Identical *de novo* Mutation in the Type 1 Ryanodine Receptor Gene Associated with Fatal, Stress-induced Malignant Hyperthermia in Two Unrelated Families

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ABSTRACT

Background: Mutations in the type 1 ryanodine receptor gene (*RYR1*) result in malignant hyperthermia, a pharmacogenetic disorder typically triggered by administration of anesthetics. However, cases of sudden death during exertion, heat challenge, and febrile illness in the absence of triggering drugs have been reported. The underlying causes of such drug-free fatal "awake" episodes are unknown.

Methods: *De novo* R3983C variant in *RYR1* was identified in two unrelated children who experienced fatal, nonanesthetic awake episodes associated with febrile illness and heat stress. One of the children also had a second novel, maternally inherited D4505H variant located on a separate haplotype. Effects of all possible heterotypic expression conditions on *RYR1* sensitivity to caffeine-induced Ca^{2+} release were determined in expressing *RYR1*-null myotubes.

Results: Compared with wild-type *RYR1* alone (EC_{50} = 2.85 ± 0.49 mM), average (±SEM) caffeine sensitivity of Ca^{2+} release was modestly increased after coexpression with either R3983C (EC_{50} = 2.00 ± 0.39 mM) or D4505H (EC_{50} = 1.64 ± 0.24 mM). Remarkably, coexpression of wild-type *RYR1* with the double mutant in *cis* (R3983C-D4505H) produced a significantly stronger sensitization of caffeine-induced Ca^{2+} release (EC_{50} = 0.64 ± 0.17 mM) compared with that observed after coexpression of the two variants on separate subunits (EC_{50} = 1.53 ± 0.18 mM).

Conclusions: The R3983C mutation potentiates D4505H-mediated sensitization of caffeine-induced *RYR1* Ca^{2+} release when the mutations are in *cis* (on the same subunit) but not when present on separate subunits. Nevertheless, coexpression of the two variants on separate subunits still resulted in a ~2-fold increase in caffeine sensitivity, consistent with the observed awake episodes and heat sensitivity.

What We Already Know about This Topic

- A subset of patients with malignant hyperthermia susceptibility can develop malignant hyperthermia-like symptoms in response to nonanesthetic stimuli
- The interactions between genetic risk factors and environmental triggers for such "awake" episodes are unclear

What This Article Tells Us That Is New

- Examination of the type 1 ryanodine receptor gene in two unrelated children who experienced fatal, nonanesthetic awake episodes revealed the presence of an identical new variant in both and a second unique variant in one
- Functional analyses of the two variants in myotubes demonstrate that allelic segregation and genetic background play a critical role in the expression of symptoms

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MALIGNANT hyperthermia (MH) manifests as a drug-induced severe metabolic reaction in susceptible individuals that occurs with administration of potent inhalation anesthetics and/or depolarizing muscle relaxants.[1] MH susceptibility (MHS) is a genetic predisposition that is usually inherited as an autosomal dominant trait. Approximately 70% of MHS families carry mutations in the gene that encodes the type1 ryanodine receptor (*RYR1*),[2] which functions as the Ca^{2+} release channel in the sarcoplasmic reticulum of skeletal muscle. With the exception of patients with *RYR1* mutations associated with congenital myopathies, it is commonly thought that most individuals with MHS have no symptoms and lack clinical manifestations of muscle disease until challenged by anesthetic drugs.[1]

Increasing evidence indicates that a subset of individuals with MHS develop MH-like symptoms during exercise, emotional stress, exposure to environmental heat stress, or a combination of these triggers. Indeed, several confirmed fulminant nonanesthetic or "awake" episodes that resulted in sudden death have been reported during the past decade.[3–7] Such variability of MH expressivity has been attributed to complex interactions among genetic, environmental, and other modulatory factors.[8] However, the exact nature of the interaction between genetic MH predisposition with environmental triggers and modulators remains unknown. We report the clinical history and genetic analysis of two unrelated children who experienced fatal nonanesthetic awake episodes triggered by a viral prodrome or exposure to environmental heat stress. Functional studies of the identified *RYR1* variants homologously expressed in myotubes derived from *RYR1*-null mice demonstrate that allelic segregation and genetic background can be a critical, and heretofore unappreciated, modifying factor in the variable expressivity of MH.

Materials and Methods

Patients and Samples

Clinical histories and specimens from two children who died of nonanesthetic awake events were studied. The first patient (case 1) experienced an anesthetic event suspicious for MH, followed by numerous nonanesthetic awake episodes. He had a previous diagnosis of MHS by the caffeine-halothane contracture test.[9] The second patient (case 2) had not had anesthesia but experienced a previous awake episode in the absence of anesthesia. Molecular genetic studies were approved for both cases by the Institutional Review Board of the Uniformed Services University of the Health Sciences, Bethesda, Maryland. After obtaining consents, family members of the two probands were also enrolled in genetic studies.

