Malignant hyperthermia susceptibility arising from altered resting coupling between the skeletal muscle L-type Ca$^{2+}$ channel and the type 1 ryanodine receptor

Jose Miguel Elitita,1, Roger A. Bannisterc,1, Ong Mouad, Francisco Altamiranoe, Philip M. Hopkinsf, Isaac N. Pessahg, Tadeusz F. Molinskih, Jose R. López, Kurt G. Beamma, and Paul D. Allena

Malignant hyperthermia (MH) susceptibility is a dominantly inherited disorder in which volatile anesthetics trigger aberrant Ca$^{2+}$ release in skeletal muscle and a potentially fatal rise in perioperative body temperature. Mutations causing MH susceptibility have been identified in two proteins critical for excitation–contraction (EC) coupling, the type 1 ryanodine receptor (RyR1) and CaV1.1, the principal subunit of the L-type Ca$^{2+}$ channel. All of the mutations that have been characterized previously augment EC coupling and/or increase the rate of L-type Ca$^{2+}$ entry. The CaV1.1 mutation R174W associated with MH susceptibility occurs at the innermost basic residue of the IS4 voltage-sensing helix, a residue conserved among all CaV channels [Carpenter D, et al. (2009) BMC Med Genet 10:104–115.]. To define the functional consequences of this mutation, we expressed it in dysgenic (CaV1.1 null) myotubes. Unlike previously described MH-linked mutations in CaV1.1, R174W ablated the L-type current and had no effect on EC coupling. Nonetheless, R174W increased sensitivity of Ca$^{2+}$ release to caffeine (used for MH diagnostic in vitro testing) and to volatile anesthetics. Moreover, in CaV1.1, R174W-expressing myotubes, resting myoplasmic Ca$^{2+}$ levels were elevated, and sarcoplasmic reticulum (SR) stores were partially depleted, compared with myotubes expressing wild-type CaV1.1. Our results indicate that CaV1.1 functions not only to activate RyR1 during EC coupling, but also to suppress resting RyR1-mediated Ca$^{2+}$ leak from the SR, and that perturbation of CaV1.1 negative regulation of RyR1 leak identifies a unique mechanism that can sensitize muscle cells to MH triggers.

1,4-dihydropyridine receptor | αS

Malignant hyperthermia (MH) is a potentially fatal, pharmacogenetic disorder that can be triggered by depolarizing muscle relaxants or halogenated anesthetics (1). Following exposure to one of these triggers, MH-susceptible individuals enter a hypermetabolic crisis characterized by tachycardia, muscle rigidity, rhabdomyolysis, increased oxidative/nitrosative stress, mitochondrial damage, acidosis, hypercapnia, and renal failure leading ultimately to death. Currently, the only means to pharmacologically terminate an MH crisis is immediate administration of the hydantoin derivative dantrolene (2).

MH has long been linked to defective excitation–contraction (EC) coupling in skeletal muscle (3). Ordinarily, EC coupling in skeletal muscle occurs in response to depolarization of the plasma membrane, which causes conformational changes of L-type Ca$^{2+}$ channels containing CaV1.1 as the principal subunit (4–6). These voltage-dependent conformational changes result in (i) activation of the L-type Ca$^{2+}$ current and (ii) the activation of Ca$^{2+}$ release from the sarcoplasmic reticulum (SR) via type 1 ryanodine receptors (RyR1). Importantly, activation of RyR1 in skeletal muscle does not depend on the L-type Ca$^{2+}$ current but, rather, is linked directly to the conformational changes of the L-type channel (“conformational coupling”) (4, 7, 8).

The vast majority of mutations (>180) associated with MH lie in the gene encoding RyR1 (9), but three individual mutations have been identified in the gene encoding CaV1.1 (10–13). Previously characterized mutations in either channel have been found to promote activation of RyR1 during EC coupling and/or to enhance Ca$^{2+}$ entry via CaV1.1 (13–21). Thus, it has been proposed that augmented Ca$^{2+}$ movements via the EC coupling pathway or via the L-type current underlie the fulminant response to MH triggers.

In this study, we describe the unexpected functional consequences of the recently identified R174W MH-causing mutation in CaV1.1 (10). In particular, we have found that the R174W mutation ablates activation of the L-type Ca$^{2+}$ current but has surprisingly little effect on the CaV1.1-dependent activation of RyR1 Ca$^{2+}$ release in response to depolarization. Strikingly, however, dysgenic (CaV1.1 null) myotubes expressing CaV1.1 R174W had elevated resting myoplasmic Ca$^{2+}$ levels, depressed SR Ca$^{2+}$ store content, and a greatly increased sensitivity to volatile anesthetics. Thus, it appears that the R174W mutation perturbs resting coupling between CaV1.1 and RyR1, promoting RyR1 leak (22), and that altered resting coupling represents a unique mechanism for the pathogenesis of MH.

Results

R174W Mutation in CaV1.1 Ablates L-Type Ca$^{2+}$ Current. Because the R174W mutation occurs at a highly conserved position in a region of CaV1.1 (IS4) known to be important in sensing changes in membrane potential (23, 24), we first examined whether the R174W mutation affected the ability of CaV1.1 to function as an L-type Ca$^{2+}$ channel. For this, we constructed a YFP-CaV1.1 R174W fusion construct and expressed the mutant channel in dysgenic

www.pnas.org/cgi/doi/10.1073/pnas.1119207109

PNAS | May 15, 2012 | vol. 109 | no. 20 | 7923–7928
(effectively Cav1.1 null) myotubes. Remarkably, YFP-Cav1.1 R174W yielded no inward Ca^{2+} current under our standard recording conditions (n = 7; Fig. 1A) even though the fluorescence arising from the YFP tag indicated normal expression and targeting of the mutant channel (Fig. S1).

