maximal Q_{on} , V_Q is the potential causing movement of half the maximal charge, and k_Q is a slope parameter.

Analysis

Figures were made using the software program SigmaPlot (version 7.0, Systat Software, Inc., Chicago, IL). All data are presented as mean $\pm$ SEM. Statistical comparisons were by unpaired, two-tailed *t*-test, with $p < 0.05$ considered significant.

Results

Skeletal muscle L-type Ca^{2+} currents are potentiated by ryanodine

Since long exposures to a high concentration of ryanodine persistently block RyR1 (Zimányi *et al.* 1992) and cause rearrangements of DHPRs within tetrads (Paolini *et al.* 2004), we probed whether a similar exposure to ryanodine would impact the properties of L-type currents. We found that 200 μM ryanodine for > 1 hour at 37°C caused a small, but consistent, hyperpolarizing shift in the voltage-dependence of activation (Figure 1A and B; Table 1) resulting in an increase in L-type current density at less depolarized test potentials in comparison to myotubes similarly exposed to EtOH vehicle. For example, the treatment with ryanodine caused the average current elicited by a depolarization to +20 mV to increase from -7.6 ± 0.8 pA/pF ($n = 16$) to -11.3 ± 1.2 pA/pF ($n = 28$; $p < 0.05$; Figure 1C). Similar effects were observed in experiments in which myotubes were exposed to 20 μM ryanodine for 12–18 hours at 37°C (data not shown). However, the magnitude and voltage-dependence of L-type currents were not affected by the application of a lesser concentration of ryanodine (10 μM) for 30 minutes at room temperature (Figure 1D; Table 1).

Ryanodine shifts the voltage-dependence of skeletal muscle charge movements

Intramembrane charge movements were measured to investigate further the effects of high ryanodine treatment on DHPR gating. The maximal charge movement (Q_{max}) in myotubes exposed to 200 μM ryanodine did not differ significantly ($p > 0.5$) from that of myotubes exposed to the vehicle ($Q_{\text{max}} = 4.8 \pm 0.4$ nC/ μF ; $n = 10$ vs. 5.3 ± 0.7 nC/ μF ; $n = 7$, respectively; Figure 2A–C). However, ryanodine pre-treatment induced a substantial hyperpolarizing shift in the voltage-dependence of charge movement compared to control myotubes (-15.4 ± 2.2 mV vs. -2.3 ± 2.2 mV, respectively; $p < 0.005$; Figure 2C). Thus, high ryanodine pre-treatment caused hyperpolarizing shifts in both charge movements and activation of L-type Ca^{2+} current

Ryanodine-induced potentiation of L-type current requires RyR1

As a test of whether the effects of ryanodine treatment on L-type channel gating were a consequence of altered RyR1 conformation or of a direct action of ryanodine on the DHPR, the effects of ryanodine pre-treatment on L-type currents were assessed in *dyspedic* (RyR1 null) myotubes. At +30 mV, the L-type current density of ryanodine-treated *dyspedic* myotubes (-2.0 ± 0.4 pA/pF; $n = 5$; Figure 3B) was similar to that of vehicle-treated *dyspedic* myotubes (-1.9 ± 0.3 pA/pF; $n = 7$; $p = 0.9$; Figure 3A). Moreover, no shift in channel activation was evident in ryanodine-treated *dyspedic* myotubes (Figure 3C; Table 1). Thus, ryanodine-mediated potentiation of skeletal muscle L-type currents required the presence of RyR1.

Discussion

In the present study, we have shown that a > 1 hr exposure to 200 μM ryanodine causes hyperpolarizing shifts in both the activation of skeletal muscle L-type Ca^{2+} current (Figure 1) and in voltage-dependent charge movement (Figure 2). Importantly, these effects depended on the presence of RyR1 because ryanodine had no effect on L-type Ca^{2+} current in *dyspedic* myotubes (Figure 3).

The hypothesis that EC coupling depends on physical linkages between the DHPR and RyR1 implies that manipulations directly affecting the conformation of RyR1 might influence conformational changes of the DHPR (although there is no *a priori* way to estimate the magnitude or polarity of such possible effects). Electrophysiologically, DHPR conformational changes are reflected in both Ca^{2+} ionic current (Tanabe *et al.* 1988) and membrane-bound charge movements (Schneider and Chandler 1973; Ríos and Brum 1987; Adams *et al.* 1990). In regard to L-type Ca^{2+} currents, previous studies of amphibian skeletal muscle have reported

no effects of ryanodine (García *et al.* 1991a; Gonzalez and Caputo 1996), except for a for a ~5 mV depolarizing shift in activation (Squecco *et al.* 2004). This opposite effect (as opposed to the hyperpolarizing shift observed in the present study) may be a consequence of the difference in preparations (amphibian muscle *vs.* mammalian myotubes). Alternatively, the paradigm used for application of ryanodine in the studies of amphibian muscle ($\leq 100 \mu\text{M}$ at 10–17°C for ≤ 40 minutes) may not have caused the ryanodine receptor to enter into the deeply inactivated state produced by our use of 200 μM ryanodine applied for >1 hr at 37°C. In this regard, we also observed little effect of 10 μM ryanodine applied to mouse myotubes for 30 minutes at ~25°C (Figure 1D).

