Gene Dose Influences Cellular and Calcium Channel Dysregulation in Heterozygous and Homozygous T4826I-RYR1 Malignant Hyperthermia-susceptible Muscle*

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Background: Muscle from heterozygous and homozygous T4826I-RYR1 MH-susceptible mice is investigated for biochemical and cellular abnormalities.

Results: T4826I-RYR1 gene dose determines severity of [Ca2+]rest, mitochondrial, EC coupling, and Ca2+ channel impairments.

Conclusion: T4826I-RYR1 channel dysfunction is regulated in vivo but imparts susceptibility to environmental triggers.

Significance: T4826I-RYR1 is sufficient to confer MHS strongly dependent on gene dose.

Malignant hyperthermia susceptibility (MHS) is primarily conferred by mutations within ryanodine receptor type 1 (RYR1). Here we address how the MHS mutation T4826I within the S4-S5 linker influences excitation-contraction coupling and resting myoplasmic Ca2+ concentration ([Ca2+]rest) in flexor digitorum brevis (FDB) and vastus lateralis prepared from heterozygous (Het) and homozygous (Hom) T4826I-RYR1 knock-in mice (Yuen, B. T., Boncompagni, S., Feng, W., Yang, T., Lopez, J. R., Matthaei, K. I., Goth, S. R., Protasi, F., Franzini-Armstrong, C., Allen, P. D., and Pessah, I. N. (2011) FASEB J. doi:22131268). FDB responses to electrical stimuli and acute halothane (0.1%, v/v) exposure showed a rank order of Hom > Het > WT. Release of Ca2+ from the sarcoplasmic reticulum and Ca2+ entry contributed to halothane-triggered increases in [Ca2+]rest in Hom FDBs and elicited pronounced Ca2+ oscillations in ~30% of FDBs tested. Genotype contributed significantly elevated [Ca2+]rest (Hom > Het > WT) measured in vivo using ion-selective microelectrodes. Het and Hom oxygen consumption rates measured in intact myotubes using the Seahorse Bioscience (Billericia, MA) flux analyzer and mitochondrial content measured with MitoTracker were lower than WT, whereas total cellular calpain activity was higher than WT. Muscle membranes did not differ in RYR1 expression nor in Ser2844 phosphorylation among the genotypes. Single channel analysis revealed highly divergent gating behavior with Hom and WT favoring open and closed states, respectively, whereas Het exhibited heterogeneous gating behaviors. [3H]Ryanodine binding analysis revealed a gene dose influence on binding density and regulation by Ca2+, Mg2+, and temperature. Pronounced abnormalities inherent in T4826I-RYR1 channels confer MHS and promote basal disturbances of excitation-contraction coupling, [Ca2+]rest, and oxygen consumption rates. Considering that both Het and Hom T4826I-RYR1 mice are viable, the remarkable isolated single channel dysfunction mediated through this mutation in S4-S5 cytoplasmic linker must be highly regulated in vivo.

Fulminant malignant hyperthermia (MH)3 is a pharmacogenetic, life-threatening syndrome triggered in susceptible individuals by volatile general anesthetics, depolarizing neuromuscular blocking agents, and heat stress (2). An MH episode is characterized by increased expired CO2, rapid onset of metabolic acidosis, elevated core temperature, and sustained muscle contraction. Such episodes are often associated with ventricular tachycardia and cardiac arrest. The prevalence of MH has been estimated as 1 in 10,000 anesthetic procedures; however, this is likely to be an underestimate of MH susceptibility in the general population, which has been estimated as high as 1:2,000 (3–5).

Although seven genomic loci have been linked to the disorder (6, 7), mutations in only two skeletal muscle proteins have been confirmed to confer MH susceptibility. At least 250 mutations within the RYR1 locus (19q13.1) that encodes for the type 1 ryanodine receptor (RYR1), a Ca2+ channel that localizes within skeletal muscle junctional sarcoplasmic reticulum (SR), have been associated with human MH susceptibility (4, 8–10). RYR1 mutations currently account for more than 50% of the families identified (4). More recently, a small number of mutations in CACNA1S (1q32) that encode for the pore-forming

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The abbreviations used are: MH, malignant hyperthermia; MHS, MH susceptibility; SR, sarcoplasmic reticulum; EC, excitation-contraction; Het, heterozygous; Hom, homozygous; OCR, oxygen consumption rate; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; t-Roc, tert-Roc-L-leucyl-L-methionine amide; Ry, ryanodine; [Ca2+]rest, resting Ca2+ concentration; RC, respiratory capacity; PP1, protein phosphatase 1; DHPR, dihydropyridine receptor; MHS, BLM, bilayer lipid membrane.
subunit (Ca\textsubscript{v.1.1}; \alpha_{1s}\textsubscript{DHPR}) of the L-type voltage-dependent Ca\textsuperscript{2+} channels that localize within the skeletal muscle T-tubule membrane were also confirmed to confer MH susceptibility. Confirmed cases include three families with R1086H (11, 12), one family with R1086S (13), and another with the R174W mutation (14). One theory currently being tested is that MH mutations in either Ca\textsubscript{v.1.1} or RYR1 alter the fidelity of bidirectional signaling across T-tubule SR junctions that is essential for normal skeletal muscle excitation-contraction (EC) coupling (15) and regulation of SR Ca\textsuperscript{2+} leak (16).

