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RGK protein-mediated impairment of slow depolarization-dependent Ca^{2+} entry into developing myotubes

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Abbreviations: EC, excitation-contraction; ECCE, excitation-coupled Ca^{2+} entry; NFAT, nuclear factor of activated T cells; RGK, Rad-Rem-Rem2-Gem/Kir; RyR1, type 1 ryanodine-sensitive intracellular Ca^{2+} release channel; SR, sarcoplasmic reticulum

Three physiological functions have been described for the skeletal muscle 1,4-dihydropyridine receptor ($Ca_V1.1$): (1) voltage-sensor for excitation-contraction (EC) coupling, (2) L-type Ca^{2+} channel, and (3) voltage-sensor for slow depolarization-dependent Ca^{2+} entry. Members of the RGK (Rad, Rem, Rem2, Gem/Kir) family of monomeric GTP-binding proteins are potent inhibitors of the former two functions of $Ca_V1.1$. However, it is not known whether the latter function that has been attributed to $Ca_V1.1$ is subject to modulation by RGK proteins. Thus, the purpose of this study was to determine whether Rad, Gem and/or Rem inhibit the slowly developing, persistent Ca^{2+} entry that is dependent on the voltage-sensing capability of $Ca_V1.1$. As a means to investigate this question, Venus fluorescent protein-fused RGK proteins (V-Rad, V-Rem and V-Gem) were overexpressed in "normal" mouse myotubes. We observed that such overexpression of V-Rad, V-Rem or V-Gem in myotubes caused marked changes in morphology of the cells. As shown previously for YFP-Rem, both L-type current and EC coupling were also impaired greatly in myotubes expressing either V-Rad or V-Gem. The reductions in L-type current and EC coupling were paralleled by reductions in depolarization-induced Ca^{2+} entry. Our observations provide the first evidence of modulation of this enigmatic Ca^{2+} entry pathway peculiar to skeletal muscle.

Introduction

Three distinct functions for the skeletal muscle 1,4-dihydropyridine receptor ($Ca_V1.1$) have been described: (1) it serves as the voltage-sensor for the excitation-contraction (EC) coupling,[1-3] (2) it conducts L-type Ca^{2+} current,[3] and (3) it supports slow, depolarization-dependent Ca^{2+} entry that has been proposed to permeate independently of the conventional channel pore.[4,5] Of these functions, the fundamental abilities of $Ca_V1.1$ to engage EC coupling by gating the type 1 ryanodine receptor (RyR1) and to conduct L-type current are well-documented.[6] However, the specific route of Ca^{2+} permeation for depolarization-dependent entry remains a matter of debate.[7,8]

Depolarization-dependent Ca^{2+} entry, also referred to as Excitation-Coupled Ca^{2+} Entry (ECCE), was first identified in *dysgenic* ($Ca_V1.1$ null) myotubes expressing a pore mutant with a minimal ability to conduct Ca^{2+}.[4] Since this type of Ca^{2+} entry was absent in naïve *dysgenic* and *dyspedic* (RyR1 null) myotubes,[4,5,9] the existence of a distinct Ca^{2+} channel whose gating was coupled to depolarization-dependent rearrangements in the EC coupling apparatus was proposed. Later on, the abundance of genetic (i.e., absence in *dysgenic*, *dyspedic* and $Ca_V\beta_1$ null myotubes) and pharmacological (i.e., sensitivity to Gd^{3+}, La^{3+}, 2-aminoethyl diphenylborate, SKF 96356, nifedipine and dantrolene) characteristics shared by depolarization-dependent Ca^{2+} entry and the conventional L-type means of Ca^{2+} entry led to the idea that these pathways were, for the most part, one and the same.[10,11] Paradoxically, these genetic and pharmacological commonalities between the Ca^{2+} entry pathways have confounded a definitive test of the latter view and, for this reason, the molecular basis of depolarization-dependent Ca^{2+} entry remains unsettled.[7,8]