In contrast to YFP-Cav1.1, which predictably produced robust L-type current (−6.1 ± 0.7 pA/pF at +50 mV, n = 32; Fig. 1B), YFP-Cav1.1 R174W had an I–V relationship that was nearly superimposable on that of naive dysgenic myotubes (Fig. 1C and Table S1). To determine whether the absence of L-type current in YFP-Cav1.1 R174W-expressing myotubes was a consequence of fewer channels present in the membrane, we recorded intramembrane charge movements. Myotubes expressing YFP-Cav1.1 R174W produced maximal charge movement (6.5 ± 0.7 nC/μF, n = 7; Fig. 1D) that was similar to that of YFP-Cav1.1-expressing myotubes (7.6 ± 0.9 nC/μF, n = 12; P > 0.05, unpaired t test; Fig. 1E and Table S1) and was substantially larger than that of naive dysgenic myotubes (1.6 ± 0.2 nC/μF, n = 6; P < 0.001, ANOVA; Fig. 1F and Table S1). Thus, the absence of L-type current in YFP-Cav1.1 R174W-expressing myotubes was not a consequence of poor membrane expression of the mutant channel. We observed no clear difference in the voltage dependence of charge movement between YFP-Cav1.1– and YFP-Cav1.1 R174W-expressing myotubes (V0 = −7.4 ± 2.0 vs. −6.4 ± 2.4 mV, respectively; P > 0.05, unpaired t test; Fig. 1F and Table S1).

**EC Coupling Is Little Affected by the Ca_{1.1} R174W Mutation.** To determine whether the R174W mutation altered EC coupling, as has been demonstrated for other MH-causing mutations in either RyR1 (14–20, 25–27) or Cav1.1 (21), we measured myoplasmic Ca^{2+} transients in the whole-cell configuration. As shown in Fig. 2A, dysgenic myotubes expressing YFP-Cav1.1 produced robust Ca^{2+} transients with a mean ΔF/ΔF_{max} of 1.82 ± 0.63 (n = 11) and an activation midpoint (V_0) of 14.0 ± 2.5 mV. Dysgenic myotubes expressing YFP-Cav1.1 R174W yielded Ca^{2+} transients that were similar both in magnitude (ΔF/ΔF_{max} = 1.85 ± 0.49, n = 6; P > 0.05, unpaired t test; Fig. 2B and C and Table S2) and in voltage dependence (V_0 = 10.1 ± 1.5 mV; P > 0.05, unpaired t test; Table S2).

As an additional means to study possible alterations in the ability of Cav1.1 R174W to engage EC coupling, we created cell lines stably expressing either wild-type Cav1.1 or Cav1.1 R174W from dysgenic progenitor cells (Fig. S2). Myoplasmic Ca^{2+} transients in these cells were measured in response to depolarization

**Fig. 1.** Ca^{2+} currents and charge movements recorded from primary dysgenic myotubes expressing either YFP-Cav1.1 or YFP-Cav1.1 R174W. (A and B) Representative currents evoked from −50 mV to −10, 10, 30, and 50 mV are shown for dysgenic myotubes expressing either YFP-Cav1.1 R174W (A) or YFP-Cav1.1 (B). (C) Peak I–V relationships for naive dysgenic myotubes (○, n = 8) or dysgenic myotubes expressing either YFP-Cav1.1 (●, n = 32) or YFP-Cav1.1 R174W (□, n = 7). In the case of YFP-Cav1.1 R174W, I–V data were collected only from the 7 myotubes where channel expression was confirmed by charge movement; no inward current was observed in 10 additional dysgenic myotubes displaying yellow fluorescence. Currents were evoked at 0.1 Hz by test potentials ranging from −20 mV through +90 mV in 10-mV increments following a prepulse protocol (41). Currents were normalized by linear cell capacitance (pA/pF). The smooth curve for YFP-Cav1.1 is plotted according to Eq. 1, with best-fit parameters presented in Table S1. (D and E) Representative charge movements evoked from −50 mV to −30, −10, 10, and 30 mV are shown for dysgenic myotubes expressing either YFP-Cav1.1 R174W (D) or YFP-Cav1.1 (E). Charge movements were evoked at 0.1 Hz by test potentials ranging from −40 mV through +50 mV in 10-mV increments following a prepulse protocol (41). Q–V relationships for naive dysgenic myotubes (n = 6) or dysgenic myotubes expressing either YFP-Cav1.1 (n = 12) or YFP-Cav1.1 R174W (n = 7). The smooth curves are plotted according to Eq. 2, with respective best-fit parameters presented in Table S1.