Knock-in mice heterozygous (Het) for missense mutation R163C-RYR1 (17) or Y522S-RYR1 (18), two of the more common mutations conferring MH susceptibility in humans, exhibit fulminant MH when exposed to either an inhaled volatile general anesthetic (e.g. halothane) or heat stress. Homozygous (Hom) R163C-RYR1 and Y522S-RYR1 mice are not viable, whereas their Het counterparts maintain MH susceptibility throughout a normal life span. Both mouse models have contributed valuable information about how N-terminal mutations affect basal RYR1 channel dysfunction and alter pharmacological responses of intact muscle cells (19, 20). Y522S-RYR1 mice show temporal development of skeletal muscle lesions resembling central core disease in humans (21), whereas Het R163C-RYR1 mice appear to have minimal muscle pathology. These observations in mice are not consistent with clinical evidence indicating that both analogous mutations cause MH susceptibility in humans and that both are associated with central core disease, although the onset and patterns of muscle damage can differ (7, 22). Importantly muscle cells expressing either RYR1 mutation led to three fundamentally important findings about MH susceptibility: 1) both show evidence of altered patterns of bidirectional signaling between Ca\textsubscript{v.1.1} and RYR1, with activation of L-type Ca\textsuperscript{2+} current shifted to more negative potentials (23–25); 2) both have chronically elevated cytoplasmic resting Ca\textsuperscript{2+} measured both \textit{in vitro} (26) and \textit{in vivo} (19, 27); and 3) both have basal alterations in mitochondrial functions that increase production of reactive oxygen species (20, 28). Why Y522S-RYR1 and R163C-RYR1 promote different patterns of skeletal muscle damage is not understood.

Recently, we completed phenotyping a new MHS mouse expressing a mutation within the C-terminal region of RYR1, T4826I-RYR1 (1), a mutation first described in a New Zealand Maori pedigree with MH susceptibility but no clinical evidence of central core disease (29). T4826I-RYR1 mice have several notable phenotypic differences compared with mice expressing N-terminal mutations. Both Het and Hom T4826I-RYR1 mice survive to maturity and show marked genotype and gender differences in susceptibility to triggered MH with halothane and/or heat stress throughout their life span. Electron microscopic assessment of soleus indicates late onset alterations, including abnormally distributed and enlarged mitochondria, deeply infolded sarcolemma, and frequent z-line streaming regions, which are more severe in males (1). Collectively, these data indicate that the location of a mutation within the RYR1 sequence influences not only the penetrance and gender dependence of MH susceptibility but also the patterns of basal changes in muscle bioenergetics and the progression and extent of muscle damage.

Here we report a detailed analysis of basal functional differences in EC coupling and resting cytoplasmic Ca\textsuperscript{2+} that confer heightened sensitivity to halothane in single FDB fibers prepared from Het and Hom T4826I-RYR1 knock-in mice and how these differences relate to inherent biochemical and biophysical alterations in RYR1 Ca\textsuperscript{2+} channels. We also find that intact myotubes derived from either Het or Hom T4826I-RYR1 have altered oxygen consumption rates when compared with WT.

Experimental Procedures

**Animals**—WT, Het, and Hom skeletal muscle tissue, FDB fibers, and myotubes were obtained from Het × Het crosses of the T4826I-RYR1 knock-in mouse line (back-bred at least 10 generations to the C57BL/6J line) as described previously (1). All cellular and biochemical experiments were performed from tissues collected from mice whose genotype was determined by PCR screening. The PCR primer sequences were as follows: T4826I-RYR1 (forward), TTT GGA GAC ACG GAA ACA GAA; T4826I-RYR1 (reverse), AGG GAG GTA CCT GGC ACT CA; WT-RYR1 (forward), TCT CAC TGT CCA TAG CTG; WT-RYR1 (reverse), ATC CAG CTT CTC CTA CAG. All experiments on animals and collections of animal tissues for the studies were conducted using protocols approved by the institutional animal care and use committees at the University of California at Davis.

**Preparation of Primary Myotubes and Adult FDB Fibers**—Primary skeletal myoblast lines were isolated from newborn WT mice or mice verified Het or Hom for T4826I-RYR1 as described previously (30–32). The myoblasts were expanded in 10-cm cell culture-treated Corning dishes coated with collagen (Calbiochem) and were plated onto 96-well \(\mu\)-clear plates (Greiner) coated with Matrigel (BD Biosciences) for detection of calpain or mitochondrial content or were alternatively seeded onto proprietary 12-well plates for measuring oxygen consumption (Seahorse Biosciences). Upon reaching ~80% confluence, growth factors were withdrawn, and the cells were allowed to differentiate into myotubes over a period of 3 days for these analyses (see below).

Flexor digitorum brevis (FDB) muscles were dissected from male 3–6-month-old mice verified by PCR as WT, Het or Hom T4826I-RYR1 genotype. Single intact myofibers were enzymatically isolated from newborn WT mice or mice verified Het or Hom for T4826I-RYR1 as described previously (19, 33). The myoblasts were expanded in 10-cm cell culture-treated Corning dishes coated with collagen (Calbiochem) and were plated onto 96-well \(\mu\)-clear plates (Greiner) coated with Matrigel (BD Biosciences) for detection of calpain or mitochondrial content or were alternatively seeded onto proprietary 12-well plates for measuring oxygen consumption (Seahorse Biosciences). Upon reaching ~80% confluence, growth factors were withdrawn, and the cells were allowed to differentiate into myotubes over a period of 3 days for these analyses (see below).

