Rem uncouples excitation–contraction coupling in adult skeletal muscle fibers

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In skeletal muscle, excitation–contraction (EC) coupling requires depolarization-induced conformational rearrangements in L-type Ca2+ channel (CaV1.1) to be communicated to the type 1 ryanodine-sensitive Ca2+ release channel (RYR1) of the sarcoplasmic reticulum (SR) via transient protein–protein interactions. Although the molecular mechanism that underlies conformational coupling between CaV1.1 and RYR1 has been investigated intensely for more than 25 years, the question of whether such signaling occurs via a direct interaction between the principal, voltage-sensing α1s subunit of CaV1.1 and RYR1 or through an intermediary protein persists. A substantial body of evidence supports the idea that the auxiliary β1s subunit of CaV1.1 is a conduit for this intermolecular communication. However, a direct role for β1s has been difficult to test because β1s serves two other functions that are prerequisite for conformational coupling between CaV1.1 and RYR1. Specifically, β1s promotes efficient membrane expression of CaV1.1 and facilitates the tetradic ultrastructural arrangement of CaV1.1 channels within plasma membrane–SR junctions. In this paper, we demonstrate that overexpression of the RGK protein Rem, an established β subunit–interacting protein, in adult mouse flexor digitorum brevis fibers markedly reduces voltage-induced myoplasmic Ca2+ transients without greatly affecting CaV1.1 targeting, intramembrane gating charge movement, or releasable SR Ca2+ store content. In contrast, a β1s-binding–deficient Rem triple mutant (R200A/L227A/H229A) has little effect on myoplasmic Ca2+ release in response to membrane depolarization. Thus, Rem effectively uncouples the voltage sensors of CaV1.1 from RYR1-mediated SR Ca2+ release via its ability to interact with β1s. Our findings reveal Rem-expressing adult muscle as an experimental system that may prove useful in the definition of the precise role of the β1s subunit in skeletal-type EC coupling.

INTRODUCTION

Excitation–contraction (EC) coupling is the physiological event in which muscle converts an electrical signal (plasma membrane depolarization) into mechanical work (muscle contraction). In the case of skeletal muscle, depolarization-induced conformational rearrangements within the L-type Ca2+ channel complex (CaV1.1) are coupled to gating of the type 1 ryanodine-sensitive Ca2+ release channel (RYR1) of the SR (Schneider and Chandler, 1973; Rios and Brum, 1987; Tanabe et al., 1988). The resultant Ca2+ efflux from the SR into the myoplasm via RYR1 activates the contractile filaments. Because SR Ca2+ release occurs rapidly in response to depolarization and independently of transient Ca2+ fluctuations, a conformational coupling mechanism appears to support communication between the two channels (see Bannister and Beam, 2013).

Although the roles of CaV1.1 and RYR1 as voltage sensor and SR Ca2+ release channel, respectively, have been established for quite some time (Tanabe et al., 1988; Nakai et al., 1996), the molecular mechanisms that support conformational coupling between these two channels remain undefined. One candidate structure to mediate such coupling is the intracellular segment that links repeats II and III of the principal α1s subunit of CaV1.1 (Tanabe et al., 1996; Lu et al., 1994; Nakai et al., 1998; Wilkens et al., 2001). Another viable candidate is the auxiliary β1s subunit of the CaV1.1 heteromultimer. In this regard, β1s is firmly established as being essential for EC coupling, as genetic deletion of β1 abolishes voltage-dependent SR Ca2+ release in both mammals and bony fish (Gregg et al., 1996; Ono et al., 2004; Schredelseker et al., 2005, 2009). Moreover, purified β1s subunits and β1s peptide fragments bind RYR1 in vitro and/or activate RYR1 in planar lipid bilayers (Cheng et al., 2005; Rebbeck et al., 2011; Karunasekara et al., 2012; Hernández-Ochoa et al., 2014). Still, the key roles of β1s in trafficking...
CaV1.1 to the plasma membrane (Gregg et al., 1996; Strube et al., 1996) and in the ultrastructural organization of CaV1.1 into the tetradic arrays requisite for EC coupling (Schredelseker et al., 2005, 2009; Daval et al., 2010, 2013; Eliot et al., 2014) have made testing a direct role for \( \beta_{1A} \) in communication between the voltage-sensing components of CaV1.1 and RyR1-mediated SR Ca\(^{2+} \) release highly problematic.

Members of the RGK (Rad, Rem, Rem2, Gem/Kir) family of monomeric G proteins inhibit L-type Ca\(^{2+} \) channels in a variety of physiological systems via interactions that occur primarily with the \( \beta \) subunit (Béguin et al., 2001, 2007; Finlin et al., 2003, 2006; Murata et al., 2004; Bannister et al., 2008; Yang et al., 2012; Romberg et al., 2014; Xu et al., 2015; reviewed recently by Yang and Colecraft, 2013). In the present study, we have examined the impact of Rem on EC coupling in adult mouse flexor digitorum brevis (FDB) fibers overexpressing Rem via in vivo electroporation (DiFranco et al., 2007). Using this approach, we have found that Rem effectively uncouples the CaV1.1 voltage sensor from RyR1-mediated SR Ca\(^{2+} \) release through its interaction with \( \beta_{1A} \). Specifically, Rem markedly reduces voltage-induced myoplasmic Ca\(^{2+} \) transients without appreciable effects on CaV1.1 targeting, intramembrane charge movement, or SR Ca\(^{2+} \) store content.

**MATERIALS AND METHODS**

**Molecular biology**

**CFP-CaV1.1.** A cDNA encoding a CFP-rabbit CaV1.1 \( \alpha_{1S} \) subunit (GenBank accession no. X05921) fusion construct was created by swapping out YFP for CFP in an existing YFP–\( \alpha_{1S} \) fusion construct (Papadopoulos et al., 2004). The cDNA segment encoding CFP was excised from the parent pECFP-C1 vector (Takara Bio Structural (Papadopoulos et al., 2004). The cDNA segment encoding CFP was excised from the parent pECFP-C1 vector (Takara Bio Inc.) using NheI and HindIII (761 bp). Likewise, YFP was removed from the YFP–\( \alpha_{1S} \) fusion construct using the same restriction enzymes, linearizing the pEYFP-C1 backbone and the \( \alpha_{1S} \)-coding sequence (9,555 bp). The CFP-encoding segment was then ligated into the linearized vector carrying the \( \alpha_{1S} \)-coding sequence (final, 10,316 bp).