**Fig. 2.** The R174W mutation has little effect on the ability of Cav1.1 to function as the voltage sensor for EC coupling. (A and B) Recordings of myoplasmic Ca^{2+} transients elicited by 50-ms depolarizations from −50 mV to −20, −10, 0, 10, 20, and 30 mV are shown for dysgenic myotubes expressing either YFP-Cav1.1 (A) or YFP-Cav1.1 R174W (B). (C) Average ΔF/ΔF_{max} relationships for dysgenic myotubes expressing either YFP-Cav1.1 (●, n = 11) or YFP-Cav1.1 R174W (○, n = 7) fitted by Eq. 3 with the parameters presented in Table S1. (D and E) Representative elevated K^{+}-induced Ca^{2+} transients in dysgenic myotubes stably expressing wild-type Cav1.1 (D) or Cav1.1 R174W (E). (F) K^{+} dose–response relationships for dysgenic myotubes stably expressing either wild-type Cav1.1 (n = 19) or Cav1.1 R174W (n = 32) fitted by Eq. 4. (G and H) Representative caffeine-induced Ca^{2+} transients in dysgenic myotubes stably expressing Cav1.1 (G) or Cav1.1 R174W (H). At the beginning of each experiment, myotubes were exposed to K^{+} (60 mM) for 10 s to confirm either wild-type or mutant Cav1.1 expression. (J) Caffeine dose–response relationships for dysgenic myotubes stably expressing either wild-type Cav1.1 (n = 24) or Cav1.1 R174W (n = 28) fitted by Eq. 4.
by elevated extracellular K⁺. In these experiments, myotubes expressing either CaV1.1 or CaV1.1 R174W displayed virtually identical responses to elevated K⁺ (EC₅₀ = 18.9 ± 0.6 mM, n = 19, and 18.0 ± 0.9 mM, n = 31, respectively; P > 0.05, t test; Fig. 2 D–F). Thus, the R174W mutation ablates the ability of CaV1.1 to conduct L-type Ca²⁺ current but has little, if any, effect on its function to serve as the voltage sensor for EC coupling.

Hypersensitivity to the RyR1 agonist caffeine in the in vitro contracture test (IVCT) is a benchmark diagnostic assay for MH susceptibility (3, 28). For this reason, we assessed the caffeine sensitivities of wild-type CaV1.1- and CaV1.1 R174W-expressing myotubes. At the beginning of each experiment, myotubes were first exposed to a 60-mM K⁺ challenge to completely deplete SR Ca²⁺ store was associated with the elevation in resting myoplasmic Ca²⁺ (120 ± 2 mM, n = 15 and 121 ± 2 mM, n = 12, respectively; P > 0.05, ANOVA; Fig. 3). In stark contrast, the CaV1.1 R174W-expressing myotubes to increase by nearly 20-fold (863 ± 21 nM, n = 8 and 1,234 ± 21 nM, n = 10, respectively; P < 0.001, ANOVA; Table 1). We next investigated whether a partially depleted SR Ca²⁺ store was associated with the elevation in resting myoplasmic Ca²⁺ by exposing myotubes to ionomycin (5 μM) in a nominally Ca²⁺-free medium (22). As shown in Fig. 4, the content of the SR Ca²⁺ store was less in CaV1.1 R174W-expressing myotubes than in those expressing wild-type CaV1.1 and CaV1.1 (Table 1) (31).

Fig. 3. Dysgenic myotubes expressing CaV1.1 R174W (Right) display elevated resting myoplasmic Ca²⁺ and hypersensitivity to MH triggers compared with dysgenic myotubes expressing wild-type CaV1.1 (Left). Myoplasmic Ca²⁺ levels measured with Ca²⁺-sensitive microelectrodes are shown before (control) and after exposure to either isoflurane (0.1% vol/vol; Right, hatched, bar) or halothane (0.1% vol/vol; Left, hatched bar). ###, significant difference (P < 0.001, ANOVA) in resting Ca²⁺ between wild-type CaV1.1 and CaV1.1 R174W control groups. ***, significant (P < 0.001, ANOVA) difference between control and anesthetized-treated CaV1.1 R174W-expressing myotubes. In dysgenic myotubes expressing wild-type CaV1.1, no significant changes in myoplasmic Ca²⁺ were observed following exposure to the volatile anesthetics relative to control.

Fig. 4. SR Ca²⁺ store is partially depleted in CaV1.1 R174W-expressing dysgenic myotubes. (A) Representative ionomycin (5 μM)-induced Ca²⁺ transients in dysgenic myotubes stably expressing either wild-type CaV1.1 (shaded trace) or CaV1.1 R174W (solid trace). (B) Quantification of peak transient amplitude (Left) and integrated transient area (Right) for ionomycin-induced Ca²⁺ transients in dysgenic myotubes expressing CaV1.1 (n = 33) and CaV1.1 R174W (n = 39). Asterisks indicate significant differences (***P < 0.001; **P < 0.01; ***P < 0.001; unpaired t test).
MH crises, restores resting Ca$^{2+}$ to normal levels in that in naive in MH pathogenesis is that dantrolene, the clinical antidote for MH susceptibility fundamentally different from those ascribed to other MH-causing mutations, which either enhance entry of Ca$^{2+}$ via the L-type channel or shift the equilibrium of RyR1 toward the activated (open) state. Importantly, we find that the R174W mutation does not alter the voltage dependence of EC coupling (Fig. 2), which indicates that this mutation does not shift the equilibrium of RyR1 toward the activated state. Furthermore, the mutation obviously does not promote L-type Ca$^{2+}$ entry (Fig. 1). However, our results are consistent with the idea that the R174W mutation in CaV1.1 derepresses Ca$^{2+}$ efflux from the SR in resting muscle cells. Thus, the CaV1.1–RyR1 interaction appears to have three manifestations: (i) CaV1.1-mediated engagement of EC coupling via RyR1, (ii) RyR1-dependent retrograde enhancement of L-type current via CaV1.1, and (iii) CaV1.1-mediated suppression of a resting SR Ca$^{2+}$ leak via RyR1. Evidence for the suppression of a resting Ca$^{2+}$ leak from the SR via RyR1 (Fig. 5), leading to elevated resting myoplasmic Ca$^{2+}$ and increasing the number of myotubes tested. Control cells were exposed only to Imaging Solution (IS). Statistical differences: ***P < 0.001 vs. CaV1.1 (one-way ANOVA); **P < 0.01 vs. CaV1.1 R174W control. NS, no significant difference vs. CaV1.1 (P > 0.05, one-way ANOVA).