Consistent with our present results, Balog and Gallant (1999) observed a hyperpolarizing shift in L-type current activation, and a small increase in current density, when ryanodine (1 mM in the patch pipette) was dialyzed into normal mouse myotubes. Our current results both confirm and extend their results in that: *i*) the ryanodine-induced effects on the L-type current were dependent on the expression of RyR1 (Figure 3), and *ii*) the increased density was not due to insertion of additional DHPRs into the plasma membrane (Figure 2; see below). Balog and Gallant (1999) postulated that the ryanodine-induced shift in L-type current activation resulted from a decrease in triadic Ca^{2+} concentration since dialysis of BAPTA produced a similar hyperpolarizing shift. It should be noted, however, that in our experiments on both control and ryanodine-treated myotubes, EGTA (10 mM) was dialyzed for >5 minutes prior to recording L-type currents (and even longer for charge movements). Consequently, resting Ca^{2+} levels in both experimental groups should have been nearly identical. Thus, we propose an alternative explanation based on previous work showing that treatment with high ryanodine results in a ~2 nm decrease in distance between adjacent tetradic particles (Paolini *et al.* 2004). Specifically, this altered disposition of tetrads, which is one indicator of altered protein-protein interactions between RyR1 and the DHPR, also affects the gating behaviour of the DHPR. The observation that the effects of ryanodine on the L-type current required the presence of RyR1 also supports the view that the alterations in DHPR gating were a consequence of altered conformational coupling between the two channels.

Several previous studies have examined the effects of ryanodine on charge movements in both amphibian and mammalian preparations. With respect to Q_{β} the major component of charge movement in amphibian muscle, a number of studies (García *et al.* 1991a; Gonzalez and Caputo 1996; Huang 1996; Huang 1998; Squecco *et al.* 2004) have reported no appreciable effect of ryanodine ($\leq 200 \mu\text{M}$ at 2–17°C for 25–120 minutes). However, Gonzales and Caputo (1996) reported that the recovery from depolarization-induced immobilization of Q_{β} is inhibited by ryanodine treatment (100 μM , ~25 minutes at 10°C). García *et al.* (1991a) reported that ryanodine (100 μM , ~40 minutes at 17°C) eliminated Q_{γ} , the “hump” of extra charge that is superimposed on the falling phase of Q_{β} at voltages near the threshold for eliciting Ca^{2+} release. If, as some have hypothesized (Csernoch *et al.* 1991; García *et al.* 1991b), Q_{γ} is a result of the released Ca^{2+} causing the movement of additional Q_{β} , then the block of Q_{γ} could be explained by ryanodine block of Ca^{2+} release. In contrast to the block of Q_{γ} observed by García *et al.* (1991a), Squecco *et al.* (2004) reported that 100 μM ryanodine shifted amphibian Q_{γ} in the depolarizing direction without abolishing it. In rat *extensor digitorum longus* fibres, where only a single component of charge movement is evident, 10 μM ryanodine had little effect on the total charge moved (Fryer *et al.* 1989). Our current results are consistent with this observation in that a higher concentration of ryanodine did not significantly affect Q_{max} (Figure 2; Table 2). In addition, we observed a substantial (~13 mV) hyperpolarizing shift in gating charge relative to control myotubes (Figure 2; Table 2). Such a shift provides a mechanistic basis for the concurrent shift in the I-V relationship (Figure 1; Table 1) and larger L-type current amplitude at weaker test potentials.

Our work now shows that opening transitions of the skeletal L-type Ca^{2+} channel are directly affected by the conformational state of RyR1 such that the ryanodine-inactivated RyR1 enhances entry of the $\text{Ca}_v1.1$ -containing channel into the open state as reflected both in shifted voltage-dependence of activation and increased steady-state current at weaker test depolarizations. The observation that pharmacological modulation of RyR1 conformation affects DHPR gating raises the possibility that physiological modulation of RyR1 (e.g., by Ca^{2+} , CaM, CaMK, PKA, redox potential; for a review, see Meissner 2002) may also influence DHPR activity and thus the cellular processes which are dependent on voltage-driven conformational changes of the DHPR.