Rem, a member of the RGK (Rad, Rem, Rem2, Gem/Kir) family of small GTP-binding proteins, profoundly inhibits skeletal muscle EC coupling by interacting with the L-type Ca^{2+} channel complex. Early work showed that exogenous expression of Rem in C_2C_{12} cells almost ablated voltage-dependent SR Ca^{2+} release.[12] Subsequent work performed with cultured myotubes demonstrated that such inhibition occurs without affecting SR Ca^{2+} store content or function of RyR1, and is accompanied a substantial reduction in L-type current amplitude.[13] Even though the inhibitory effects of Rem on the EC coupling voltage-sensor and L-type channel functions of $Ca_V1.1$ are well documented,

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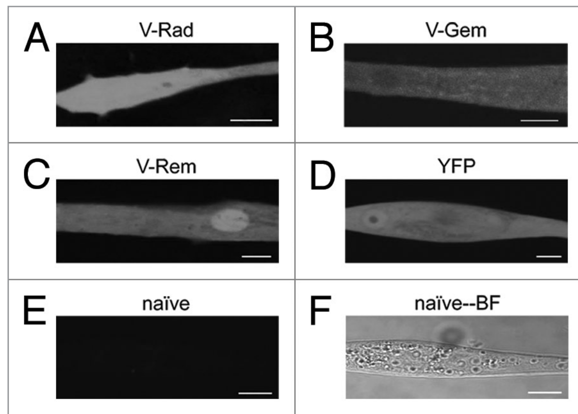

Figure 1. Exogenous expression of Venus-tagged RGK protein constructs in cultured myotubes. Confocal fluorescence images of normal mouse myotubes overexpressing V-Rad, V-Gem, V-Rem and YFP are shown as labeled in panels (**A–D**). Fluorescence and brightfield images of a naïve normal myotube are shown in panels (**E and F**), respectively. The image in (**E**) was acquired with identical laser settings as the image in (**A**). Bars-10 μm.

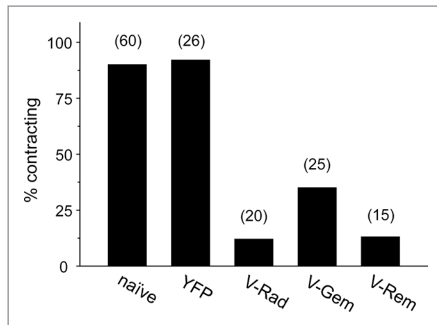

Figure 2. RGK proteins blunt EC coupling in cultured myotubes. Overexpression of a V-RGK protein construct reduces the fraction of myotubes contracting in response to a 100 V, 10 ms electrical stimulus. For each group, the total number of myotubes tested is indicated above each bar.

the ability of RGK proteins to modulate slow depolarization-dependent Ca^{2+} entry remains unknown. In this study, we have found that Rad, Gem and Rem all profoundly reduce depolarization-dependent Ca^{2+} entry in developing myotubes.

Results

RGK proteins alter myotube morphology and inhibit EC coupling

As a means to investigate the effects of the RGK proteins thought to be endogenously expressed in skeletal muscle (i.e., Rad, Gem and Rem)[14-18] on the known functions of $Ca_V1.1$, we overexpressed one of these muscle RGK protein isoforms in cultured "normal" myotubes and confirmed expression via a fused Venus fluorescent protein tag. When viewed with confocal microscopy, V-Rad, V-Gem and V-Rem each displayed a diffuse fluorescence distribution similar to that of unfused YFP (**Fig. 1A–D**); very little, if any, background fluorescence was present in naïve myotubes (**Fig. 1E and F**). Interestingly, the V-RGK proteins all appeared to have deleterious effects on myotube morphology. In general, V-RGK protein expressing myotubes had central protuberances and were flat and narrow compared with adjacent non-transfected myotubes.

We, with others, previously demonstrated that YFP-Rem reduces the frequency of myotube contractions without altering SR Ca^{2+} stores or RyR1 function, indicating a specific effect on $Ca_V1.1$.[13] To test whether Rad and/or Gem are also capable of inhibiting EC coupling in myotubes, we elicited contractions with electrical field stimulation (100 V, 10 ms). Naïve normal myotubes and normal myotubes expressing non-fused YFP both readily contracted upon stimulation (54 of 60 and 24 of 26 myotubes tested, respectively; **Fig. 2**). In contrast, exogenous expression of V-Rad, V-Gem or V-Rem each caused a marked reduction in the fraction of contracting myotubes (3 of 25, 7 of 20, and 2 of 15 myotubes tested, respectively).