Discussion

The results reported here identify a mechanism for MH susceptibility fundamentally different from those ascribed to other MH-causing mutations, which either enhance entry of Ca$^{2+}$ via the L-type channel or shift the equilibrium of RyR1 toward the activated (open) state. Importantly, we find that the R174W mutation does not alter the voltage dependence of EC coupling (Fig. 2), which indicates that this mutation does not shift the equilibrium of RyR1 toward the activated state. Furthermore, the mutation obviously does not promote L-type Ca$^{2+}$ entry (Fig. 1). However, our results are consistent with the idea that the R174W mutation in CaV1.1 derepresses Ca$^{2+}$ efflux from the SR in resting muscle cells. Thus, the CaV1.1–RyR1 interaction appears to have three manifestations: (i) CaV1.1-mediated engagement of EC coupling via RyR1, (ii) RyR1-dependent retrograde enhancement of L-type current via CaV1.1, and (iii) CaV1.1-mediated suppression of a resting SR Ca$^{2+}$ leak via RyR1. Evidence for the last of these is provided by our previous work, which showed that resting SR Ca$^{2+}$ stores are reduced, and myoplasmic Ca$^{2+}$ levels are higher, in naive dysgenic (i.e., CaV1.1-lacking) myotubes compared with wild-type myotubes (22), suggesting that CaV1.1 represses a resting Ca$^{2+}$ leak from the SR via RyR1 (Fig. 5A). The increase in resting myoplasmic Ca$^{2+}$ in CaV1.1 R174W-expressing myotubes is also accompanied by a reduction in the resting SR Ca$^{2+}$ store (Fig. 4). This leak pathway in dysgenic myotubes appears to be distinct from the conventional activated state of RyR1 because it is blocked by bastadin 5 (or bastadin 5 in the presence of ryanodine) but not by ryanodine alone (32, 33); bastadin 5 (B5) application also causes a partial reduction in resting myoplasmic Ca$^{2+}$ in dysgenic myotubes expressing CaV1.1 (Table 1). As shown in Fig. 5B, expression of wild-type CaV1.1 in dysgenic myotubes represses the resting Ca$^{2+}$ leak (22) and restores both EC coupling and L-type current (6). The R174W mutation alters the resting Ca$^{2+}$ leak from the SR via RyR1 (Fig. 5A). Thus, the R174W mutation alters the CaV1.1 such that it does not suppress resting SR Ca$^{2+}$ leak, leading to an increase in resting Ca$^{2+}$ and enhanced sensitivity to MH triggers (Left; Fig. 3); the R174W mutation also ablates the ability of CaV1.1 to produce L-type current but has little, if any, effect on the ability of CaV1.1 to engage EC coupling (Right).

Table 1. Myoplasmic Ca$^{2+}$ measurements

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<th>[Ca$^{2+}$]$_{i}$, nM</th>
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<tr>
<td></td>
<td>CaV1.1</td>
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<tr>
<td>Control</td>
<td>120 ± 1 (18)</td>
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<tr>
<td>Nifedipine, 25 μM</td>
<td>118 ± 3 (6)</td>
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<tr>
<td>Dantrolene, 50 μM</td>
<td>89 ± 1 (10)***</td>
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<tr>
<td>Bastadin 5, 10 μM</td>
<td>98 ± 2 (8)***</td>
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Data are given as mean ± SEM, with the numbers in parentheses indicating the number of myotubes tested. Control cells were exposed only to Imaging Solution (IS). Statistical differences: ***P < 0.001 vs. CaV1.1 (one-way ANOVA); **P < 0.01 vs. CaV1.1 R174W control. NS, no significant difference vs. CaV1.1 (P > 0.05, one-way ANOVA).

Fig. 5. The R174W mutation in CaV1.1 disrupts the resting interaction between CaV1.1 and RyR1 that suppresses Ca$^{2+}$ leak from the SR (A). Our previous work in dysgenic myotubes has demonstrated that the absence of CaV1.1 reveals a leak state of RyR1 (Left) that increases resting Ca$^{2+}$ (22). Additionally, the absence of the CaV1.1 voltage sensor renders dysgenic myotubes incapable of EC coupling or generating L-type Ca$^{2+}$ current during plasma membrane depolarization (Right) (42). (B) When present, CaV1.1 inhibits Ca$^{2+}$ leak from the SR via RyR1 at rest (Left) and responds to depolarization by engaging EC coupling in Ca$^{2+}$ release and producing L-type current (Right) (6). (C) The R174W mutation alters the configuration of CaV1.1 such that it does not suppress resting SR Ca$^{2+}$ leak, leading to an increase in resting Ca$^{2+}$ and enhanced sensitivity to MH triggers (Left; Fig. 3); the R174W mutation also ablates the ability of CaV1.1 to produce L-type current but has little, if any, effect on the ability of CaV1.1 to engage EC coupling (Right).

RyR1 MH-linked mutations (30). Thus, other mutations in either CaV1.1 or RyR1 may potentially lead to increased resting Ca$^{2+}$ leak. Although increased SR Ca$^{2+}$ leak may represent a sensitizing mechanism, it is almost certain that multiple mechanisms are at work in MH susceptibility. In this regard, any mechanism that causes sustained, partial SR Ca$^{2+}$ store depletion would presumably result in a sustained increase in store-operated Ca$^{2+}$ entry (34, 35) and thus increased resting myoplasmic Ca$^{2+}$. Simply raising myoplasmic Ca$^{2+}$ would be expected to increase the efficacy of other activators (e.g., caffeine and volatile anesthetics). Moreover, chronically elevated myoplasmic Ca$^{2+}$ levels could have downstream sequelae as a result of altered mitochondrial metabolism (17, 36).