**CFP–\( \beta_{1A} \) and YFP–\( \beta_{1A} \).** The constructions of CFP–\( \beta_{1A} \) and YFP–\( \beta_{1A} \) (both GenBank accession no. M25514) were described previously by Leuranguer et al. (2006); CFP–\( \beta_{1A} \), YFP–\( \beta_{1A} \), and CFP–CaV1.1 were all provided by K.G. Beam (University of Colorado Denver-Anschutz Medical Campus, Aurora, CO).

**V-Rem AAA.** The construction of V-Rem AAA (RefSeq accession no. NP_033073) was described previously by Begollari et al. (2015). Restriction digests and sequencing were used to verify all constructs.

In vivo electroporation and dissociation of FDB fibers

All procedures involving mice were approved by the University of Colorado Denver-Anschutz Medical Campus Institutional Animal Care and Use Committee. cDNA plasmids encoding YFP, CFP–\( \alpha_{1S} \), CFP–\( \beta_{1A} \), V-Rem, and/or V-Rem AAA were delivered to FDB fibers of anesthetized 2–3-mo-old male C57BL/6j mice (The Jackson Laboratory) via an in vivo electroporation protocol similar to that originally described by DiFranco et al. (2007). In brief, 10 µl of 2 mg/ml hyaluronidase solution was injected into the FDB muscle with a 30-gauge hypodermic needle. After 1 h, mice were re-anesthetized and 20 µl cDNA (3–5 µg/µl) was injected into the muscle. 5 min later, two gold-plated acupuncture needle electrodes (Lhasa OMS) coupled to an isolated pulse stimulator (A-M Systems) were placed subcutaneously near the proximal and distal tendons of the muscle (~1 cm apart), cDNAs were then electroporated into the FDB muscle with 20 100-V, 20-ms pulses delivered at 1 Hz. For assessment of SR Ca\(^{2+} \) stores, the transfection mixture also contained 5 µg pmCherry-C1 (Takara Bio Inc.) as a means to identify successfully transfected fibers after loading with Fluo 3-AM dye (Invitrogen; see below).

Electroporated (9–10 d after transfection) FDB muscles were dissected in cold rodent Ringer’s solution (mM: 146 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), and 10 HEPES, pH 7.4 with NaOH). Muscles were then digested in a collagenase solution (mM: 155 Cs-aspartate, 10 HEPES, and 5 MgCl\(_2\), pH 7.4 with CsOH, supplemented with 1 mg/ml BSA [Sigma-Aldrich] and 1 mg/ml collagenase type IA [Sigma-Aldrich]) with agitation at 37°C for ~1 h. Immediately after digestion, the collagenase solution was replaced with a dissociation solution (mM: 140 Cs-aspartate, 10 Cs\(_2\)-EGTA, 10 HEPES, and 5 MgCl\(_2\), pH 7.4 with CsOH, supplemented with 1 mg/ml BSA), and muscles were triturated gently with a series of firepolished glass pipettes of descending bore. Dissociated FDB fibers destined for whole-cell patch-clamp experiments were then plated onto ECL (EMD Millipore)-coated 35-mm plastic culture dishes (Falcon). For imaging, fibers were allowed to settle onto laminin (Invitrogen)-coated 35-mm culture dishes with glass coverslip bottoms (MatTek). Experiments were performed with FDB fibers 1–6 h after dissociation; successfully transfected fibers were identified by the presence of YFP or Venus fluorescence.

Measurement of intramembrane charge movements and L-type Ca\(^{2+} \) currents from FDB fibers

Patch pipettes were fabricated from borosilicate glass and had resistances of ~1.0 MΩ when filled with internal solution, which consisted of (mM): 145 TEA-methanesulfonic acid, 10 CaCl\(_2\), 10 HEPES, 2 MgSO\(_4\), 1 4-aminopyridine, 0.1 anthracene-9-carboxylic acid, and 0.002 tetrodotoxin, pH 7.4 with TEA-OH. For measurement of charge movements, the bath contained (mM): 145 TEA-methanesulfonic acid, 10 CaCl\(_2\), 10 HEPES, 2 MgSO\(_4\), 1 4-aminopyridine, 0.1 anthracene-9-carboxylic acid, 0.002 tetrodotoxin, 1 LaCl\(_3\), and 0.5 CdCl\(_2\), pH 7.4 with TEA-OH. Linear components of leak and capacitative current were corrected with \( -P/4 \) online subtraction protocols. Output filtering was at 2–5 kHz, and digitization was either at 5 kHz (currents) or 10 kHz (charge movements). Cell capacitance was determined by integration of a transient from \( -80 \) to \( -70 \) mV using Clampex 10.3 (Molecular Devices) and was used to normalize charge movement (nC/µF) and current amplitude (pA/µF). The average value of \( C_m \) was \( 2.3 \pm 0.1 \) nF (\( n = 48 \) fibers). To minimize voltage error, the time constant for decay of the whole-cell capacity transient (\( \tau_m \)) was reduced as much as possible using the analogue compensation circuit of the amplifier; the average values of \( \tau_m \) and \( R_m \) were 1.0 ± 0.02 ms and 467 ± 26 kΩ, respectively. \( Q_{ON} \) was then normalized to \( C_m \) and plotted as a function of test potential (V), and the resultant QV relationship was fitted according to:

\[
Q_{ON} = Q_{max} \left[ \frac{1}{1 + \exp \left( \frac{V - V_Q}{k_Q} \right)} \right].
\]

(1)

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.
Peak currents were normalized to $C_m$, and the resultant $I$-V was fitted according to:

$$I = G_{\text{max}} \left( \frac{V - V_{\text{rev}}}{1 + \exp \left[ \frac{-(V - V_{1/2})}{k}\right]} \right),$$

(2)

where $I$ is the normalized current for the test potential $V$, $V_{\text{rev}}$ is the reversal potential, $G_{\text{max}}$ is the maximum Ca$^{2+}$ channel conductance, $V_{1/2}$ is the half-maximal activation potential, and $k$ is the slope factor. All electrophysiological and Ca$^{2+}$-imaging experiments were performed at room temperature ($\sim$25°C).