**Ca\textsuperscript{2+} Imaging**—FDB fibers were loaded with Fluo-4/AM (10 \(\mu\)M; 40 min at room temperature) in normal Ringer solution
containing 146 mM NaCl, 4.7 mM KCl, 0.6 mM MgSO₄, 6 mM glucose, 25 mM HEPES, 2 mM CaCl₂, and 0.02% Pluronic® F-127 (Invitrogen). The cells were then washed three times with Ring-
er’s solution and transferred to the stage of an IX71 inverted microscope equipped with a 40 × 0.9 numerical aperture objec-
tive (Olympus, Center Valley, PA) and illuminated at 494 nm to excite Fluo-4 with a DeltaRam wavelength-selectable light source. Fluorescence emission at 510 nm was captured from individual fibers. Electrical field stimuli were applied using two platinum electrodes fixed to opposite sides of the well and con-
ected to an A.M.P.I. Master 8 stimulator set at 4 V, 0.5-ms bipolar pulse duration over a range of frequencies (1–20 Hz; 10-s pulse train duration). Fluo-4 fluorescence emission was measured at 30 frames/s using a Cascade Evolve 512 camera (Photometrics, Tucson, AZ). The images were acquired using the Easy Ratio Pro software (PTI). The data were analyzed using Origin 7 software (OriginLab Corp.). Transients were normal-
ized to the florescence base line (Fo) of each individual fiber, and the integrated area within the evoked responses was calculated from the number of fibers indicated in the figure legend. Statis-
tical comparisons were performed with an unpaired t test.

**In Vivo Recording of Vₘ and [Ca²⁺]ᵣest—**Measurements were performed on mice sedated with non-triggering ketamine/xyl-
lazine (100/5 mg/kg), and core temperature was maintained euthermic with an automated heating system (ATC1000 WPI). Once anesthesia was confirmed by a loss of tail pinch response, mice were intubated with a tracheal cannula and connected to a ventilator (Harvard Minivent, M-845, Holliston, MA) set at a stroke volume of 200 μl, 180 stokes/min, and ventilated with medical air. Small incisions were made to expose the vastus lateralis muscle of the left leg, and muscle fibers were impaled with the double-barreled microelectrode as described previ-
ously (19). Potentials were recorded via a high impedance amplifier (WPI FD-223, Sarasota, FL). The potential from the 3 M KCl barrel (Vₘ) was subtracted electronically from V_Ca₄₋ to produce a differential Ca²⁺-specific potential (V_Ca) that repre-
sents the [Ca²⁺]ᵣest. Vₘ and V_Ca were filtered (30–50 kHz) to improve the signal/noise ratio and stored in a computer for further analysis.

**Halothane Exposure—**Dissociated fibers were perfused with 0.1% (v/v) halothane freshly prepared in the imaging solution. The halothane concentration was confirmed by mass spec-
trometry (23).

**Seahorse XF-24 Metabolic Flux Analysis—**Myoblasts were cultured on Matrigel-coated Seahorse XF-24 plates (Seahorse Biosciences) at a density of 30,000 cells/well using the same procedure described above for Ca²⁺ imaging experiments. The oxygen consumption rate (OCR) and extracellular acidification rate were evaluated after 3 days of differentiation. Before the experiment, the myotubes were equilibrated in DMEM sup-
plemented with 1 mM pyruvate and 1 mM GlutaMAX (running medium) during 1 h at 37 °C. The oligomycin (10 μg/ml final concentration), FCCP (1 μM final concentration), and rotenone (0.1 μM final concentration) were dissolved in the running medium. The respiratory capacity is defined like the OCR after the FCCP addition. To compare the respiratory capacity among the three genotypes, we calculated the area under the curve. Values were normalized using the protein content of each well.

**Mitochondria Content—**Mitochondria content was quantified by staining myotubes with MitoTracker Green (Invitrogen), which preferentially accumulates in mitochondria regardless of the of the mitochondrial membrane potential and provides an accurate assessment of total mitochondrial mass (34, 35). Briefly, myotubes were loaded with 100 nM Mito-
Tracker Green for 30 min at 37 °C. The myotubes were trypsinized and washed by centrifugation, and the associated fluorescence was measured (excitation/emission 516/490 nm). The fluorescence values were normalized using the protein concentration.

**Calpain Activity—**The peptidase activity was evaluated using the synthetic substrate **tert-Boc-L-leucyl-L-methionine amide** (t-Boc) (Invitrogen) (36). Skeletal myotubes were loaded with 10 μM t-Boc for 30 min at 37 °C. After cleavage by peptidases, the product produces blue fluorescence with excitation and emission maxima of 351 and 430 nm, respectively. In order to compare the fluorescence among the different genotypes, we performed the assay using the Quantum View feature of an Evolve 512 digital camera, permitting electron counting of each pixel in the image. Measurements were captured from regions of interest of identical size for each myotube, and the intensity values were averaged for each genotype under identical illumina-
tion conditions on the same day.

**Preparations of Membrane Fractions from Mouse Skeletal Muscle—**Skeletal muscles collected from male WT-RYR1, Het, or Hom T4826I-RYR1 mice (2–3 animals/preparation, 3–6 months of age) were either prepared from freshly isolated tissue or tissue flash-frozen and stored in liquid nitrogen and stored at −80 °C. Fresh tissue was minced on ice (frozen tissue was pul-
verized) and placed in ice-cold buffer containing 300 mM sucrose, 5 mM imidazole, 0.1 mM PMSF, and 10 μg/ml leupep-
tin, pH 7.4, and homogenized with three sequential bursts (30 s each) of a PowerGen 700D (Fisher), at 9,000, 18,000, and 18,000 rpm. Homogenates were centrifuged at 10,000 × g for 20 min. Supernatants were saved, whereas the pellets were subjected to a second round of homogenization and centrifugation at the same settings described above. The remaining pellets were discarded, and the supernatants were combined and poured through four layers of cheesecloth. The filtrate was centri-
fuged at 110,000 × g for 60 min at 4 °C. Pellets were resus-
pended in 300 mM sucrose, 10 mM Hepes, pH 7.4, aliquoted into microcentrifuge tubes (100 μl/sample), and either stored at −80 °C for biochemical analyses or subjected to further purification to obtain membranes enriched in junctional SR as described previously by Saito et al. (37). Protein concentration for each preparation was determined using the DC protein assay kit (Bio-Rad).