In conclusion, we have described a unique mechanism whereby a mutation of CaV1.1 promotes MH susceptibility by causing an increase in resting SR Ca$^{2+}$ leak. It will be important to determine the extent to which an enhanced resting leak contributes to MH susceptibility for other CaV1.1 and RyR1 mutations.

Materials and Methods

Molecular Biology. YFP-CaV1.1 R174W was derived from the plasmid YFP-CaV1.1 (37), using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies).

Primary Culture and cDNA Expression. All procedures involving mice were approved by the Institutional Animal Care and Use Committees of University of Colorado Denver and Harvard Medical School. Primary cultures of dysgenic myotubes were prepared as described previously (38). Single nuclei of
differentially myotubes were microinjected with 200 ng/mL plasmid cDNA encoding either YFP-CaV1.1 or YFP-CaV1.1 R174W.

Generation of Myoblasts Stably Expressing Wild-Type CaV1.1 or CaV1.1 R174W. cDNAs encoding either wild-type CaV1.1 (22) or CaV1.1 R174W were subcloned between the LTRs of a lentiviral vector driven by an EF1α human promoter (22, 39). The lentiviral particles were packaged by transfection of HEK 293T cells as described previously (40). Dysgenic myoblasts were transduced with lentiviral particles and were replated with one cell per well in 96-well culture plates 24 h later. After 2–3 wk, myoblasts from 20–30 clones were plated in 96-well plates and differentiated into myotubes. To screen for expression of either CaV1.1 or CaV1.1 R174W, myotubes were loaded with Fluor-4 AM and challenged by K+ depolarization (see below); positive myotubes were identified by the ability to support Ca2+ transients in response to depolarization.

Electrophysiology. Electrophysiological experiments were performed with dysgenic myotubes expressing either YFP-CaV1.1 or YFP-CaV1.1 R174W 2 d postmicroinjection. Pipettes were fabricated from borosilicate glass and had resistances of ~2.0 MΩ when filled with internal solution, which consisted of 140 mM Cs-aspartate, 10 mM Cs3EGTA, 5 mM MgCl2, and 10 mM Hepes, pH 7.4, with CsOH. The external solution contained 145 mM tetraethylammonium (TEA)-Cl, 10 mM CaCl2, 0.002 mM tetrodotoxin, and 10 mM Hepes, pH 7.4, with CsOH. For measurement of charge movements, 0.5 mM CdCl2 and 0.1 mM LaCl3 were added to the external solution. Filtering was at 2 kHz (eight-pole Bessel filter; Frequency Devices) and digitization was either at 10 kHz (the currents) or 20 kHz (charge movements). Current-voltage (I–V) curves were fitted according to

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

where $I$ is the current for the test potential $V$, $V_{\text{rev}}$ is the reversal potential, $G_{\text{max}}$ is the maximum Ca++ channel conductance, $V_{1/2}$ is the half-maximal activation potential, and $k_{G}$ is the slope factor. Plots of the integral of $Q_{\text{m}}$, as a function of test potential $(V)$ were fitted according to

$$Q_{\text{m}} = \frac{Q_{\text{m,max}}}{1 + \exp\left[-\left(V - V_{1/2}\right)/k_{Q}\right]},$$

where $Q_{\text{m,max}}$ is the maximal $Q_{\text{m}}$, $V_{1/2}$ is the potential causing movement of half the maximal charge, and $k_{Q}$ is a slope parameter.

Whole-Cell Measurement of Ca2+ Transients. Changes in intracellular Ca2+ were recorded with Fluor-3 (F-3715; Invitrogen). The dye was added to the internal solution for a final concentration of 200 μM. After entry into the whole-cell configuration, a period of >5 min was used to allow the dye to diffuse into the cell interior. The peak fluorescence change ($\Delta F/F$) for each test potential (V) was fitted according to

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

where ($\Delta F/F_{\text{max}}$) is the maximal fluorescence change, $V_{1/2}$ is the potential causing half the maximal change in fluorescence, and $k_{F}$ is a slope parameter.

Intact Myotube Ca2+ Imaging. Differentiated dysgenic myotubes stably expressing wild-type CaV1.1 or CaV1.1 R174W were loaded with 5 μM Fluor-4 AM (Invitrogen) for 15 min at 37 °C and washed with Imaging Solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 5.5 mM glucose, 10 mM Hepes, pH 7.4, with NaOH). The Ca2+ signal was monitored using 480/30-nm excitation and 535/40-nm emission wavelengths. The peak response to a given K+ or caffeine concentration ($\Delta F/F_{\text{norm}}$) was quantified as the integrated area under the curve normalized by the maximal response to either agent (60 or 40 mM, respectively). Dose-response relationships were fit by the equation

$$\Delta F/F_{\text{norm}} = \frac{1}{1 + \exp\left[-\left(V - V_{1/2}\right)/k_{\text{EC50}}\right]}$$

where $\text{EC50}$ is the concentration that produces the half-maximal response, $X$ is the concentration of $K^+$ or caffeine applied, and $k_{\text{EC50}}$ is a slope parameter.

Resting Myoplasmic Ca2+ Measurements. Resting myoplasmic Ca2+ levels in intact myotubes were assessed with double-barreled Ca2+-selective microelectrodes as described previously in ref. 22.