Measurement of intracellular Ca$^{2+}$ transients in the whole-cell configuration

Voltage-induced changes in myoplasmic Ca$^{2+}$ were recorded from FDB fibers with Fluo 3 single-wavelength Ca$^{2+}$ indicator dye (Invitrogen). The pentapotassium salt form of the dye was added to the standard internal solution (see above) for a final concentration of 200 µM. The external solution contained (mM): 145 TEA-methanesulfonic acid, 10 CaCl$_2$, 10 HEPES, 2 MgSO$_4$, 1 4-aminopyridine, 0.1 anthracene-9-carboxylic acid, and 0.002 tetrodotoxin, pH 7.4 with TEA-OH. After entry into the whole-cell configuration, a waiting period of no less than 20 min was used to allow the dye to diffuse into the cell interior. A 100-W mercury illuminator and a set of fluorescin filters were used to excite the dye present in the voltage-clamped fiber. A computer-controlled shutter was used to block illumination in the intervals between test pulses. Fluorescence emission was measured by means of a fluorometer (Biomedical Instrumentation Group, University of Pennsylvania). Fluorescence data were expressed as $\Delta F/F$, where $\Delta F$ represents the change in peak fluorescence from baseline during the test pulse, and $F$ is the fluorescence immediately before the test pulse minus the average background fluorescence. The peak value of the fluorescence change ($\Delta F/F$) for each test potential ($V$) was fitted according to:

$$\frac{\Delta F}{F} = \frac{\Delta F/F_{\text{max}}}{1 + \exp \left[ \frac{(V - V_{1/2})}{k}\right]},$$

(3)

where ($\Delta F/F_{\text{max}}$) is the maximal fluorescence change, $V_1$ is the potential causing half the maximal change in fluorescence, and $k$ is a slope parameter. Only cells with transients that could be fit with Eq. 3 were used for analysis.

Live cell imaging

Dissociated FDB fibers were examined in rodent Ringer’s solution using a confocal laser-scanning microscope (LSM 510 META; Carl Zeiss). A Plan-Apochromat 63× oil-immersion objective (1.4 NA) was used to view the fiber of interest. CFP and Venus were excited with separate sweeps of the 458- and 514-nm lines, respectively, of an argon laser (30-milliwatt maximum output, operated at 50% or 6.3 A) directed to the cell via a 458/514-nm dual dichroic mirror. The emitted fluorescence was split via a 515-nm long-pass filter; CFP was directed to a photomultiplier equipped with a 465-495-nm band-pass filter, and Venus was directed to a photomultiplier equipped with a 500-530-nm band-pass filter. SR Ca$^{2+}$ release was induced by 1 mM 4-chloro-m-cresol (4-CmC; Pfaltz & Bauer) delivered via a manually operated, gravity-driven global perfusion system. Fluorescence amplitude data are expressed as $\Delta F/F$, where $F$ represents the baseline fluorescence before application of 4-CmC, and $\Delta F$ represents the change in peak fluorescence during the application of 4-CmC.

tsa201 cell culture and expression of CDNA

Low (<$\sim$20) passage tsA201 cells were propagated in culture medium containing 90% DMEM (Thermo Fisher Scientific), 10% defined fetal bovine serum (GE Healthcare), and 100 µg/ml penicillin-streptomycin (Life Technologies). Cells were trypsinized twice weekly and replated onto 35-mm culture dishes at $\sim$20% confluence. Lipofectamine 2000 (Life Technologies) was used to transfect these cells within 3–5 d of plating. The transfection mixture contained expression plasmids encoding rat Ca$^{2+}$,1.3, rabbit $\beta_1$, and rat $\alpha_6$1 channel subunits at 1 µg of each cDNA per dish. The transfection mixture also contained a plasmid-encoding Venus–Rem construct (1 µg/dish; see above) or YFP (30 ng/dish; Takara Bio Inc.). The day after transfection, cells were trypsinized and replated onto 35-mm plastic for experiments the next day.

Coimmunoprecipitation

TsA201 cells expressing YFP-$\beta_1$, YFP-$\beta_2$, Y-Rem, or YFP-$\beta_1$/Y-Rem were lysed into 300 µl of lysin buffer (mM: 50 Tris-HCl, pH 7.5, 100 NaCl, 10 MgCl$_2$, 1 DTT, and 0.2% Tween-20) supplemented with 0.1 mM iodoacetamide and 1 mM phenylmethylsulfonyl fluoride (both from Thermo Fisher Scientific). After insoluble material was removed by centrifugation, the homogenates were incubated with a monoclonal antibody directed to Rem (1:200; Santa Cruz Biotechnology, Inc.) for 4–6 h with gentle agitation followed by an overnight incubation with protein A agarose beads (Santa Cruz Biotechnology, Inc.). The agarose beads were then washed twice with lysin buffer and collected after gentle centrifugation at 2,500 rpm. The beads were then resuspended in 30 µl of 1% SDS buffer (Bio-Rad Laboratories) and subjected to SDS-PAGE analysis. Proteins were transferred into a nitrocellulose membrane, blocked with 3% nonfat dry milk (Kroger) in PBS-Tween, and incubated overnight at 4°C with monoclonal antibodies directed to either X(G)FP (1:1,500; Antibodies Inc.) or Rem (1:500). The nitrocellulose membrane was then washed three times with PBS-Tween and incubated at room temperature for 1 h with horseradish peroxidase–conjugated goat anti-mouse IgG (1:10,000; SouthernBiotech). Protein bands were visualized with the SuperSignal West Femto kit (Thermo Fisher Scientific) and viewed on a FluorChem HD2 scanner (Alpha Innotech). Blots were stripped using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific).

L-type Ca$^{2+}$ current recordings from tsA201 cells

Borosilicate pipettes (2.0–3.0 MΩ) were filled with internal solution, which consisted of (mM): 140 Cs-aspartate, 10 Cs$^2$-EGTA, 5 MgCl$_2$, and 10 HEPES, pH 7.4 with CsOH. The bath solution contained (mM): 145 NaCl, 10 CaCl$_2$, and 10 HEPES, pH 7.4 with NaOH. Electronic compensation was used to reduce the effective series resistance, and linear components of leak and capacitive current were corrected with $-P/4$ online subtraction protocol. Filtering and digitation were at 2 and 5 kHz, respectively. For tsA201 cell experiments, the average values of $C_m$, $v_m$, and $R$ were 20.0 ± 1.4 pF, 202.0 ± 18.9 µs, and 10.9 ± 0.9 MΩ, respectively ($n = 28$ cells).
Rem inhibits EC coupling in FDB fibers without affecting intramembrane charge movement

Recently, we described the effects of the RGK proteins Rad and Rem on L-type Ca\textsuperscript{2+} currents and intramembrane charge movement in adult FDB muscle fibers (Beqollari et al., 2014). Although both Rad and Rem inhibited L-type currents by ~60 and ~45%, respectively, charge movement was only reduced in fibers transfected with Rad; charge movement for Rem-expressing fibers was virtually identical to charge movement observed in naive fibers. To confirm the latter observation, we used in vivo electroporation (DiFranco et al., 2007) to transfect FDB muscles of otherwise normal 2–3-mo-old C57Bl/6J mice with either YFP or a Venus-fused wild-type mouse Rem construct (V-Rem). As expected, FDB fibers overexpressing V-Rem again displayed maximal charge movement virtually identical to YFP-expressing fibers in both amplitude and voltage dependence (Fig. 1, A–C and Table 1). Both Q-V relationships were similar to that reported by Prosser et al. (2009) when La\textsuperscript{3+} was included in the extracellular recording solution.