**Measurements of [³H]Ryanodine Binding—**The apparent association or equilibrium binding of [³H]ryanodine ([³H]Ry) to RYR1-enriched membrane preparation (0.1–0.15 mg/ml) was measured at 25 or 37 °C for 0–3 h with constant shaking in buffer consisting of 2–5 mM [³H]Ry (PerkinElmer Life Sciences), 250 mM KCl, 20 mM HEPES, pH 7.4 (38), and defined free [Ca²⁺] as indicated in each specific experiment. Free Ca²⁺ was obtained by the addition of EGTA calculated according to the software Bound-and-Determined (39). RYR1 channel modula-
tors Ca²⁺ and/or Mg²⁺ were titrated in specific experiments as
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described in the figure legends. Nonspecific [3H]Ry binding was determined in the presence of a 1,000-fold excess of unlabeled ryandine. Bound and free ligand were separated by rapid filtration through Whatman GF/B glass fiber filters using a Brandel cell harvester (Whatman, Gaithersburg, MD) with three washes with 5 ml of ice-cold buffer (250 mM KCl, 20 mM HEPES, 15 mM NaCl, and 50 μM Ca2+, pH 7.4). [3H]Ry retained in filters was quantified by liquid scintillation spectrometry using a scintillation counter (Beckman model 6500). Each experiment was performed on at least two independent skeletal muscle preparations, each in triplicate. Linear or non-linear curve fitting was performed using Origin® software (Northampton, MA).

Measurements and Analyses of Single RYR1 Channels in Bilayer Lipid Membrane (BLM)—Skeletal muscle membrane preparation was used in order to induce fusion with the planar BLM. BLM was formed by 30 mg/ml phosphatidylethanol-amine/phosphatidylserine/phosphatidylcholine in decane (5:3:2 (w/w/w); Avanti Polar Lipids, Inc., Alabaster, AL). A 10-fold amine/phosphatidylserine/phosphatidylcholine mixture was built from cis to trans (500 to 50 μM). The recording baths were buffered to pH 7.4 by 20 mM Hepes. The holding potential was held at −40 mV by bilayer clamp BC 525C (Warner Instruments, Hampden, CT) on the trans side, where cis was virtually grounded. The cis chamber was where membrane protein was added and thus was actually the cytosolic face of the incorporated channel. The acquired current signals, filtered at 1 kHz (low pass Bessel Filter 8 Pole; Warner Instruments) were digitized and acquired at a sampling rate of 10 kHz (Digidata 1320A, Molecular Devices (Sunnyvale, CA)). All current recordings were made with Axoscope 10 software (Northampton, MA). Representative responses of FDB to electrical pulse trains applied at 1–20 Hz for a 10-s duration isolated from WT (black trace) and Hom T4826I-RYR1 (gray trace) mice. For clarity, the Het T4826I-RYR1 trace is not superimposed on the traces. B, summary data for all three genotypes relating integrated areas of the Ca2+ transient at each stimulus frequency. WT-RYR1 results are mean data ± S.E. from n = 162 fibers from five animals; Het results are from n = 180 fibers from three animals, and Hom results are from n = 203 fibers from four animals. **, p < 0.001 compared with WT; ***, p < 0.001 compared with WT. * *, p < 0.05 compared with WT.

RESULTS

T4826I-RYR1 FDB Fibers Display Enhanced Ca2+ Transient Properties and Heightened Sensitivity to Halothane—Differences in EC coupling in FDB fibers isolated from male WT mice and knock-in Het or Hom T4826I-RYR1 mice were loaded with Fluo-4 and tested for electrically evoked EC coupling in the Ca2+ -replete external buffer ([Ca2+]O = 2 mM). A, representative responses of FDB to electrical pulse trains applied at 1–20 Hz for a 10-s duration isolated from WT (black trace) and Hom T4826I-RYR1 (gray trace). For clarity, the Het T4826I-RYR1 trace is not superimposed on the traces. B, summary data for all three genotypes relating integrated areas of the Ca2+ transient at each stimulus frequency. WT-RYR1 results are mean data ± S.E. from n = 162 fibers from five animals; Het results are from n = 180 fibers from three animals, and Hom results are from n = 203 fibers from four animals. **, p < 0.001 compared with WT; ***, p < 0.001 compared with WT. * *, p < 0.05 compared with WT.

Properties and Heightened Sensitivity to Halothane—Differences in EC coupling in FDB fibers isolated from male WT mice and knock-in Het or Hom T4826I-RYR1 mice were measured by imaging electrically evoked Ca2+ transients with Fluo-4. FDB fibers were sequentially stimulated at frequencies ranging from 1 to 20 Hz, and the magnitude of the Ca2+ transients was quantified as the integrated area of the response. Fig. 4A shows representative traces of electrically evoked Ca2+ transients measured in WT and Hom T4826I-RYR1 myotubes superimposed.
Compared with WT-RYR1, both Het and Hom T4826I-RYR1 FDB fibers consistently displayed accentuated responses to a 10-s pulse train of electrical stimuli at 10 Hz. Hom, but not Het, exhibited significantly larger Ca$^{2+}$ transients with pulses delivered at 20 Hz (Fig. 1B).