Rad, Rem and Gem inhibit L-type currents in myotubes

Although YFP-Rem is known to reduce L-type current density in myotubes,[13] the abilities of Rad and Gem to blunt skeletal muscle L-type current have not been investigated. For this reason, we recorded L-type currents from myotubes expressing either V-Rad or V-Gem; we also deemed it necessary to assess the effects of V-Rem on the L-type current in order to compare with its effects on depolarization-dependent Ca^{2+} entry (see below). In control experiments, naïve normal myotubes produced large, slowly activating L-type currents that were not different than those observed in normal myotubes expressing YFP (-12.4 ± 0.9 pA/pF; n = 16 and -11.7 ± 0.7 pA/pF; n = 16, respectively, at +30 mV; $P > 0.05$, unpaired t test; **Fig. 3A**). By comparison, L-type currents were reduced by ~90%, ~85% and ~65% in normal myotubes expressing V-Rad (-1.3 ± 0.5 pA/pF; n = 9; $P < 0.001$, unpaired t test; **Fig. 3B**), V-Gem (-1.9 ± 1.1 pA/pF; n = 9; $P < 0.001$, unpaired t test; **Fig. 3C**) or, as expected, V-Rem (-4.5 ± 1.7 pA/pF; n = 11; $P < 0.001$, unpaired t test; **Fig. 3D**). We also observed small, but significant, depolarizing shifts in $Ca_V1.1$ activation in myotubes expressing V-Rad, V-Gem and V-Rem (**Table 1**).

RGK proteins inhibit depolarization-dependent Ca^{2+} entry in myotubes

Previous work[12,13] and the experiments described above have established that all RGK isoforms thought to be expressed in skeletal muscle are capable of inhibiting the ability of $Ca_V1.1$ to serve as the voltage-sensor for EC coupling and to conduct L-type Ca^{2+} current. Yet, it remains unknown whether RGK proteins affect depolarization-dependent Ca^{2+} entry into skeletal muscle. For this reason, we examined the effects of V-Rad, V-Rem and V-Gem expression on Ca^{2+} entry elicited by long, weak depolarizations. As expected, control myotubes expressing an mcherry transfection marker displayed substantial slowly activating Ca^{2+} transients in response to elevation of extracellular K^+ from 5 mM to 80 mM (1.27 ± 0.13 ΔF/F; n = 7; **Fig. 4A–D**). The transients were almost certainly indicative of Ca^{2+} entry because: (1) SR Ca^{2+} release was blocked by preincubation with 200 μM ryanodine,[10,11] and (2) no quantifiable transients were observed in nine naïve myotubes exposed to 100 μM Gd^{3+} prior

to and during depolarization ($P < 0.001$, unpaired t test *vs*. control; **Fig. 4D**). In contrast, the entry mediated-transients were nearly ablated by overexpression of either V-Rad (0.23 ± 0.11 ΔF/F; n = 6; **Fig. 4A**), V-Gem (0.19 ± 0.08 ΔF/F; n = 11; **Fig. 4B**) or V-Rem (0.02 ± 0.01 ΔF/F; n = 7; all $P < 0.001$, *ANOVA vs.* control; **Fig. 4C and D**).

Discussion

The most significant finding of this study is that Rad, Rem and Gem each inhibit slow depolarization-dependent Ca^{2+} influx into skeletal muscle cells (**Fig. 4**) in such a way that parallels their abilities to dampen EC coupling and to reduce L-type Ca^{2+} current (**Figs. 2 and 3**, respectively). Thus, RGK proteins inhibit all three physiological processes that are dependent on the voltage-sensing ability of $Ca_V1.1$.