Because skeletal muscle EC coupling is coupled directly to translocation of Ca\textsubscript{v.1.1}'s voltage-sensing structures (Schneider and Chandler, 1973; Ríos and Brun, 1987; García et al., 1994; Tanabe et al., 1988), we next investigated the impact of Rem on EC coupling by recording myoplasmic Ca\textsuperscript{2+} transients in response to membrane depolarization (as in Wang et al., 1999; Wu et al., 2012). Ca\textsuperscript{2+} transients recorded from fibers transfected with V-Rem were substantially reduced compared with the transients of YFP-expressing fibers (0.6 ± 0.1 \( \Delta F/F \), \( n = 8 \) vs. 1.6 ± 0.4 \( \Delta F/F \), \( n = 6 \), respectively; \( P < 0.001 \); Fig. 1, D–F). No significant effect on the voltage dependence of SR Ca\textsuperscript{2+} release was observed between the two groups (\( P > 0.05 \); Table 1).

**SR Ca\textsuperscript{2+} store content is not significantly affected by overexpression of Rem**

As a means to determine whether the V-Rem–mediated reduction in voltage-induced Ca\textsuperscript{2+} release was a consequence...
of an altered SR Ca\textsuperscript{2+} store, we exposed intact fibers loaded with Fluo-3 AM dye to the RYR agonist 4-CmC. In these experiments, 1 mM 4-CmC elicited SR Ca\textsuperscript{2+} release that was nearly indistinguishable between FDB fibers overexpressing V-Rem and fibers expressing YFP only (5.7 ± 1.0 ΔF/F, n = 9 vs. 6.8 ± 1.8 ΔF/F, n = 5, respectively; P > 0.05; Fig. 2, A–C). The equivalent responses of YFP- and V-Rem–expressing fibers to 4-CmC suggest that depletion of SR Ca\textsuperscript{2+} store is an unlikely explanation for the ~65% reduction in Ca\textsuperscript{2+} transient amplitude observed in V-Rem–expressing fibers.

Rem overexpression does not alter targeting of Ca\textsubscript{v}1.1 \(\alpha_{15}\) or \(\beta_{12}\) subunits

Because Rem has been reported to alter high voltage-activated Ca\textsuperscript{2+} channel trafficking in heterologous systems (Béguin et al., 2007; Mahalakshmi et al., 2007; Flynn and Zamponi, 2010; Yang et al., 2010) and in cardiac myocytes (Jhun et al., 2012), one possible explanation for the disruption of EC coupling by V-Rem (Fig. 1, D and F) is that the small G protein redirects Ca\textsubscript{v}1.1 away from triad junctions. For this reason, we examined the subcellular distribution of Ca\textsubscript{v}1.1 \(\alpha_{15}\) and \(\beta_{12}\) subunits in the absence and presence of coexpressed V-Rem. When expressed in FDB fibers, CFP-tagged \(\alpha_{15}\) subunits of Ca\textsubscript{v}1.1 were targeted to transverse tubules as shown previously for YFP-tagged \(\alpha_{15}\) subunits (DiFranco et al., 2011; Fig. 3 A). The tubular distribution of CFP-\(\alpha_{15}\) was unaffected by coexpression of V-Rem (Fig. 3 B). Likewise, coexpression of V-Rem had little, if any, effect on the subcellular distribution of CFP-\(\beta_{12}\) (Fig. 3, C and D). Interestingly, the V-Rem fluorescence extended from the transverse tubules into the region of the I band. In this regard, the subcellular distribution of V-Rem overlapped, but did not completely match, the transverse tubular distributions of Ca\textsubscript{v}1.1 \(\alpha_{15}\) and \(\beta_{12}\) subunits. We do not consider the presence of Rem in the I band to be an artifact of overexpression, as the related RGK protein Rad clearly targets to transverse tubules when expressed in FDB fibers via electroporation (see Beqollari et al., 2014). Moreover, this observation does not affect our interpretation of the data shown in Fig. 3: coexpression

### Table 1

| Construct   | \(Q_{max}\) \(\text{nC/µF}\) | \(V_Q\) \(\text{mV}\) | \(k_Q\) \(\text{mV}\) | \(\Delta F/F_{max}\) \(\text{mV}\) | \(V_F\) \(\text{mV}\) | \(k_F\) \(\text{mV}\) |
|------------|----------------|----------------|----------------|----------------|----------------|----------------|
| YFP        | 22.8 ± 3.4 (5) | -7.8 ± 3.5 | 12.0 ± 1.7 | 1.6 ± 0.2 (6) | -13.0 ± 1.2 | 8.4 ± 0.8 |
| V-Rem      | 22.4 ± 1.2 (7) | -8.1 ± 1.6 | 11.8 ± 1.6 | 0.6 ± 0.1* (8) | -20.2 ± 4.7 | 14.7 ± 2.6 |
| V-Rem AAA  | 24.6 ± 3.4 (5) | -8.5 ± 1.0 | 8.1 ± 1.0 | 1.4 ± 0.2 (6) | -15.5 ± 1.3 | 9.9 ± 0.9 |

Data are given as mean ± SEM, with the numbers in parentheses indicating the number of FDB fibers tested. Charge movement and EC coupling data were fit by Eqs. 1 and 3, respectively. Only cells with Ca\textsuperscript{2+} transients that could be fit with Eq. 3 were used for analysis; two Rem-expressing fibers lacking quantifiable ΔF/F were dropped. One significant difference between the three groups is indicated.

\*P < 0.001; one-way ANOVA.