Fig. 2 shows three representative traces from each WT (A), Het (B), and Hom (C) FDB fiber responding to electrical pulse trains and subsequent responses to perfusion of 0.1% halothane. Although perfusion of halothane had negligible influence on [Ca$^{2+}$]$_{rest}$ in WT, the anesthetic induced a pronounced rise in [Ca$^{2+}$]$_{rest}$ in Het (92% responded, $n = 39$ fibers tested) and a much larger rise in Hom (100% response, $n = 31$ fibers tested) T4826I-RYR1 FDBs. The massive rise in [Ca$^{2+}$]$_{rest}$ observed with Hom T4826I-RYR1 fibers exposed to continuous halothane perfusion was invariably transitory and returned toward the base line, whereas the rise in resting Ca$^{2+}$ was...
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FIGURE 3. \([\text{Ca}^{2+}]_e\) contributes to halothane-triggered peak \([\text{Ca}^{2+}]_e\) rise in Hom T4826I-RYR1 FDB. Fibers dissociated from WT-RYR1 and Hom T4826I-RYR1 mice were loaded with Fluo-4 and tested for electrically evoked EC coupling in \([\text{Ca}^{2+}]_e\)-replete external buffer (\([\text{Ca}^{2+}]_e = 2 \text{ mM}\)). Fibers were then challenged with halothane in the same external buffer whose \([\text{Ca}^{2+}]_e\) was reduced with EGTA (\([\text{Ca}^{2+}]_e = 0.1 \text{ mM}\)). A, representative traces from two individual Hom T4826I-RYR1 fibers. B, comparison of the halothane-triggered peak \([\text{Ca}^{2+}]_e\) amplitude (mean ± S.E. (error bars)) for Hom T4826I-RYR1 fibers with \([\text{Ca}^{2+}]_e = 2 \text{ mM}\) (0.9 ± 0.2, n = 8) and \([\text{Ca}^{2+}]_e = 0.1 \text{ mM}\) (3.7 ± 0.7; n = 4) (p < 0.001).

slower and sustained with Het T4826I-RYR1 fibers. In 29% of the Hom T4826I-RYR1 fibers (eight fibers), halothane perfusion initially induced a massive \([\text{Ca}^{2+}]_e\) rise that was followed by regenerative \([\text{Ca}^{2+}]_e\) waves having a mean ± S.D. frequency of 3.7 ± 2.1 min\(^{-1}\) (Fig. 2D and supplemental Movie 1). Neither WT nor Het T4826I-RYR1 FDBs showed oscillatory behavior in response to halothane (not shown). In the absence of halothane, spontaneous \([\text{Ca}^{2+}]_e\) waves were not observed in 180 Het and 203 Hom T4826I fibers analyzed.

To further evaluate the contribution of \([\text{Ca}^{2+}]_e\) entry to halothane-triggered \([\text{Ca}^{2+}]_e\) dysregulation, we first verified the integrity of electrically evoked EC coupling of Hom T4826I-RYR1 FDB fibers in the external solution replete with \([\text{Ca}^{2+}]_e\) (\([\text{Ca}^{2+}]_e = 2 \text{ mM}\)) and then exchanged the external solution with \([\text{Ca}^{2+}]_e = 0.1 \text{ mM}\) in the presence of 0.1% halothane (Fig. 3A). Fig. 3B shows that halothane-triggered peak elevation of myoplasmic resting \([\text{Ca}^{2+}]_e\) is attenuated ~3.5-fold in \([\text{Ca}^{2+}]_e = 0.1 \text{ mM}\), indicating that significant \([\text{Ca}^{2+}]_e\) entry contributes to the halothane response in resting Hom T4826I-RYR1 FDB fibers.

**Chronically Elevated Cytoplasmic \([\text{Ca}^{2+}]_e\)\(^{\text{rest}}\) in Adult T4826I-RYR1 Fibers**—We previously reported that RYR null IB5 myotubes transduced with T4826I-RYR1 cDNA results in significantly elevated \([\text{Ca}^{2+}]_e\)\(^{\text{rest}}\) compared with WT (42). Here we measured \([\text{Ca}^{2+}]_e\)\(^{\text{rest}}\) in the vastus lateralis of ketamine/xylazine-anesthetized WT, Het, and Hom T4826I-RYR1 mice (results from both sexes combined). Compared with WT muscles, in vivo microelectrode measurements showed that myoplasmic \([\text{Ca}^{2+}]_e\)\(^{\text{rest}}\) was 2.4- and 3.0-fold higher than WT in Het and Hom T4826I-RYR1 muscle fibers (mean ± S.D. \([\text{Ca}^{2+}]_e\)\(^{\text{rest}}\): 114 ± 3.8, 278 ± 21, and 337 ± 20 nM, respectively, from \(n = 20−34\) fibers/genotype; \(p < 0.001\) among the three genotypes). In contrast to \([\text{Ca}^{2+}]_e\)\(^{\text{rest}}\), the corresponding \(V_m\) of these \(vastus\) lateralis fibers did not differ among the three genotypes (WT = −82 ± 1.3; \(n = 20\) from two mice; Het = −82 ± 1.4; \(n = 34\) from five mice; Hom = −82 ± 1.5; \(n = 25\) from three mice).

Collectively, these data indicate that adult FDB fibers isolated from Het and Hom T4826I-RYR1 mice exhibit heightened sensitivity to halothane, whose magnitude is a function of gene dose, consistent with their relative sensitivities to triggering fulminant MH \textit{in vivo} (1). Moreover, both MH-susceptible genotypes have two basal abnormalities: 1) enhanced gain of EC coupling and 2) chronically elevated \([\text{Ca}^{2+}]_e\)\(^{\text{rest}}\). We therefore investigated if chronic dysregulation of cellular \([\text{Ca}^{2+}]_e\) dynamics due to the T4826I-RYR1 mutation in the absence of triggering agents affected mitochondrial bioenergetics and calpain activity, biomarkers known to mediate muscle damage.