Assessment of SR Ca2+ Stores. Dysgenic myotubes stably expressing either wild-type CaV1.1 or CaV1.1 R174W were loaded with Fluor-4 AM (5 μM) for 30 min at 37 °C and then incubated with nominally 0 mM Ca2+ Krebs Ringer solution supplemented with 2 mM EGTA. Ca2+ release from the SR was induced by the application of Krebs Ringer containing ionomycin (5 μM) and quantified by the integrated area under the resultant Ca2+ transient. The Ca2+ signal was monitored using 480/30-nm excitation and 535/40-nm emission wavelengths; the emission signal was acquired at 30 frames per second.

Statistical Analysis. Figures were made using SigmaPlot 11.0 (SPSS). All data are presented as mean ± SEM. Comparisons were made by unpaired, two-tailed t test or one-way ANOVA coupled with Tukey’s t test (as appropriate), with $P < 0.05$ considered significant. A more complete description of experimental methods is included in SI Materials and Methods.

ACKNOWLEDGMENTS. This work was supported by National Institutes of Health Grants AR055104 (to K.G.B.), AR052534 (to P.D.A. and I.N.P.), and AG038778 (to R.A.B.) and Muscular Dystrophy Association Grant MDA 176448 (to K.G.B.).
Supporting Information

Eltit et al. 10.1073/pnas.1119207109

SI Materials and Methods

**Molecular Biology.** YFP-Cav1.1 R174W was derived from the plasmid YFP-Cav1.1 (referred to as YFP-d1g in ref. 1). Briefly, the point mutation was introduced using the QuikChange II XL site-directed mutagenesis kit (200523; Agilent Technologies) with the forward primer 5′-GTGTCCAGACCCCTCTGCTGTTGTG-3′ and the reverse primer 5′-CACCCTGGACACCA-GCCAGGGGTCTGGACAC-3′. Sequencing of the final construct verified the presence of the point mutation.

**Primary Dysgenic Myotube Culture and cDNA Expression.** All procedures involving mice were approved by the Institutional Animal Care and Use Committees of the University of Colorado Denver–Anschutz Medical Campus and Harvard Medical School. Primary cultures of dysgenic (mdg/mdg) myotubes were prepared as described previously (2). Cultures were grown for 6–7 d in a humidified 37 °C incubator with 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM) (15-017-CM; Media- tetch), supplemented with 10% (vol/vol) FBS/10% (vol/vol) horse serum (HyClone Laboratories). This medium was then replaced with differentiation medium (DMEM supplemented with 2% horse serum). Two to four d following the shift to differentiation medium, single nuclei were microinjected with 200 ng/µL plasmid cDNA encoding either YFP-Cav1.1 or YFP-Cav1.1 R174W.

**Generation of Myoblasts Stably Expressing Wild-Type Cav1.1 or Cav1.1-R174W.** cDNAs encoding either wild-type Cav1.1 or Cav1.1 R174W were subcloned between the LTRs of a lentiviral vector driven by an EF1α human promoter (3, 4). The lentiviral particles were subsequently packaged into HEK 293T cells by the addition of 0.5 mM CdCl2. Positive myoblasts were identified by the presence of the point mutation.

**Measurement of Ionic Currents and Charge Movements.** Electrophysiological experiments were performed with dysgenic myotubes expressing either YFP-Cav1.1 or YFP-Cav1.1 R174W 2 d following microinjection; expressing myotubes were identified by YFP fluorescence. Pipettes were fabricated from borosilicate glass and had resistances of ∼2.0 MΩ when filled with internal solution, which consisted of 140 mM Cs-aspartate, 10 mM Cs-EGTA, 5 mM MgCl2, and 10 mM Hepes, pH 7.4, with CsOH. The external solution contained 145 mM tetraethylammonium chloride, 10 mM CaCl2, 0.002 M tetrodotoxin, and 10 mM Hepes, pH 7.4, with tetraethylammonium-OH. For measurement of intracellular charge movements, ionic currents were blocked by the addition of 0.5 mM CdCl2 and 0.1 mM LaCl3 to the external solution. Electronic compensation was used to reduce the effective series resistance (usually to <1 MΩ) and the time constant for charging the linear cell capacitance (usually to <0.5 ms). L-type currents were corrected for linear components of leak and capacitive current by digital scaling and subtraction of the average of 11, 30-mV hyperpolarizing pulses from a holding potential of −80 mV. Charge movements were corrected for linear cell capacitance and leakage currents using a −P/8 subtraction protocol. Filtering was at 2 kHz (eight-pole Bessel filter; Frequency Devices) and digitization was either at 10 kHz (L-type currents) or at 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 25-ms repolarization to −50 mV was administered before the test pulse (prepulse protocol) (6), to inactivate endogenous Cav1.3 and Nav channels. Cell capacitance was determined by integration of a transient from −80 mV to −70 mV, using Clampex 8.0 (Molecular Devices), and was used to normalize charge movements (nC/pF) and current amplitudes (pA/pF). Current–voltage (I–V) curves were fitted according to

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

[S1]

where \(I\) is the 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 \(k_G\) is the slope factor. Plots of the integral of the ON charge movement \(Q_{\text{on}}\) as a function of test potential \((V)\) were fitted according to

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

[S2]

where \(Q_{\text{max}}\) is the maximal \(Q_{\text{on}}\) \(V_{1/2}\) is the potential causing movement of half the maximal charge, and \(k_Q\) is a slope parameter. All electrophysiological experiments were performed at room temperature (∼25 °C).