Depolarization-dependent Ca^{2+} entry is influenced by expression of various constituents of the EC coupling apparatus including the α_{1S} and β_{1a} subunits of $Ca_V1.1$,[4,5,10] RyR1,[4,5,9-11] JP-45,[19] and calsequestrin1.[19] In the present work, we present the first example of modulation of this mysterious mode of Ca^{2+} influx into skeletal muscle by dynamically-regulated cell signaling molecules (**Fig. 4**). In this regard, RGK proteins may regulate a variety of cellular processes that have been linked to depolarization-dependent Ca^{2+} entry such as maintenance of myoplasmic Ca^{2+} levels during activity,[4] activation of transcription via NFAT translocation[20] and regulation of muscle strength[19] and mass (see below). Moreover, altered RGK protein-mediated modulation of depolarization-dependent Ca^{2+} entry may potentially be involved in the pathophysiology of malignant hyperthermia,[21-23] central core disease,[20] and rippling muscle disease[24] since enhanced depolarization-dependent Ca^{2+} entry is characteristic of these muscle disorders.

The similar degree of inhibition between depolarization-dependent Ca^{2+} entry and L-type current represents yet another commonality between these 2 modes of Ca^{2+} flux into skeletal muscle (please see Table 4 of ref. 10). Such parallel inhibition provides further support for the idea that L-type current is, in fact, the predominant means of Ca^{2+} entry upon depolarization.[10,11] However, the evidence presented here is still not conclusive because it is plausible that RGK proteins may stem depolarization-dependent Ca^{2+} entry through another channel which shares a common gating mechanism with $Ca_V1.1$.[4] Even so, the mounting circumstantial evidence in favor of depolarization-dependent Ca^{2+} entry passing though the conventional $Ca_V1.1$ pore casts heavy doubt on the existence of a coupled 2-channel mechanism.