**Figure 2.** SR Ca\textsuperscript{2+} store content is not significantly affected by overexpression of Rem. SR Ca\textsuperscript{2+} store content as assessed by changes in Fluo-3 AM fluorescence (ΔF/F) in response to the application of 1 mM 4-CmC to fibers expressing either YFP (A) or V-Rem (B). Insets show images of loaded fibers before 4-CmC application (left) and at the peak of fluorescence (right). Bars, 100 µm. (C) A comparison of the average peak ΔF/F values for YFP- and V-Rem–expressing fibers is shown. Error bars represent ±SEM.
of V-Rem did not alter targeting of the channel subunits to the transverse tubules.

Simultaneous introduction of alanines at Rem positions R200, L227, and H229 disrupts interactions with β1a
So far, our data indicate that Rem uncouples the CaV1.1 voltage sensor from RYR1-mediated SR Ca²⁺ release. However, it is unclear whether this effect of Rem is dependent on the ability of the small GTP-binding protein to interact with the β₁a subunit of the CaV1.1 channel complex. In this regard, three highly conserved residues of Rem (R200, L227, or H229) have been identified as being critical for interactions with the β₃-subunit isoform (Béguin et al., 2007; Puhl et al., 2014); conversion of any one of these residues to alanine severely impairs binding to β₃-subunit isoforms in both yeast-2-hybrid and coimmunoprecipitation assays. To specifically test whether Rem binds to the β₁a subunit isoform, we engineered a V-Rem–based construct with alanines introduced at positions R200, L227, and H229 (V-Rem AAA) and compared its ability to coimmunoprecipitate with a YFP-fused β₁a construct (YFP-β₁a). In these experiments, a commercially available monoclonal Rem antibody failed to immunoprecipitate YFP-β₁a in lysates obtained from tsA201 cells transfected with only YFP-β₁a (shown in duplicate in Fig. 4 A, lanes 2 and 6). In contrast, the antibody efficiently immunoprecipitated YFP-β₁a subunits when V-Rem was coexpressed with YFP-β₁a (Fig. 4 A, lanes 3 and 7). Consistent with the earlier report of Béguin et al. (2007) showing the disruption of the Rem-β₃ interaction with alanine single-point mutants, an interaction between V-Rem AAA and β₁a was not detectable (Fig. 4 A, lanes 4 and 8). In control experiments, the Rem antibody detected similar levels of immunoprecipitated V-Rem and V-Rem AAA (Fig. 4 B, lanes 3–4 and 7–8). Comparable expression levels for YFP-β₁a, V-Rem, and V-Rem AAA mutant were confirmed in a Western blot from total lysates collected before coimmunoprecipitation (Fig. 4 C).

V-Rem AAA fails to inhibit L-type channels expressed in tsA201 cells
We next determined the functional consequences of the disruption of the Rem-β₁a interaction. In these experiments, we coexpressed YFP, V-Rem, or V-Rem AAA with CaV1.3 α₁D, β₁a, and α₂δ₁-subunits to detect interactions that occur within a functional L-type channel complex (we used CaV1.3 as a surrogate for CaV1.1 because of its highly efficient and consistent membrane expression in tsA201 cells; see Meza et al., 2013). Predictably, tsA201 cells expressing CaV1.3, β₁a, α₂δ₁, and V-Rem displayed virtually no L-type current (−3.8 ± 0.7 pA/pF...
V-Rem AAA fails to inhibit CaV1.1 function in FDB fibers

To establish β1a as the mechanistic target of Rem in our experimental system, we overexpressed V-Rem AAA in FDB fibers and assayed its effects on L-type Ca\(^{2+}\) currents, intramembrane charge movement, and EC coupling. Successful expression of V-Rem AAA in FDB fibers was confirmed by Venus fluorescence, which was comparable to the fluorescence generated by V-Rem at 0 mV; \(n = 4\); Fig. 5 A). In contrast, cells expressing Ca\(_{\alpha}1.3\), β1a, α\(\delta\)-1, and V-Rem AAA had L-type currents nearly identical in amplitude (−78.3 ± 16.0 pA/pF, \(n = 15\); Fig. 5, B and D) to control cells expressing the same channel subunits with a YFP transfection marker (−71.0 ± 20.0 pA/pF, \(n = 9\); \(P > 0.05\); Fig. 5, C and D). Successful expression of V-Rem and V-Rem AAA in tsA201 cells was indicated by Venus fluorescence (Fig. 5 E).
In this study, we found that the RGK family small G protein Rem profoundly inhibits skeletal muscle EC coupling in adult mouse FDB muscle fibers (Fig. 1, D–F). Because the observed reduction in voltage-induced SR Ca\textsuperscript{2+} release was not likely a consequence of altered Ca\textsubscript{V}1.1 targeting (Fig. 3), impaired voltage sensing (Fig. 1, A–C) or a greatly depleted SR Ca\textsuperscript{2+} store (Fig. 2), a “communication breakdown” must have occurred between Ca\textsubscript{V}1.1 and RYR1. An intuitive candidate locus for such EC uncoupling is the auxiliary \( \beta \textsubscript{1a} \) subunit of the Ca\textsubscript{V}1.1 heteromultimer because RGK proteins are established \( \beta \)-subunit–interacting partners (Béguin et al., 2001, 2007; Finlin et al., 2003, 2006; Yang and Colecraft, 2013; Puhl et al., 2014; Xu et al., 2015). Earlier work by Colecraft and colleagues has established that Rem can inhibit L-type Ca\textsubscript{V}1.2 channels expressed in HEK 293 cells without affecting intramembrane charge movement (Yang et al., 2007, 2010), and that this particular mode of Rem action is largely dependent on structural elements that are important for contact(s) with \( \beta \textsubscript{1a} \).