**Reduced Mitochondrial Respiration and Higher Calpain Activities in Resting T4826I-RYR1 Myotubes**—We tested if T4826I-RYR1 mutations influenced OCR and respiratory capacity (RC) in intact muscle cells using a Seahorse XF-24 metabolic flux analyzer. Technical challenges, including inconsistent plating density, precluded making these measurements on FDB fibers; therefore, primary myotubes were used as an alternative. Myotubes from all three genotypes were plated on separate wells of a Seahorse plate at a density of 30,000 cells/well and differentiated into myotubes for 3 days, at which time OCR and RC were measured. In parallel cultures, MitoTracker was used to measure mitochondrial mass. Fig. 4A shows a representative OCR experiment performed on the three genotypes. The OCR values are expressed as a percentage of the respective base lines (before the addition of oligomycin). Both Het and Hom T4826I-RYR1 myotubes displayed a significantly lower basal OCR (\(p < 0.001\)) compared with WT (Fig. 4B), and basal OCR was significantly lower with Hom compared with Het T4826I-RYR1 myotubes (\(p = 0.0167\)). In order to compare RC across the three genotypes (OCR values after FCCP injection), the results were expressed as area under the curve (corrected for non-mitochondrial rotenone-insensitive OCR). The mean ± S.E. respiratory capacities of Het and Hom T4826I-RYR1 myotubes were significantly lower than that of WT (Fig. 4C). Interestingly, the Hom T4826I-RYR1 RC was higher than that of Het (\(p < 0.01\)).

MitoTracker green, a dye shown to bind mitochondria regardless of membrane potential and a quantitative method for measuring total mitochondrial mass (34, 35), was used to test if the lower RC of the myotubes expressing T4826I-RyR1 was associated with altered mitochondrial content. Fig. 4D shows that Het and Hom T4826I-RYR1 myotubes have significantly lower mitochondrial mass than WT myotubes (83.0 ± 1.9 and 67.5 ± 6.6%, respectively).

\(t\)-BOC fluorescence was used to determine if chronically elevated myoplasmic \([\text{Ca}^{2+}]_e\)\(^{\text{rest}}\) levels could promote calpain...
activities associated with muscle damage and degeneration (43). Fig. 5 (left) shows representative micrographs, and bar graphs (Fig. 5, right) show that both Het and Hom T4826I-RYR1 myotubes have elevated calpain activities that are 170 and 145% of WT (mean \( t \)-BOC fluorescence of 100 myotubes/genotype normalized to WT).

Expression and Phosphorylation of T4826I-RYR1 Channels Do Not Account for Increased \(^{3}H\)Ry Binding Capacity—The influence of PKA phosphorylation of RyR1 on channel activity has been debated in the literature (19, 41, 44, 45). Our recent study of R163C-RYR1 isolated from Het mouse muscle indicated higher Ser\(^{2844}\) phosphorylation (~31%) compared with
WT–RYR1, although dephosphorylation with protein phosphatase did not restore R163C–RYR1 channel behavior to that observed with WT–RYR1 channels (19). We therefore measured the level of total RYR1 expression and the degree of phosphorylation of Ser\(^{2844}\) (Ser(P)\(^{2844}\)) in whole membrane fractions from skeletal muscle prepared from WT, Het, and Hom T4826I–RYR1. Fig. 6A shows representative Western blots of skeletal muscle membranes probed with monoclonal antibody 34C that recognizes RYR1, indicating that no significant differences were detected in the level of RYR1 protein expression (normalized to GAPDH) among the three genotypes, regardless of whether male and female muscle samples were pooled or separated prior to preparing membranes for Western blotting (Fig. 6A, left). Blots were probed with both 34C and an antibody that recognizes Ser(P)\(^{2844}\), which showed that unlike in muscles expressing R163C–RYR1, there were no significant differences among the three genotypes in the degree to which RYR1 was phosphorylated (Fig. 6B, top) and C (bar plot of densitometry); \(n = 16\) blots from five different preparations.

Het and Hom T4826I–RYR1 preparations display a significantly higher capacity to specifically bind \[^{3}H\]Ry than WT–RYR1 preparations under defined assay conditions (Fig. 6B, bottom), which is likely to reflect their inherently higher open probabilities (see below). Although muscle preparations from T4826I–RYR1 mice show similar levels of total RYR1 protein and Ser(P)\(^{2844}\) as those from WT, we determined if the level of phosphorylation influences the capacity to bind \[^{3}H\]Ry to a greater extent in T4826I–RYR1 than WT by exposing membrane preparations to PP1 (see “Experimental Procedures”). Western blotting with and without PP1-treated membranes showed nearly complete dephosphorylation of Ser(P)\(^{2844}\) in all three genotypes (Fig. 6B, top), although the level of \[^{3}H\]Ry binding remained unchanged regardless of the degree of phosphorylation (Fig. 6B, bottom).

Het and Hom T4826I–RYR1 Channels Have Inherently Higher Open Probability than WT—WT–RYR1, Het, or Hom T4826–RYR1 channels were incorporated into BLM by induced fusion of SR vesicles. In the presence of 1 \(\mu\)M free cytosolic (cis) Ca\(^{2+}\), 2 \(\mu\)M Na\(_{2}\)ATP, and 100 \(\mu\)M free luminal (trans) Ca\(^{2+}\), the single channel activity was recorded at a holding potential of \(-40\) mV (applied to trans). Fig. 7A shows representative current traces from a WT–RYR1 (top trace), a Het (middle trace), and a Hom (bottom trace) channel. Compared with WT–RYR1, the Het T4826I–RYR1 channel exhibited 9-fold higher \(P_o\) with 2.5-fold greater \(\tau_o\) and \(-6\)-fold shortened \(\tau_c\). Hom T4826–RYR1 channels demonstrated even greater deviations compared with WT–RYR1. For example, the Hom T4826–RYR1 shown in Fig. 7A displayed \(-15\)-fold higher \(P_o\), \(-5\)-fold greater \(\tau_o\), and \(-10.5\)-fold shortened \(\tau_c\), compared with WT–RYR1.

