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_{1.1}): (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_{1.1}. However, it is not known whether the latter function that has been attributed to Ca_{1.1} 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_{1.1}. 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_{1.1}) 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_{1.1} 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_{1.1} 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_{1.1} 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 C2C12 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_{1.1} are well documented,
we have found that Rad, Gem and Rem all profoundly reduce YFP (a diffuse fluorescence distribution similar to that of unfused 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 Ca2+ stores or RyR1 function, indicating a specific effect on CaV1.1.13 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-Rad on the L-type current in order to compare with its effects on depolarization-dependent Ca2+ 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 CaV1.1 activation in myotubes expressing V-Rad, V-Gem and V-Rem (Table 1).

RGK proteins inhibit depolarization-dependent Ca2+ entry in myotubes

Previous work12,13 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 CaV1.1 to serve as the voltage-sensor for EC coupling and to conduct L-type Ca2+ current. Yet, it remains unknown whether RGK proteins affect depolarization-dependent Ca2+ entry into skeletal muscle. For this reason, we examined the effects of V-Rad, V-Rem and V-Gem expression on Ca2+ entry elicited by long, weak depolarizations. As expected, control myotubes expressing an mcherry transfection marker displayed substantial slowly activating Ca2+ 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 Ca2+ entry because: (1) SR Ca2+ 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 Gd3+ prior

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.

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), on the known functions of CaV1,1,1,1 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.

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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 $\Delta F/F$; $n = 6$; Fig. 4A), V-Gem (0.19 ± 0.08 $\Delta F/F$; $n = 11$; Fig. 4B) or V-Rem (0.02 ± 0.01 $\Delta F/F$; $n = 7$; 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$_{1.1}$.

Depolarization-dependent Ca$^{2+}$ entry is influenced by expression of various constituents of the EC coupling apparatus including the $\alpha_{1S}$ and $\beta_{1a}$ subunits of Ca$_{1.1}$, RyR1, JP-45, and calsequestrin. 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, activation of transcription via NFAT translocation and regulation of muscle strength 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, central core disease, and rippling muscle disease 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. 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$_{1.1}$. Even so, the mounting circumstantial evidence in favor of depolarization-dependent Ca$^{2+}$ entry passing though the conventional Ca$_{1.1}$ pore casts heavy doubt on the existence of a coupled 2-channel mechanism.

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$_{1.1}$’s 3 functions. In this regard, in vivo siRNA-mediated knockdown of Ca$_{1.1}$ expression is known to cause profound atrophy and fibrosis in mouse hindlimb muscle. 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, amyotrophic lateral sclerosis patients, dystrophic (mdx) mice and denu...
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.

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 Ca²⁺ 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.

Table 1. Conductance fit parameters

|          | Gᵥₑₑₑₑ (nS/nF) | Vᵥₑₑₑₑ (mV) | kᵥₑₑₑₑ (mV) |
|----------|----------------|-------------|-------------|
| naive    | 248 ± 15       | 10.3 ± 1.2  | 4.9 ± 0.4   |
| YFP      | 241 ± 16       | 12.9 ± 1.4  | 4.8 ± 0.2   |
| V-Rad    | 83 ± 17        | 18.9 ± 2.9* | 4.6 ± 0.9   |
| V-Gem    | 73 ± 23        | 17.5 ± 4.1* | 6.8 ± 1.9   |
| V-Rem    | 148 ± 31       | 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 Ca²⁺ 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).

Figure 4. RGK proteins reduce slow depolarization-dependent Ca²⁺ entry into cultured myotubes. Representative Ca²⁺ transients evoked by global perfusion of 80 mM K⁺ Ringer’s solution for naïve normal myotubes 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 Ca²⁺ transient. A summary is shown in panel (D). In one set of experiments, naïve myotubes were exposed to Gd³⁺ (100 μM) continuously in the bath to confirm that the Ca²⁺ signal was generated by extracellular Ca²⁺ influx, as opposed to SR Ca²⁺ 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).
KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, pH 7.4 with NaOH) using 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 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, pH 7.4 with NaOH) using 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 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 ΔF/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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