**DISCUSSION**

Figure 6. Expression of V-Rem AAA in FDB fibers has very little effect on native Ca\textsubscript{V}1.1 function. Representative recordings of skeletal muscle L-type Ca\textsuperscript{2+} currents elicited by 500-ms depolarizations from \(-50\) to \(-20, 0, 20, \) and \(40\) mV are shown for FDB fibers expressing V-Rem AAA (A; left). The peak I-V relationship for fibers expressing V-Rem AAA (\( n = 6 \); gray circles) is shown with the peak I-V relationship for fibers expressing unfused YFP (\( n = 5 \); black circles) and V-Rem (\( n = 5 \); white circles) in the right panel. L-type currents were evoked at 0.1 Hz by test potentials ranging from \(-40\) through \(80\) mV in 10-mV increments. The smooth curves are plotted according to Eq. 2 with the following respective parameters for V-Rem AAA–, V-Rem–, and YFP-expressing fibers: \( G_{\text{max}} = 212 \pm 26, 128 \pm 19, \) and \(205 \pm 12\) nS/nF; \( V_{1/2} = 4.3 \pm 3.1, 8.3 \pm 3.2, \) and \(4.0 \pm 2.0\) mV; \( k_{G} = 5.0 \pm 0.4, 5.4 \pm 0.5, \) and \(5.1 \pm 0.5\) mV; \( V_{\text{rev}} = 70.0 \pm 1.7, 70.0 \pm 3.4, \) and \(67.0 \pm 1.4\) mV. Representative recordings of intramembrane charge movements elicited by 25-ms depolarizations from \(-80\) to \(-40, -20, 0, \) and \(20\) mV are shown for transfected FDB fibers expressing V-Rem AAA (B; left). The Q-V relationships for fibers expressing V-Rem (\( n = 7 \); white circles), V-Rem AAA (\( n = 5 \); gray circles), or YFP (\( n = 5 \); black circles) are shown in the right panel. Charge movements were evoked at 0.1 Hz by test potentials ranging from \(-70\) through \(30\) mV in 10-mV increments. The smooth curves for V-Rem–, V-Rem AAA–, or YFP-expressing fibers are plotted according to Eq. 1 with the respective fit parameters shown in Table 1. Representative recordings of myoplasmic Ca\textsuperscript{2+} transients elicited by 25-ms depolarizations from \(-80\) to \(-40, -20, 0, \) and \(20\) mV are shown for FDB fibers overexpressing V-Rem AAA (C; left). The peak \( \Delta F/F-V \) relationships for YFP- and V-Rem–expressing fibers are reproduced from Fig. 1. Error bars represent ±SEM.
of Rem-mediated inhibition is dependent solely on an interaction with the β subunit (Yang et al., 2012; Yang and Colecraft, 2013). Because Rem exclusively uses this “low Po” gating mode to inhibit CaV1.1 channel function in differentiated muscle fibers (Beqollari et al., 2014), the observed impairment of EC coupling by Rem is almost certainly dependent on a Rem-β1 interaction. The inability of V-Rem AAA, a Rem construct lacking key structural elements for β binding and channel inhibition (Figs. 4 and 5, respectively), to reduce EC coupling provides additional support for this assertion (Fig. 6).

In addition to inhibiting EC coupling, V-Rem also reduced L-type current in FDB fibers (Fig. 6A; Beqollari et al., 2014). Because L-type Ca2+ entry has been found to contribute to SR Ca2+ store refilling in myotubes (Cherednichenko et al., 2004) and in differentiated muscle fibers (Lee et al., 2015), it is not beyond possibility that SR stores may be partially depleted in V-Rem–expressing fibers. However, the nearly equivalent responses of YFP- and V-Rem–expressing fibers to 4-CmC (Fig. 2) support the idea that such a mechanism is unlikely to account for the observed effect of Rem on voltage-induced SR Ca2+ release. Likewise, an acute contribution from L-type Ca2+ flux via the channel is also improbable, as the F-V curves for YFP- and Rem AAA–expressing fibers both displayed sigmoidal dependencies on voltage, a hallmark indication of skeletal-type EC coupling (see Fig. 6C, right). If Ca2+ flux were making a small contribution to the transients, its loss could not likely explain the nearly 65% decrease in SR Ca2+ release resulting from coexpression of V-Rem.

Strong circumstantial, but by no means definitive, evidence exists supporting the hypothesis that β1s is directly involved in CaV1.1–RYR1 communication (see Rebbeck et al., 2014). In particular, expression of β1s is essential for EC coupling and enhances L-type current amplitude considerably (Gregg et al., 1996; Strube et al., 1996). Unfortunately, these early results obtained with myotubes cultured from β1 null mice have been difficult to interpret because membrane expression of the principal α1S subunit of CaV1.1 was severely compromised. The confounding obstacle of poor α1S trafficking in β1 null mice was overcome by elegant work with the effectively β1 null relaxed zebrafish mutant line. In the relaxed system, unaughtened α1S subunits trafficked somewhat more effectively to plasma membrane–SR junctions than in mice (Schredelseker et al., 2005). The improved membrane expression of CaV1.1 enabled meticulous ultrastructural examination of relaxed junctions, revealing that β1s is required to organize CaV1.1 into the tetrad arrays that are prerequisite for EC coupling.

Beyond ultrastructure, the zebrafish model system poses nearly the same challenges to deciphering the function of β1s, as does the β1 null mouse model. Specifically, the introduction of chimeric β1s constructs or other CaV1β isoforms has been highly useful in the identification of functionally important domains, but information regarding essential intermolecular interactions remains frustratingly difficult to glean (Beam and Bannister, 2010). In efforts to avoid such ambiguity, in vitro approaches have been used to identify interactions of potential functional significance between β1s and RYR1. Indeed, purified full-length β1s subunits do bind fragments of RYR1 in vitro (Cheng et al., 2005; Rebbeck et al., 2011), and a peptide corresponding to α1S residues V490–M524 increases RYR1 Po when applied to lipid layers (Karunasekara et al., 2012). Likewise, dialysis of FDB fibers with a slightly shorter peptide (V490–M508) to the periphery of the tetrad (Szpyt et al., 2012). If ultrastructure is preserved in Rem-overexpressing fibers (as depicted in C), the binding of Rem to β1s within the intact CRU would most likely induce conformational rearrangements within β1s that deter transmission of the EC coupling signal from the membrane-bound, voltage-sensing regions of CaV1.1 to RYR1.