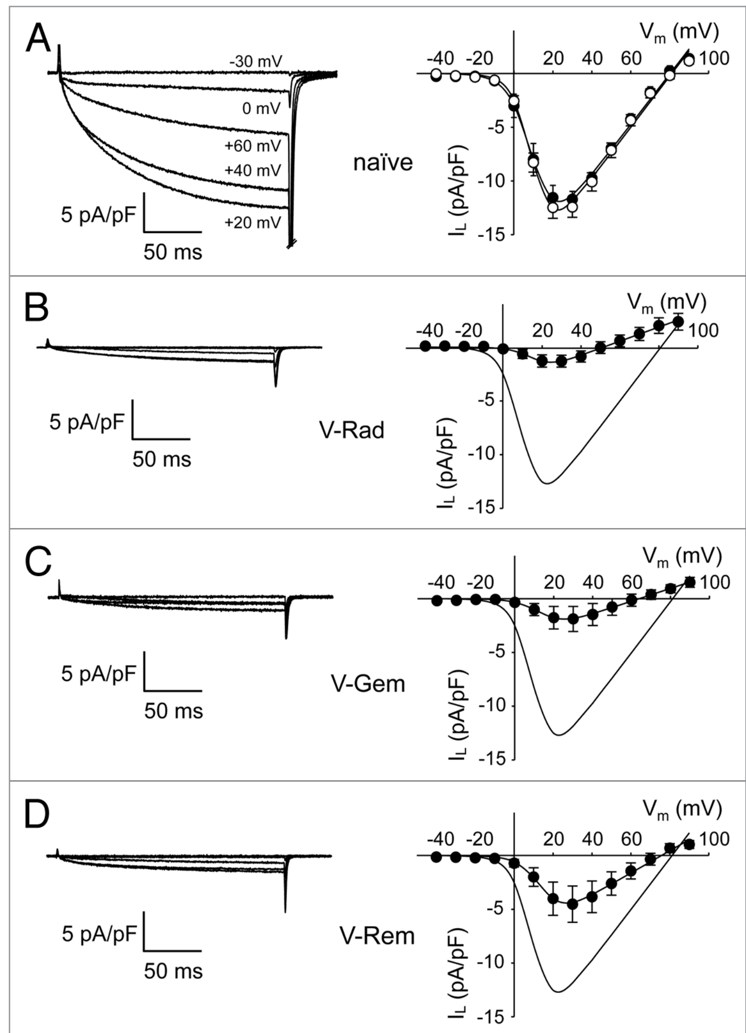

Figure 3. RGK proteins reduce L-type Ca^{2+} current amplitude in cultured myotubes. Representative recordings of skeletal muscle L-type currents elicited by 200 ms depolarizations from -50 mV to the indicated potentials are shown for a naïve normal myotube (**A**, left) and normal myotubes expressing V-Rad (**B**, left), V-Gem (**C**, left) or V-Rem (**D**, left). The peak I-V relationships corresponding to each current family are shown in the *right* panels. In panel (**A**, right), the I-V relationship for naïve normal myotubes expressing unfused YFP is also shown. In panels (**B–D**) the smooth line is the fit of I-V relationship for naïve myotubes. Currents were evoked at 0.1 Hz by test potentials ranging from -40 mV through +90 mV in 10 mV increments following a prepulse protocol (Adams et al., 1991; please see Materials and Methods). The smooth curves are plotted according to Eq. 1 with fit parameters displayed in **Table 1**. Throughout, error bars represent ± SEM.

We also observed that overexpression of Rad, Rem or Gem had adverse effects on the morphology of cultured myotubes (**Fig. 1A–C**). Unfortunately, we were unable to quantify the impact of RGK protein overexpression on morphology because developing myotubes in a culture dish exhibit a broad range of morphological characteristics. Even so, our qualitative observations raise the possibility that RGK proteins may engage atrophic signaling through their ability to downregulate one or more of $Ca_V1.1$'s 3 functions. In this regard, in vivo siRNA-mediated knockdown of $Ca_V1.1$ expression is known to cause profound atrophy and fibrosis in mouse hindlimb muscle.[25] By the same token, one may speculate that pathological increases in RGK protein expression may have similar atrophic effects in a broad spectrum of disorders that affect skeletal muscle. In particular, Rad expression is substantially enhanced in muscle obtained from type II diabetics,[14,16] amyotrophic lateral sclerosis patients,[18,26] dystrophic (*mdx*) mice[17] and dennervated

Table 1. Conductance fit parameters

	G-V		
	G_{max} (nS/nF)	$V_{1/2}$ (mV)	k_G (mV)
naïve	248 ± 15 (16)	10.3 ± 1.2	4.9 ± 0.4
YFP	241 ± 16 (16)	12.9 ± 1.4	4.8 ± 0.2
V-Rad	83 ± 17‡ (7)	18.9 ± 2.9*	4.6 ± 0.9
V-Gem	73 ± 23‡ (7)	17.5 ± 4.1*	6.8 ± 1.9
V-Rem	148 ± 31† (7)	16.7 ± 2.3*	5.5 ± 0.4

Data were fit by Eq. 1 and are given as mean ± SEM, with the numbers in parentheses indicating the number of myotubes tested. Since some individual current-voltage relationships for V-RGK protein expressing myotubes could not be fit with Eq. 1 because of the absence of inward Ca^{2+} current, these experiments have been omitted from the data presented in **Table 1**. However, these experiments were included in the calculation of peak current density presented in the Results section. For all the data given, the calculated average voltage error was < 5 mV. Asterisks indicate significant differences (* denotes *P* < 0.05; † denotes *P* < 0.01; ‡ denotes *P* < 0.001; *t* test vs. naïve control).

mice.[18] Thus, the potential role for RGK proteins as agents of pathological muscle atrophy highlights the importance of understanding the mechanisms that these small GTP-binding proteins employ to regulate L-type channel activity in skeletal muscle.