Case 1

A 7-month-old male infant underwent bilateral ptosis repair with general anesthesia. While breathing oxygen-halothane (0.5–1%)–nitrous oxide, he became "dusky and rigid." The halothane was discontinued, and 100% oxygen was given. Surgery was canceled. Muscle rigidity resolved promptly, but tachycardia and tachypnea persisted. The rectal temperature peaked at 100.1°F (38°C). Creatine kinase was 1,883 U/l 8 h later. Neurologic examination revealed mild hypotonia in the upper extremities without weakness. Histopathologic findings were normal except for mild variation in fiber size with several atrophic type 1 and 2 fibers. Electron microscopic study detected no ultrastructural abnormalities.

At 20 months, he experienced his first nonanesthetic awake episode. On a warm day, he awoke hot, flushed, and restless. Gradually his legs became extended and remained in extension for 60–80 min. At the local emergency department, he was alert and oriented but extremely warm and in respiratory distress. Vital signs were: heart rate = 176 beats/min, respiratory rate = 62 breaths/min, temperature = 104°F (40°C). Laboratory results were normal, except serum bicarbonate concentration of 18 mEq/L. He received intravenous fluids, oxygen, external cooling, and antipyretics. Later the same day, he experienced a recurrence with temperature of 105.1°F (40.6°C) and rigidity of his lower limbs. Resolution followed aggressive cooling and administration of antipyretics (paracetamol) over 60–80 min. Electrolytes were normal, and creatine kinase was 7,525 U/l 24 h later. The presence of short stature, congenital ptosis, mild hypotonia of the upper extremities, and MHS suggested central core disease and King-Denborough syndrome be considered in the differential diagnosis. However, the child did not appear dysmorphic, a key feature of King-Denborough syndrome, to an experienced pediatric neurologist.[10]

During the next 3 yr, the child experienced numerous episodes, starting with leg cramping, tachycardia and tachypnea, and increased creatine kinase, that varied in severity. Mild upper respiratory and/or gastrointestinal infections often were present, but some episodes occurred in the absence of infection. Mild episodes usually resolved within 30 min after prompt administration of acetaminophen and cooling. Severe episodes required hospitalization and intravenous dantrolene. One emergency department report noted that he was "profusely diaphoretic, with stiff extended limbs and exaggerated lordosis." Episodes, marked by increased temperature as high as 105°F (40.6°C), serum creatine kinase (100,000 U/l), myoglobinuria, and serum potassium (8.0 mEq/L) required IV dantrolene. On occasion, muscle cramping involved the abdominal, neck, wrist, and hand muscles. Because these episodes responded well to dantrolene, his mother requested that he be placed on a regimen of low-dose oral dantrolene as prophylaxis when he experienced febrile illness. This approach appeared to reduce the frequency of episodes.

At age 5 yr, he exhibited marked muscle hypertrophy. Results of nerve conduction studies of the peroneal and sural nerves were within normal limits. Electromyography examination of the anterior tibialis muscle was consistent with a congenital myopathic pattern of muscle discharges. Lumbar lordosis with truncal weakness was noted.

When he underwent another ptosis surgery, muscle was obtained for caffeine-halothane contracture testing and histopathologic examination of the muscle samples. Results of the caffeine-halothane contracture test were markedly positive, with a mean response of 8.5 g contracture in the presence of halothane (3%) and 2.4 g contracture in the presence of 2 mM caffeine. This second histologic analysis revealed nonspecific changes, such as scattered type 2 atrophic fibers, a few angular fibers, increased internal nuclei, and occasional subsarcolemmal crescents. There was no evidence of cores. Differential diagnoses included atypical carnitine palmitoyltransferase 2 deficiency and an incomplete form of King-Denborough syndrome. Muscle stiffness with high fever in conjunction with mild infections continued throughout his life. At 9 yr, he died on the way to the hospital during another episode. There was no family history of MH or other neuromuscular disorders.

Case 2

A 6-yr-old girl presented to the hospital with fever (102.7°F or 39.3°C), muscular rigidity, masseter spasm, and vomiting. Over a few hours, her symptoms worsened. Seizures appeared. Diazepam and ketorolac were administered, but muscle rigidity persisted. Oxygen saturation decreased as her breathing became labored. The rectal temperature increased to 108°F (42.2°C). Asystole was treated with cardiopulmonary resuscitation. The rigidity was not reversed by the administration of 10 mg/kg IV dantrolene. Acidosis (pH 6.6) and hyperkalemia (more than 10.0 mM) were severe. She died after 2 h of cardiopulmonary resuscitation. Autopsy was unremarkable except for mild chronic upper airway inflammation, indicative of a recent viral illness.

Her medical history was notable for mild facial nerve palsy (Bell's) in infancy. She experienced a spontaneous episode of high fever (more than 105°F or 40.6°C) at 4 yr of age, accompanied by total body and jaw muscle rigidity after a day playing at the beach. Symptoms were reversed promptly by immersion in an ice bath, followed by administration of oral antipyretics and IV fluids. In a local hospital that day, she experienced an explosive bout of diarrhea. Stool testing was positive for rotavirus. She had hypertrophy of the lower extremity muscles. There was no family history of MH or other neuromuscular disorders.