**Figure 7.** Schematic depicting potential mechanisms for Rem-mediated EC uncoupling. (A) The diagram represents the intact CaV1.1–RYR1 ultrastructure requisite for skeletal-type EC coupling. Four CaV1.1 α1S (red circles)–β1s (white ovals) channel complexes are shown coupled to each subunit of a single RYR1 (gray tetramer) from a transverse-tubular vantage point. For clarity, the β1s subunits are superimposed on the α1S subunits, and the αβ1–1 subunits, γ1 subunits, and other nonessential components of the junction have been omitted. The orientation of β1s within the tetrad follows on previous work (Leumanguer et al., 2006; Sheridan et al., 2012). In the right panels (B and C), we present two potential mechanisms by which Rem (black ovals) may disrupt EC coupling. In B, Rem displaces the CaV1.1 channel complex from RYR1 sufficiently to disrupt the tetrad ultrastructure that is required for CaV1.1–RYR1 communication by interacting with the conserved guanlyate kinase–like domain of β1s (Finlin et al., 2006; Béguin et al., 2007) on the...
peptide potentiates both EC coupling and L-type Ca\(^{2+}\) current by nearly 50% (Hernández-Ochoa et al., 2014). Although the use of \(\beta_{1a}\)-based peptide approaches has provided support for the idea that \(\beta_{1a}\) residues V490–M508 are involved in transmitting the signal between CaV1.1 and RYR1, the interpretation of these results has been somewhat limited because of uncertainty of substrate and lack of peptide specificity; one must take into account that a variety of small peptides binds to the ginormous \(~2.3\text{-MDa}\) RYR1 tetramer and/or modulates RYR1 \(P_o\), in lipid bilayers (e.g., peptides corresponding to the A domain of the CaV1.1 II–III linker, Imperatoxin A, Ca\(^{2+}\) release unit (CRU). Obviously, the next step in this peptide sequences of the endogenous components of the changes in to be involved in transmission of the EC coupling signal means for the investigation of the structures (e.g., II–III loop of the which in turn would imply that conformational changes in the domain of the CaV1.1 II–III linker, Imperatoxin A, Maurocalcine; El-Hayek and Ikemoto, 1998; Gurrola et al., 1999; Fajloun et al., 2000; Nabhani et al., 2002; Chen et al., 2003; Cui et al., 2009).

In light of the frustrating limitations of the experimental approaches described above, new strategies are needed to further investigate the role of \(\beta_{1a}\) in skeletal-type EC coupling. The use of wild-type Rem or modified Rem constructs to probe junctional architecture represents such an advance because the small G protein disrupts CaV1.1–RYR1 communication in intact, differentiated muscle fibers without deleting or altering the peptide sequences of the endogenous components of the Ca\(^{2+}\) release unit (CRU). Obviously, the next step in this line of investigation is to determine the precise mechanism by which Rem cuts communication between CaV1.1 and RYR1. Based on what is currently known, \(\beta_{1a}\) coordinates the juxtaposition of CaV1.1 with RYR1 in tetrad (Fig. 7 A). So, it is quite possible that the Rem–\(\beta_{1a}\) interaction merely impairs the ability of \(\beta_{1a}\) to facilitate the ultrastructural configuration of CaV1.1 and RYR1 that is requisite for conformational coupling (Fig. 7 B). However, the preservation of tetrad arrays in fibers overexpressing Rem would indicate that the RGK protein is exerting its inhibitory influence on \(\beta_{1a}\) within the intact CRU, which in turn would imply that conformational changes in \(\beta_{1a}\) are involved in CaV1.1–RYR1 coupling (Fig. 7 C). A correlate of the latter interpretation would be that other structures (e.g., II–III loop of the \(\alpha_{1s}\) subunit) thought to be involved in transmission of the EC coupling signal are adversely impacted by Rem-induced conformational changes in \(\beta_{1a}\). Of course, these ideas remain to be tested. In this regard, our current observations provide a new means for the investigation of the \(\beta_{1a}\) subunit as mediator of the communication between CaV1.1 and RYR1 that underlies EC coupling skeletal muscle.

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References

Bannister, R.A., and K.G. Beam. 2013. CaV1.1: The atypical prototypical voltage-gated Ca\(^{2+}\) channel. Biochim. Biophys. Acta 1828:1587–1597. http://dx.doi.org/10.1016/j.bbabio.2012.09.007

Bannister, R.A., H.M. Colecraft, and K.G. Beam. 2008. Rem inhibits skeletal muscle EC coupling by reducing the number of functional L-type Ca\(^{2+}\) channels. Biophys. J. 94:2631–2638. http://dx.doi.org/10.1529/biophysj.107.116467

Beam, K.G., and R.A. Bannister. 2010. Perspectives on: SGP Symposium on Muscle in Health and Disease: Looking for answers to EC coupling’s persistent questions. J. Gen. Physiol. 136:7–12. http://dx.doi.org/10.1085/jgp.201010461

Béguin, P., K. Nagashima, T. Gomoi, T. Shibasaki, K. Takahashi, Y. Kashima, N. Ozaki, K. Geering, T. Iwanaga, and S. Seino. 2001. Regulation of Ca\(^{2+}\) channel expression at the cell surface by the small G-protein kir. J. Biol. Chem. 276:7101–7106. http://dx.doi.org/10.1074/jbc.276.11.7101

Béguin, P., V.J. Ng, C. Krause, R.N. Mahalakshmi, M.Y. Ng, and W. Hunziker. 2007. RGK small GTP-binding proteins interact with the nucleotide kinase domain of Ca\(^{2+}\) channel \(\beta\)-subunits via an uncommon effector binding domain. J. Biol. Chem. 282:11509–11520. http://dx.doi.org/10.1074/jbc.M606423200

Beqollari, D., C.F. Romberg, U. Meza, S. Papadopoulos, and R.A. Bannister, 2014. Differential effects of RGK proteins on L-type channel function in adult mouse skeletal muscle. Biophys. J. 106:1950–1957. http://dx.doi.org/10.1016/j.bpj.2014.03.033

Beqollari, D., C.F. Romberg, D. Filipova, S. Papadopoulos, and R.A. Bannister. 2015. Functional assessment of three Rem residues identified as critical for interactions with Ca\(^{2+}\) channel \(\beta\)-subunits. Pflugers Arch. In press.

Chen, L., E. Estève, J.M. Sabatier, M. Ronjat, M. De Waard, P.D. Allen, and I.N. Pessah. 2003. Maurocalcine and peptide A stabilize distinct subconductance states of ryanodine receptor type 1, revealing a proportional gating mechanism. J. Biol. Chem. 278:16095–16106. http://dx.doi.org/10.1074/jbc.M209501200

Cheng, W., X. Alafaj, M. Ronjat, and R. Coronado. 2005. Interaction between the dihydropyridine receptor Ca\(^{2+}\) channel \(\beta\)-subunit and ryanodine receptor type 1 strengthens excitation-contraction coupling. Proc. Natl. Acad. Sci. USA 102:19225–19230. http://dx.doi.org/10.1073/pnas.0504334102

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

Cui, Y., H.S. Tae, N.C. Norris, Y. Karunasekara, P. Pouliquin, P.G. Board, A.F. Dhumney, and M.G. Casarotto. 2009. A dihydropyridine receptor \(\alpha_1\) loop region critical for skeletal muscle contraction is intrinsically unstructured and binds to a SPRY domain of
movement is present in mammalian muscle fibres, but suppressed in the absence of S100A1. J. Physiol. 587:4523–4541. http://dx.doi.org/10.1113/jphysiol.2009.177238