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Abbreviations used in this paper

DHPR	1,4 dihydropyridine receptor
EC	excitation-contraction
RyR	ryanodine-sensitive intracellular Ca^{2+} release channel
SR	sarcoplasmic reticulum

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Fig. 1.

Skeletal muscle L-type Ca^{2+} currents are affected by 200 μ M ryanodine. Representative currents evoked from -50 mV to the indicated test potentials are shown for myotubes treated with EtOH vehicle (A) or 200 μ M ryanodine (B) for >1 hour at 37°C . Peak I-V relationships are shown in (C). (D) Peak I-V relationships for myotubes treated with 10 μ M ryanodine or EtOH (0.02%) vehicle for 0.5 hour at room temperature ($\sim 25^\circ\text{C}$). Currents were evoked at 0.1 Hz by test potentials ranging from -20 mV through $+80$ mV in 10 mV increments following a prepulse protocol (Adams *et al.*, 1990). Current amplitudes were normalized by linear cell capacitance (pA/pF). The smooth curves are plotted according to Equation 1, with best fit parameters presented in Table 1. Throughout, error bars represent $\pm$ SEM.

Fig. 2.

Charge movements are shifted to more hyperpolarized potentials by 200 μ M ryanodine. Representative charge movements evoked from -50 mV to -30, -10, 10 and 30 mV are shown for myotubes treated with EtOH vehicle (A) or 200 μ M ryanodine (B) for >1 hour at 37°C. Q-V relationships are shown in (C). The smooth curves are plotted according to Equation. 2, with best fit parameters presented in Table 2.

Fig. 3.

L-type currents in *dyspedic* myotubes are not potentiated by 200 μ M ryanodine. Representative current families evoked from -50 mV to -50 mV to -10 , 0 , 10 , 20 and 30 mV for *dyspedic* myotubes treated with EtOH vehicle (A) or 200 μ M ryanodine (B) for >1 hour at 37°C . Peak I-V relationships are shown in (C). The smooth curves represent best fits of Equation 1, which yielded the parameters presented in Table 1.

Table 1

L-type current conductance fit parameters

	<i>G-V</i>		
	<i>G</i> _{max} (nS/nF)	<i>V</i> _{1/2} (mV)	<i>k</i> _G (mV)
normal + high EtOH vehicle	234 ± 13 (16)	19.9 ± 1.2	5.4 ± 0.3
normal + 200 μM ryanodine	275 ± 22 (28)	15.1 ± 1.6 *	5.6 ± 0.2
normal + low EtOH vehicle	271 ± 19 (5)	21.3 ± 2.0	5.3 ± 0.7
normal + 10 μM ryanodine	238 ± 22 (6)	18.2 ± 2.0	4.9 ± 0.2
<i>dyspedic</i> + high EtOH vehicle	56 ± 6 (7)	20.6 ± 1.5	6.0 ± 0.7
<i>dyspedic</i> + 200 μM ryanodine	48 ± 9 (5)	20.3 ± 1.3	8.0 ± 1.0

Ryanodine-treated cells were exposed to 200 μM ryanodine in differentiation medium for >1 hour at 37°C or to 10 μM ryanodine in differentiation medium for 0.5 hour at room temperature (~25°C). Normal control cells were exposed to vehicle for an equal duration and the *G-V* values were not significantly different from those recently published for untreated, normal myotubes (Bannister *et al.* 2008; $p > 0.05$, *t*-test). Data are fit according to Equation 1 (see Methods). Data are given as mean ± SEM, with the numbers in parentheses indicating the number of myotubes tested.

The asterisk indicates a significant difference (

*, $p < 0.05$; *t*-test) between ryanodine-treated cells and the matched vehicle control group.

Table 2

Charge movement fit parameters

	<i>Q-V</i>		
	<i>Q_{max}</i> (nC/μF)	<i>Q_{1/2}</i> (mV)	<i>k_Q</i> (mV)
normal + high EtOH vehicle	5.3 ± 0.7 (7)	-2.3 ± 2.2	9.3 ± 0.7
normal + 200 μM ryanodine	4.8 ± 0.4 (10)	-15.4 ± 2.2**	9.8 ± 1.4

Ryanodine-treated cells were exposed to 200 μM ryanodine in differentiation medium for >1 hour at 37°C. Data are fit according to Equation 2 (see Methods). Data are given as mean ± SEM, with the numbers in parentheses indicating the number of myotubes tested.

Asterisks indicate a significant difference (

**, $p < 0.002$; *t*-test).