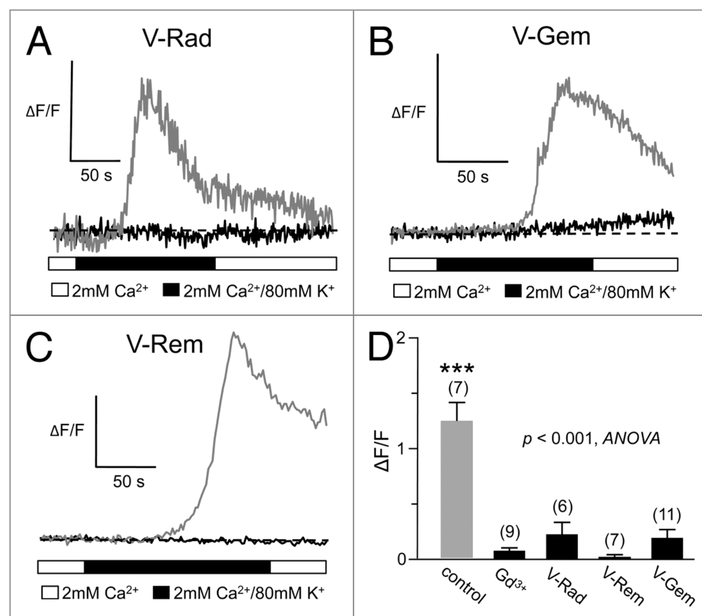

Figure 4. RGK proteins reduce slow depolarization-dependent Ca^{2+} entry into cultured myotubes. Representative Ca^{2+} transients evoked by global perfusion of 80 mM K^+ Ringer's solution for naïve normal mytoubes V-Rad (**A**), V-Gem (**B**) or V-Rem (**C**). In panels (**A–C**), the fluorescence record for the RGK protein of interest (gray) is accompanied by a fluorescence record obtained from a presumably naïve (i.e., mcherry negative) normal myotube in the same field (black). The sampling rate for the experiments shown in panels (**A and B**) was 1.3 Hz and the sampling rate for the experiment shown in (**C**) was 0.45 Hz. Myotubes were exposed to 200 μM ryanodine for > 1 hour at 37 °C prior to experiments in order to block the EC coupling component of the Ca^{2+} transient. A summary is shown in panel (**D**). In one set of experiments, naïve myotubes were exposed to Gd^{3+} (100 μM) continuously in the bath to confirm that the Ca^{2+} signal was generated by extracellular Ca^{2+} influx, as opposed to SR Ca^{2+} release. The number of cells tested per experiment is indicated in parentheses. Asterisks indicate significant differences relative to control mcherry-expressing myotubes (*** denotes *P* < 0.001; *ANOVA*).

Materials and Methods

Myotube culture and microinjection of cDNA

All procedures involving mice were approved by the University of Colorado Denver-Anschutz Medical Campus Institutional Animal Care and Use Committee. Primary cultures of normal (+/+ or +/*dysgenic*) myotubes were prepared from newborn mice as described previously.[27] For electrophysiological experiments, myoblasts were plated into 35 mm, plastic culture dishes (Falcon #353801) coated with ECL (Millipore #08–100). Myoblasts destined for imaging were plated into 35 mm culture dishes with laminin (Invitrogen #23017–015)-coated glass coverslip bottoms (MatTek #P35G-1.5–14-C).

Two days following differentiation, single nuclei were microinjected with a cDNA encoding a Venus fluorescent protein-RGK protein fusion construct (V-Rad, V-Gem or V-Rem, all generously provided by Drs. S.R. Ikeda and H.L. Puhl, III; 20 ng/μl) or YFP only (Clontech; 5 ng/μl). Myotubes to be used in Ca^{2+} imaging experiments were co-transfected with the plasmid pmCherry-C1 (Clontech; 5 ng/μl) as a means to identify V-RGK positive cells following exposure to Fluo 3-AM dye (see below). N-Benzyl-P-toluensulfonamide (BTS; 20 μM; Sigma-Aldrich #S949760) was added to the culture medium during microinjection in order to prevent contractions. Fluorescent myotubes were used in experiments 2 d following microinjection.