Pühl, H.L., III, V.B. Lu, Y.-J. Won, Y. Sasso, J.A. Hirsch, F. Ono, and S.R. Ikeda. 2014. Ancient origins of RGK protein function: Modulation of voltage-gated calcium channels preceded the protostome and deuterostome split. PLoS ONE. 9:e100694. http://dx.doi.org/10.1371/journal.pone.0100694

Rebeck, R.T., Y. Karunasekara, E.M. Gallant, P.G. Board, N.A. Beard, M.G. Casarotto, and A.F. Dulhunty. 2011. The β₃ subunit of the skeletal DHPR binds to skeletal R1;R1 and activates the channel via its 35-residue C-terminal tail. Biophys. J. 100:922–930. http://dx.doi.org/10.1016/j.bpj.2011.01.022

Rebeck, R.T., Y. Karunasekara, P.G. Board, N.A. Beard, M.G. Casarotto, and A.F. Dulhunty. 2014. Skeletal muscle excitation-contraction coupling: Who are the dancing partners? Int. J. Biochem. Cell Biol. 48:28–38. http://dx.doi.org/10.1016/j.biocel.2013.12.001

Ríos, E., and G. Brum. 1987. Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle. Nature. 325:717–720. http://dx.doi.org/10.1038/325717a0

Romberg, C.F., D. Beqollari, U. Meza, and R.A. Bannister. 2014. RGK protein-mediated impairment of slow depolarization-dependent Ca²⁺ entry into developing myotubes. Channels (Austin). 8:243–248. http://dx.doi.org/10.4161/chann.27686

Schneider, M.F., and W.K. Chandler. 1973. Voltage dependent charge movement of skeletal muscle: a possible step in excitation-contraction coupling. Nature. 242:244–246. http://dx.doi.org/10.1038/242244a0

SchedelSeker, J., V. Di Biase, G.J. Obermair, E.T. Felder, B.E. Flucher, C. Franzini-Armstrong, and M. Grabner. 2005. The β₃ subunit is essential for the assembly of dihydropyridine-receptor arrays in skeletal muscle. Proc. Natl. Acad. Sci. USA. 102:17219–17224. http://dx.doi.org/10.1073/pnas.0508710102

SchedelSeker, J., A. Dayal, T. Schwerte, C. Franzini-Armstrong, and M. Grabner. 2009. Proper restoration of excitation-contraction coupling in the dihydropyridine receptor β₃-null zebrafish is relaxed: an exclusive function of the β₃ subunit. J. Biol. Chem. 284:1242–1251. http://dx.doi.org/10.1074/jbc.M807767200

Sheridan, D.C., O. Moua, N.M. Lorenzon, and K.G. Beam. 2012. Bimolecular fluorescence complementation and targeted biotinylation provide insight into the topology of the skeletal muscle Ca²⁺ channel β₃ subunit. Channels (Austin). 6:26–40. http://dx.doi.org/10.4161/chann.18916

Strube, C., M. Beurg, P.A. Powers, R.G. Gregg, and R. Coronado. 1996. Reduced Ca²⁺ current, charge movement, and absence of Ca²⁺ transients in skeletal muscle deficient in dihydropyridine receptor β₃ subunit. Biophys. J. 71:2531–2543. http://dx.doi.org/10.1016/S0006-3495(96)79446-8

Szpyt, J., N. Lorenzon, C.F. Perez, E. Norris, P.D. Allen, K.G. Beam, and M. Samsø. 2012. Three-dimensional localization of the α and β subunits and of the II-III loop in the skeletal muscle L-type Ca²⁺ channel. J. Biol. Chem. 287:43853–43861. http://dx.doi.org/10.1074/jbc.M112.419283

Tanabe, T., K.G. Beam, J.A. Powell, and S. Numa. 1988. Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. Nature. 336:134–139. http://dx.doi.org/10.1038/336134a0

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. http://dx.doi.org/10.1038/346567a0

Wang, Z.M., M.L. Messi, and O. Delbono. 1999. Patch-clamp recording of charge movement, Ca²⁺ current, and Ca²⁺ transients in adult skeletal muscle fibers. Biophys. J. 77:2709–2716. http://dx.doi.org/10.1016/S0006-3495(99)77104-3

Wilkins, C.M., N. Kasielke, B.E. Flucher, K.G. Beam, and M. Grabner. 2001. Excitation-contraction coupling is unaffected by drastic alteration of the sequence surrounding residues L720-L764 of the α₁S II-III loop. Proc. Natl. Acad. Sci. USA. 98:5889–5897. http://dx.doi.org/10.1073/pnas.101618098

Wu, F., W. Mi, E.O. Hernández-Ochoa, D.K. Burns, Y. Fu, H.F. Gray, A.F. Struyk, M.F. Schneider, and S.C. Cannon. 2012. A calcium channel mutant mouse model of hypokalemic periodic paralysis. J. Clin. Invest. 122:4580–4591. http://dx.doi.org/10.1172/JCI66091

Xu, X., F. Zhang, G.W. Zamponi, and W.A. Horne. 2015. Solution NMR and calorimetric analysis of Rem2 binding to the Ca²⁺ channel β₃ subunit: a low affinity interaction is required for inhibition of Ca₉.2 Ca²⁺ currents. FASEB J. 29:1794–1804. http://dx.doi.org/10.1096/fj.14-264499

Yang, T., and H.M. Colecraft. 2013. Regulation of voltage-dependent calcium channels by RGK proteins. Biochim. Biophys. Acta. 1828:1644–1654. http://dx.doi.org/10.1016/j.bbamem.2012.10.005

Yang, T., Y. Suhail, S. Dalton, T. Kerman, and H.M. Colecraft. 2007. Genetically encoded molecules for inducibly inactivating Ca²⁺ channels. Nat. Chem. Biol. 3:795–804. http://dx.doi.org/10.1038/nchembio.2007.42

Yang, T., X. Xu, T. Kerman, V. Wu, and H.M. Colecraft. 2010. Rem, a member of the RGK GTPases, inhibits recombinant Ca₁.2 channels using multiple mechanisms that require distinct conformations of the GTPase. J. Physiol. 588:1665–1681. http://dx.doi.org/10.1113/jphysiol.2010.187203

Yang, T., A. Puckerin, and H.M. Colecraft. 2012. Distinct RGK GTPases differentially use α₃ and auxiliary β-binding-dependent mechanisms to inhibit Ca₉.2/Ca₉.2 channels. PLoS ONE. 7:e37079. http://dx.doi.org/10.1371/journal.pone.0037079