**Measurement of Intracellular Ca2⁺ Transients.** Changes in intracellular \(Ca^{2+}\) were recorded with Fluo-3 (F-3715; Molecular Probes). The salt form of the dye was added to the standard internal solution for a final concentration of 200 µM. After entry into the whole-cell configuration, a waiting period of >5 min was used to allow the dye to diffuse into the cell interior. A 100-W mercury illuminator and a set of fluorescein filters were used to excite the dye present in a small rectangular region of the voltage-clamped myotube. A computer-controlled shutter was used to block illumination in the intervals between test pulses. Fluorescence emission was measured by means of a fluorometer apparatus (Biomedical Instrumentation Group). The average background fluorescence was quantified before bath immersion of the patch pipette. Fluorescence data are expressed as the total change in fluorescence \(\Delta F/F\), 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 (non–Fluo-3) fluorescence. The peak value of the fluorescence change \(\Delta F/F\) for each test potential \((V)\) was fitted according to

\[
\Delta F/F = [\Delta F/F]_{\text{max}}/[1 + \exp(-(V - V_{1/2})/k_F)],
\]

[S3]

where \(\Delta F/F\) is the maximal fluorescence change, \(V_{1/2}\) is the potential causing half the maximal change in fluorescence, and \(k_F\) is a slope parameter.
Ca\(^{2+}\) Imaging. Differentiated dysgenic myotubes stably expressing wild-type Cav1.1 or Cav1.1 R174W were loaded with 5 μM Fluor-4 AM (Invitrogen) for 15 min at 37 °C, washed three times with Imaging Solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 5.5 mM glucose, 10 mM Hepes, pH 7.4, with NaOH), and placed in the stage of a Nikon TE2000 microscope coupled with a digital acquisition system (Stanford Photonics). The Ca\(^{2+}\) signal was monitored using 480/30-nm excitation and 535/40-nm emission wavelengths. Elevated K\(^{+}\)-induced depolarization was assessed by exposing myotubes to 10-s pulses of 10, 20, 30, and 60 mM K\(^{+}\) solutions (Na\(^{+}\) was decreased appropriately to maintain constant osmolarity). Caffeine sensitivity was evaluated by exposing myotubes to 30-s pulses of isoflurane (IS) supplemented with 0.5, 1, 3, 5, 10, and 40 mM caffeine. In both cases, a >2-min interval in IS between pulses was used to ensure recovery of the basal Ca\(^{2+}\) level after each pulse. Peak responses to either elevated K\(^{+}\) or caffeine were quantified as the integrated area under the curve for each induced Ca\(^{2+}\) release event. The peak response of a given K\(^{+}\) or caffeine concentration was then normalized by the maximal response to either agent (60 or 40 mM, respectively) and dose–response relationships were fitted by the modified Hill equation
\[
\frac{(\Delta F/F)_{\text{norm}}}{(\Delta F/F)_{\text{norm}}} = \frac{1}{1 + 10 \exp((\log EC_{50} - \log X) \times k)},
\]
where \((\Delta F/F)_{\text{norm}}\) is the \((\Delta F/F)\) normalized by the maximal response, EC\(_{50}\) is the concentration that produces 50% of the maximal response, X is the concentration of K\(^{+}\) or caffeine applied, and k is a slope parameter.

Assessment of Sarcoplasmic Reticulum (SR) Ca\(^{2+}\) Stores. Dysgenic myotubes stably expressing either wild-type Cav1.1 or Cav1.1 R174W were loaded with Fluor-4 AM (5 μM) for 30 min at 37 °C and then incubated with nominally 0 mM Ca\(^{2+}\) Krebs Ringer solution supplemented with 2 mM EGTA. Ca\(^{2+}\) release from the SR was induced by the application of Krebs Ringer containing ionomycin (5 μM) and quantified by the integrated area under the resultant Ca\(^{2+}\) transient. Fluorescence data were obtained with a Nikon TE2000 epi-fluorescence microscope coupled to a digital acquisition system (Stanford Photonics). The Ca\(^{2+}\) signal was monitored using 480/30-nm excitation and 535/40-nm emission wavelengths. The emission signal was acquired at 30 frames per second.

Resting Myoplasmic Ca\(^{2+}\) Measurements. Double-barreled Ca\(^{2+}\)-selective microelectrodes were prepared and calibrated as previously described (3). Only those electrodes with a linear relationship between pCa 3 and pCa 8 (Nernstian response, 28.5 mV per pCa unit at 24 °C) were used experimentally. To mimic the intracellular ionic conditions more effectively, all calibration solutions were supplemented with 1 mM Mg\(^{2+}\). All electrodes were then recalibrated after making measurements of myoplasmic Ca\(^{2+}\), and the data from a microelectrode were discarded if the two calibration curves diverged >3 mV from pCa 7 to pCa 8. Myotubes were impaled with the double-barreled microelectrode and potentials were recorded via a high-impedance amplifier (WPI FD-223). The potential from the 3-M KCl barrel \((V_{\text{Ca}})\) was subtracted electronically from \(V_{\text{Ca},0}\) to produce a differential Ca\(^{2+}\)-specific potential \((V_{\text{Ca}})\) that represents the myoplasmic Ca\(^{2+}\) concentration. \(V_{\text{m}}\) and \(V_{\text{Ca}}\) were filtered (30–50 KHz) to improve the signal-to-noise ratio and stored in a computer for further analysis.