As shown in Fig. 7, B and C, channels reconstituted from Het T4826I–RYR1 mice produced more heterogeneous gating behavior with a broadly scattered \(P_o\), ranging from 0.19 to 0.71 (mean \(P_o = 0.41\); \(n = 5\); statistical analysis was not done due to limited data points). This behavior would be expected from random association of WT– and T4826I–RYR1 monomers into functional tetramers as recently described for channels isolated from Het R163C mice (19). Hom T4826–RYR1 channels showed invariantly high \(P_o\) behavior (mean \(P_o = 0.82\); \(n = 10\)), which was significantly different from WT–RYR1 (mean \(P_o = 0.10\); \(n = 12\); \(p < 0.0001\)).

Analyses of current amplitude distributions from representative channels reconstituted from each genotype are shown in Fig. 8. The most frequent transitions of WT–RYR1 were centered about zero current, the closed channel state (top). In marked contrast, the Hom T4826I–RYR1 channel (bottom) exhibited most transitions centered about the maximal unitary current amplitude, the full open channel state. Het T4826I–RYR1 channels exhibited an intermediate and broader current amplitude distribution compared with WT–RYR1 and Hom T4826I–RYR1 channels (Fig. 8, middle).
T4826I-RYR1 Exhibits Altered Sensitivities to Regulation by Ca\(^{2+}\) and Mg\(^{2+}\)—Ca\(^{2+}\) and Mg\(^{2+}\) are physiological modulators of RYR1 channel activity (46–48). Using \(^{3}\text{H}\)Ry as a probe to RYR1 channel conformational status, we investigated how the T4826I-RYR1 mutation alters Ca\(^{2+}\) regulation of \(^{3}\text{H}\)Ry binding through interactions with high affinity activation sites, and both Ca\(^{2+}\) and Mg\(^{2+}\) interact with allosterically coupled low affinity sites (49, 50). Fig. 9A shows the concentration-effect relationship across a range of Ca\(^{2+}\) concentrations from 10\(^{-8}\) to 10\(^{-2}\) M. The maximum level of \(^{3}\text{H}\)Ry binding at optimal Ca\(^{2+}\) was consistently 4- and 9-fold higher for Het and Hom T4826I-RYR1 compared with WT, respectively (Fig. 9A, left) (p < 0.01). Analysis of the Ca\(^{2+}\) curves normalized to their respective maxima (Fig. 9A, right) revealed that both Het and Hom T4826I-RYR1 exhibit higher sensitivity to Ca\(^{2+}\) activating at high affinity sites, but only Hom has reduced sensitivity to Ca\(^{2+}\) inhibition through low affinity sites (Fig. 9, A and C). Interestingly, Hom and Het T4826I-RYR1 had reduced sensitivity to Mg\(^{2+}\) inhibition through low affinity sites compared with WT (Fig. 9, B and D) (p < 0.05).

Augmented Response of T4826I-RYR1 to Temperature—Fulminant MH can be triggered in Het and Hom T4826I-RYR1 mice in response to halothane and/or heat stress (1). We therefore investigated whether temperature differentially influences the kinetics of \(^{3}\text{H}\)Ry binding to skeletal muscle prepared from the three genotypes. Fig. 10 shows that the observed rate of \(^{3}\text{H}\)Ry binding (k_\text{obs}) depends significantly on genotype, with the rank order Hom > Het > WT, and temperature (25 versus 37 °C) (A and B). Fig. 10C shows that the relative increase in binding rate (normalized to WT) is greater at 37 °C than it is at 25 °C (Fig. 10C) and that Hom is more responsive to temperature than Het.

DISCUSSION

As is the case with most RYR1 MHS mutations, heterozygosity for T4826I-RYR1 in humans is sufficient to confer MHS, and individuals homozygous for T4826I-RYR1 have not been described. Although very rare, individuals homozygous for RYR1 mutation C35R (51) and R614C (52) have been described in families with a history of MHS, and muscle biopsies from Hom individuals have increased sensitivity to halothane and caffeine compared with Het individuals (52). Recently, we described that Hom and Het T4826I-RYR1 mice are viable but display distinctly different phenotypic penetrance for triggering fulminant MH with halothane and heat stress. This difference allowed us to uncover a genotype- and sex-dependent susceptibility to pharmacological and environmental stressors that trigger fulminant MH and promote myopathy (1). In this regard, T4826I-RYR1 mice provide unique insights into understanding the genotype-phenotype relationships of MHS mutations in vivo and the mechanisms influencing muscle dysfunction and fulminant MH in adult skeletal muscle in vivo and in vitro.

One important outcome of channel dysfunction in adult muscle fibers expressing T4826I-RYR1 is a chronically elevated myoplasmic [Ca\(^{2+}\)]\text{rest} under basal (non-triggered) conditions. In this regard, there is a clear gene dose influence on myoplasmic [Ca\(^{2+}\)]\text{rest} with WT < Het < Hom. Recently Murayama et al. (53) did not detect a difference in cytoplasmic [Ca\(^{2+}\)]\text{rest} in HEK 293 cells transiently transfected with T4826I-RYR1 com-
pared with those expressing WT-RYR1. Several significant methodological differences could account for these divergent results. First, in vitro heterologous expression of RyRs in a system where there are no DHPRs to provide negative regulation versus measurements made in vivo in adult muscle fibers will decrease any difference due to fact that increased numbers of WT-RYR1 protomers will be in the leak conformation (16). This is compounded by the use of Fura 2 ratios with the inherent inaccuracy caused by using an EGTA-based buffer as an indicator versus measurements of $[\text{Ca}^{2+}]_{\text{rest}}$ using calibrated double barreled microelectrodes. Nevertheless, the present results are consistent with those previously reported in adult R163C-RYR1 Het muscle fibers (19) and indicate that chronically elevated $[\text{Ca}^{2+}]_{\text{rest}}$ may be a common outcome of MHS mutations that promote RYR1 conformations that are leaky (26).