Imaging of V-RGK constructs

Live myotubes expressing YFP or V-RGK fusion constructs were examined in Rodent Ringer solution (in mM: 146 NaCl, 5

KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4 with NaOH) using the confocal laser scanning microscope LSM 510 META with a Zeiss Plan-Apochromat 63X 1.4NA oil-immersion objective. In these experiments, YFP/Venus was excited with the 488-nm line of an argon laser (30-milliwatt maximum output, operated at 50% or 6.3A, attenuated to 12%), which was directed to the cell via a 488 nm dual dichroic mirror. The emitted YFP/Venus fluorescence was directed to a photomultiplier equipped with a 505 nm long-pass filter. Confocal fluorescence intensity data were recorded as the average of 8 line scans per pixel and digitized at 8 bits, with photomultiplier gain adjusted such that maximum pixel intensities were no more than ~70% saturated.

Myotube contractions

Myotube contractions were elicited by 10 ms, 100 V stimuli. The extracellular pipette contained 150 mM NaCl and the bath solution was Rodent Ringers solution.

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

Pipettes were fabricated from borosilicate glass (~2.0 MΩ). The pipette solution consisted of (mM): 140 Cs-aspartate, 10 Cs_2-EGTA, 5 $MgCl_2$, and 10 HEPES, pH 7.4 with CsOH. The bath contained (mM): 145 TEA-Cl, 10 $CaCl_2$, 0.002 TTX, and 10 HEPES, pH 7.4 with TEA-OH. Linear capacitative and leakage currents were eliminated by -P/4 online subtraction. Electronic compensation was used to reduce the effective series resistance and the time constant for charging the linear cell capacitance. The corresponding mean values were: 2.6 ± 0.2 MΩ; 612 ± 50 μs and 311 ± 31 pF, respectively (n = 61 myotubes). Ca^{2+} currents were filtered at 2 kHz and digitized at 5 kHz. To measure macroscopic L-type current in isolation, a 1 s prepulse to -20 mV followed by a 100 ms repolarization to -50 mV was administered before the test pulse to inactivate voltage-gated Na^+ and T-type Ca^{2+} channels (please see ref. 28). Cell capacitance was determined by integration of a transient from -80 to -70 mV using Clampex versions 9.3 and 10.3 (Molecular Devices) and was subsequently used to normalize current amplitudes (pA/pF). Current-voltage (I-V) curves were fitted using the following equation:

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

where I is the normalized 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.

Assessment of depolarization-dependent Ca^{2+} entry

Myotubes were exposed to ryanodine (200 μM; Sigma-Aldrich #R6017) for > 1 h at 37 °C prior to experiments to eliminate voltage-dependent Ca^{2+} release from the SR. During the final 30 min of ryanodine exposure, myotubes were concurrently loaded with 5 μM Fluo 3-AM (Molecular Probes #F-1242) dissolved in Rodent Ringer's solution. Myotubes were then washed 3X in Rodent Ringer's solution with gentle agitation. After, a 10 min de-esterification period, Fluo 3-AM-loaded cells bathed in Rodent Ringer solution were then placed on the stage of an LSM 510 META scanning laser confocal microscope and viewed with a Zeiss Plan-Neofluar 10X 0.3NA objective. Positively transfected mytoubes were identified by mcherry fluorescence. Fluo 3-AM was excited with the 488-nm line of an argon laser (30-milliwatt maximum output, operated at 50% or 6.3A, attenuated to 5%). The emitted fluorescence was directed through a dual 488/543 dichroic mirror to a photomultiplier equipped with either a 505 nm long-pass filter or a 490–530 nm band-pass filter. Entry-mediated myoplasmic Ca^{2+} transients were elicited by application of high K^+ Ringer solution (in mM: 71 NaCl, 80 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4 with NaOH) via a manually-operated, gravity-driven global perfusion system. Fluorescence amplitude data are expressed as $\Delta F/F$, where F represents the baseline fluorescence prior to application of high K^+ Ringer solution and ΔF represents the change in peak fluorescence during the application of high K^+ Ringer solution. All experiments were performed at room temperature (~25 °C).

Analysis

All data are presented as mean ± SEM. Statistical comparisons were made by *ANOVA* (as appropriate) with $P < 0.05$ considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author contributions

C.F.R., D.B. and U.M. performed research, analyzed data, and wrote the paper. R.A.B. designed research, performed research, analyzed data, and wrote the paper.

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