Pharmacology. Caffeine (Sigma) was diluted directly into IS, and dantrolene (Sigma) was dissolved in dry DMSO to make a 25-mM stock. Nifedipine (Sigma) was dissolved in DMSO to make a 25-mM stock. Both dantrolene and nifedipine were diluted to their respective working concentrations immediately before use in the dark. Volatile anesthetics (i.e., halothane and isoflurane) were obtained from Halocarbon and Baxter, respectively. To make 0.1% vol/vol working solutions, 100% volatile anesthetic was drawn with a Hamilton syringe using a large bore flat needle, transferred to an amber bottle containing Imaging Solution, and placed in 50 °C water bath for 10 min. The working solution was then sonicated at ~45–50 °C for another 10 min with periodic agitation. Bastadin 5 (B5) was extracted from hophylized Ianthella basta sponge and was solubilized in water (7). B5 was added to the Ringer’s solution for a final concentration of 20 μM 10 min before making measurements of resting myoplasmic Ca\(^{2+}\).

Statistical Analysis. Figures were made using the software program SigmaPlot (version 11.0; SSPS). All data are presented as mean ± SEM. Statistical comparisons were made by unpaired, two-tailed t test or one-way ANOVA coupled with Tukey’s t test (as appropriate), with P < 0.05 considered significant.

Fig. S1. Junctional targeting of YFP-Ca\textsubscript{V}1.1 and YFP-Ca\textsubscript{V}1.1 R174W in primary dysgenic myotubes. Confocal images are shown for dysgenic myotubes expressing YFP-Ca\textsubscript{V}1.1 (A), YFP-Ca\textsubscript{V}1.1 R174W (B), C1-YFP (C), and a naive dysgenic myotube (D, Upper). Except for C1-YFP, all images were obtained with similar laser settings. (D, Lower) Light image of the same myotube shown in D, Upper. Two days postinjection, myotubes were examined in Rodent Ringer’s solution (146 mM NaCl, 5 mM KCl, 2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM Hepes, 11 mM glucose, pH 7.4, with NaOH), using the confocal laser scanning microscope LSM 510 META. YFP was excited with the 514-nm line of an argon laser (30-mW maximum output, operated at 50% or 6.3 A, attenuated by 95%), which was directed to the cell via a 488/543-nm dual dichroic mirror. The emitted YFP fluorescence was directed to a photomultiplier equipped with a 530-nm long-pass filter. Confocal fluorescence intensity data were recorded as the average of four line scans per pixel and digitized at 8 bits, with photomultiplier gain adjusted such that maximum pixel intensities were no more than \(\sim 70\%\) saturated. (Scale bars, 5 \(\mu\)m.)

Fig. S2. Junctional targeting of Ca\textsubscript{V}1.1 and Ca\textsubscript{V}1.1-R174W in a dysgenic cell line. (A–C) Epi-fluorescence images of dysgenic myotubes stably expressing either Ca\textsubscript{V}1.1 (A) or Ca\textsubscript{V}1.1-R174W (B) and of a naive dysgenic myotube (C). In each case, the myotubes were immunostained with a monoclonal antibody directed to Ca\textsubscript{V}1.1 (ABR-Affinity BioReagents), which was visualized by an Alexa Fluor 568-conjugated goat anti-mouse secondary antibody (Invitrogen). Nuclei were visualized using DAPI staining. (Scale bars, 5 \(\mu\)m.)

Table S1. L-type conductance and intramembrane charge movement

<table>
<thead>
<tr>
<th>Construct</th>
<th>(G_{\text{max}}), nS/nF</th>
<th>(V_{1/2}), mV</th>
<th>(k_C), mV</th>
<th>(Q_{\text{max}}), nC/(\mu)F</th>
<th>(V_0), mV</th>
<th>(k_Q), mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>YFP-Ca\textsubscript{V}1.1</td>
<td>179 ± 18 (32)</td>
<td>35.2 ± 1.5</td>
<td>8.4 ± 0.4</td>
<td>7.6 ± 0.9 (12)</td>
<td>−7.4 ± 2.0</td>
<td>11.6 ± 0.7</td>
</tr>
<tr>
<td>YFP-Ca\textsubscript{V}1.1 R174W</td>
<td>No inward current (7)</td>
<td>6.5 ± 0.7 (7)</td>
<td>−6.4 ± 2.4</td>
<td>10.0 ± 0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninjected dysgenic myotubes</td>
<td>No inward current (17)</td>
<td>1.6 ± 0.2*** (6)</td>
<td>−19.5 ± 2.1**</td>
<td>5.8 ± 1.9**</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are given as mean ± SEM, with the numbers in parentheses indicating the number of myotubes tested. L-type current conductance was fitted by Eq. 1 and charge movements were fitted by Eq. 2. For all of the data given, the calculated average voltage error was \(<5\) mV. Asterisks indicate significant differences relative to YFP-Ca\textsubscript{V}1.1 (***\(P < 0.005\); ****\(P < 0.001\); one-way ANOVA).
### Table S2. Ability of fluorescent protein-tagged Ca\(_v\)1.1 clones to restore EC coupling in dysgenic myotubes

<table>
<thead>
<tr>
<th>Construct</th>
<th>( [\Delta F/F]_{\text{max}} )</th>
<th>( V_i ), mV</th>
<th>( k_i ), mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>YFP-Ca(_v)1.1</td>
<td>1.82 ± 0.63 (11)</td>
<td>14.0 ± 2.5</td>
<td>6.8 ± 0.7</td>
</tr>
<tr>
<td>YFP-Ca(_v)1.1 R174W</td>
<td>1.85 ± 0.49 (6)</td>
<td>10.1 ± 1.5</td>
<td>6.1 ± 1.4</td>
</tr>
</tbody>
</table>

Data are given as mean ± SEM, with the numbers in parentheses indicating the number of myotubes tested. All \( \Delta F/F-V \) data given were fitted with Eq. 2.