Arguably, the most significant finding in the present study is the uniformly high single channel $P_o$ and high capacity to bind $[^3 \text{H}]$Ry of Hom T4826I-RYR1 preparations isolated from adult mice when compared with WT. Although the unitary current level and full gating transitions of Hom T4826I-RYR1 channels remain intact, the mutation concomitantly destabilizes the full closed state and stabilizes the full open state of the channel to achieve remarkably high $P_o$ with very tight current distributions, consistent with the formation of a uniform population of T4826I-RYR1 tetramers. Channel gating behavior, rather than differences in RYR1 protein expression or phosphorylation, is responsible for the significantly higher level of $[^3 \text{H}]$Ry binding observed in Hom muscle preparations when assayed in the presence of optimal $\text{Ca}^{2+}$ and is achieved because the inherently stable open channel conformation also stabilizes all $[^3 \text{H}]$Ry binding sites in their high affinity conformation (54).

This interpretation is consistent with the fact that Het T4826I-RYR1 channels display more heterogeneous gating behavior consistent with random assembly of chimeric (WT/T4826I) tetraters and achieve a $[^3 \text{H}]$Ry occupancy level intermediate between those of WT and Hom. This behavior is similar to those recently described for preparations isolated from Het R163C-RYR1 MHS mice (19). Both Het R163C and T4826I mutations enhance sensitivity to activation by $\text{Ca}^{2+}$ (3- and 2-fold compared with WT, respectively), but only the latter mutation confers decreased sensitivity to inhibition by $\text{Mg}^{2+}$ in both Het and Hom preparations (~1.5-fold compared with WT) and a decreased sensitivity to $\text{Ca}^{2+}$ inactivation in the Hom preparation. Cytoplasmic $\text{Mg}^{2+}$ is a physiological negative control of RYR1 channel activity under resting and activating conditions, and impaired regulation by $\text{Mg}^{2+}$ has been implicated as an important contributor to MHS (42, 55–57). However, the current results indicate that subtle shifts in regulation by physiological cations cannot fully explain the profound dysfunction of Het and Hom T4826I-RYR1 channels observed here and may involve altered nitrosylation (20) or other covalent modifications.
A more significant contributor to channel dysfunction is the location of the mutation within the cytoplasmic loop linking transmembrane segments 4 and 5 (S4-S5). Recently, Murayama et al. (53) reported results of substitution scan of the N-terminal half of the putative S4-S5 linker (Thr4825–Ser4829) of RYR1, including T4825I (rabbit sequence). Consistent with our find-
ings with halothane in the current work and our finding of increased sensitivity to caffeine and 4-CmeC previously (42), HEK 293 cells expressing T4825I-RYR1 exhibited higher sensitivity to caffeine and produced higher $P_o$ channels reconstituted in BLM compared with those expressing WT (53). In this regard, our results from Hom T4826I-RYR1 channels prepared from adult skeletal muscle show even more profound stability of the open state conformation ($P_o > 0.8$ in the presence of suboptimal $1 \mu M$ cytoplasmic Ca$^{2+}$) compared with expressed T4825I-RYR1 ($P_o \sim 0.3$ in the presence of optimal $100 \mu M$ cytoplasmic Ca$^{2+}$) (53).

Although qualitatively similar, several experimental differences could contribute to the quantitative divergence of our single channel results from those of Murayama et al. (53), most notable are the BLM solutions having pH of 7.4 versus 6.8, 1 $\mu M$ versus 100 $\mu M$ cis-Ca$^{2+}$, and trans-Ca$^{2+}$ of 100 $\mu M$ versus undefined, respectively. Our experimental conditions resulted in a mean $P_o$ for reconstituted WT channels of 0.12 (Fig. 7C), which is lower than those reported by Murayama et al. (53) for WT channels (mean $P_o \sim 0.19$). Nevertheless, our Hom T4826I RYR1 channels produced a mean $P_o$ of 0.81 (nearly 8-fold higher than WT), whereas the T4825I-RYR1 channels reconstituted from HEK 293 cells by Murayama et al. had a $\sim15$-fold higher mean $P_o$ than WT ($P_o \sim 0.19$ for WT and $P_o \sim 0.29$ T4825I-RyR1; Fig. 4B in Ref. 53). Interestingly, our BLM results are consistent with both the 7–8-fold higher level of $[^3H]$ Ry binding reported in our study (Fig. 9A) and the 7–8-fold increase reported by Murayama et al. at 100 $\mu M$ Ca$^{2+}$ (Fig. 5B in Ref. 53).

This distinction is important because it suggests that in addition to the inherent dysregulation imparted by the T4826I mutation, the presence of the mutation over time in the context of its native muscle environment may lead to stable covalent modifications, other than phosphorylation, that contribute to abnormally active channel behavior, and these differences need to be explored in the future.

As for the mechanism by which the T4826I mutation causes RyR1 dysfunction, two possibilities are apparent: 1) impairment of the strong negative feedback regulation provided by RyR1 dysfunction, two possibilities are apparent: 1) impair-

$Ca^{2+}$ dysregulation triggered by acute halothane exposure of FDB fibers from Het and Hom T4826I-RYR1 male mice is clearly dependent on gene dose. A new finding is that the augmented response to halothane seen in intact Hom FDB fibers involves triggered release from SR Ca$^{2+}$ stores and a significant component of Ca$^{2+}$ entry. Thus, the fulminant MH syndrome is likely to be initiated by disinhibition of RYR1 and exaggerated Ca$^{2+}$ excitation-coupled Ca$^{2+}$ entry and store-operated Ca$^{2+}$ entry as suggested previously (19, 23, 61, 63). A better understanding of how MHS mutations impair coordinated regulation of Ca$^{2+}$ release units may have novel therapeutic implications.

The fact that Hom T4826I-RYR1 mice survive without overt clinical pathology, although consistent with the human condition when homozygosis has been identified (51, 52), raises several fundamentally important issues about the remarkable dys-

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