Candidate Gene and Haplotype Analysis

All 106 *RYR1* exons were analyzed in both cases with the use of genomic DNA extracted from blood lymphocytes using standard methods. In addition, exons 2 and 24 of the α1 subunit of voltage gated L-type calcium channel gene (*CACNA1S*) and exons 1, 3, and 4 of the carnitine palmitoyltransferase 2 gene (*CPT2*) were analyzed in case 1. The primers used for amplifying and sequencing of 106 *RYR1* exons (see table, Supplemental Digital Content 1, http://links.lww.com/ALN/A769) were designed using the Primer3 software (Broad Institute, Boston, MA). Amplified products were cleaned and sequenced. Genotyping of family members was conducted with the use of four microsatellite markers from 19q12-q13.2.[11] Haplotypes were determined using marker allele segregation in the pedigrees of both families. In case 2, a 1.8-kb fragment spanning codons encoding the identified mutation sites (corresponding 3983 and 4505) was generated using skeletal muscle message RNA. The amplified sequences were subcloned, and 30 different colonies were selected for sequencing to chromosomal origin of the R3983C and D4505H variants in case 2 (see Supplemental Digital Content 2, http://links.lww.com/ALN/A771).

Identified RYR1 *Variants in Subjects and Population Controls*

A heterozygous nucleotide substitution c.11947C>T (NM_000540.2) resulting in p.Arg3983Cys (R3983C) substitution in exon 87 was identified in both cases. A second heterozygous nucleotide substitution c.13513G>C resulting in p.Asp4505His (D4505H) substitution in exon 92 was also identified in case 2. The frequency of each *RYR1* variant was estimated in 100 subjects with MHS. Healthy, unrelated Caucasian controls (N = 150) were screened for the presence of the identified variants. All samples were collected previously for other studies and were made available for this study without personal information.[12,13]

Preparation and Nuclear cDNA Injections of RYR1*-null Myotubes*

The two identified *RYR1* variants (R3983C and D4505H) and a double variant (R3983C-D4505H) were introduced into a full-length rabbit *RYR1* complementary DNA (cDNA) (accession number, X15750) using standard two-step, site-directed mutagenesis.[14,15] All sequences generated and modified by polymerase chain reaction were checked for integrity by sequence analysis. Myotubes were prepared from primary cultures of myoblasts obtained from skeletal muscle of newborn *RYR1*-null (dyspedic) mice as described previously.[14,15] All animals were housed in a pathogen-free area at the University of Rochester, Rochester, New York, and experiments performed in accordance with procedures reviewed and approved by the local University Committee on Animal Resources. Expression of wild-type (WT) *RYR1* or either variant was achieved by direct microinjection of myotube nuclei with cDNA mixtures including CD8 (0.1 μg/μl) plus the appropriate *RYR1* expression plasmid (0.5 μg/μl).[14,15] In coexpression experiments, nuclei of dyspedic myotubes were microinjected with a 1:1 cDNA mixture (0.25 μg/μl each) of two plasmids of all four possible heterotypic combinations (WT+R3983C, WT+D4505H, R3983C+D4505H, and WT+R3983C-D4505H). Expressing myotubes were identified 2–4 days after nuclear microinjection by incubation with CD8 antibody-coated beads as described previously.[14,15]

Intracellular Ca^{2+} Measurements

Intracellular Ca^{2+} measurements were obtained from Indo-1 AM-loaded myotubes as described previously.[14,15] Briefly, myotubes grown on glass-bottom dishes were loaded with 6 μM Indo-1 AM for 1 h at 37°C in a normal rodent Ringer's solution consisting of (in mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4. Cytosolic dye within a small rectangular region of the expressing myotube was excited at 350 ± 10 nm, and fluorescence emission at 405 ± 30 nm (F_{405}) and 485 ± 25 nm (F_{485}) was recorded using a photomultiplier detection system with results presented as the ratio of F_{405} and F_{485} ($R = F_{405}/F_{485}$).

Caffeine concentration-response curves were obtained by sequential exposure of expressing myotubes to increasing concentrations of caffeine applied through a rapid (less than 5 s response time) local perfusion system. For these experiments, expressing myotubes were exposed for 30 s to different concentrations of caffeine (0.1, 0.3, 0.7, 1.0, 2.0, 3.0, 10, and 30 mM) with each concentration followed by a 30-s wash with control solution. Relative caffeine-induced changes in intracellular Ca^{2+} were expressed as changes in indo-1 ratio ($\Delta Ratio = R_{caffeine} - R_{baseline}$, where $R_{caffeine}$ is the peak indo-1 ratio observed during caffeine application and $R_{baseline}$ is the resting indo-1 ratio observed just before caffeine addition) and plotted against caffeine concentration.

Statistical Analyses

Caffeine concentration-response curves were fitted with the following three-parameter Hill equation:

$$Y = F_{max}/(1 + [EC_{50}/X]^n)$$

where F_{max} is the maximal change in indo-1 ratio, EC_{50} is the concentrations for half-maximal activation, and n is the Hill coefficient. EC_{50} values were obtained from a total of 128 myotube concentration-response experiments. After log transformation of the data, one EC_{50} value was determined to be an outlier, based on the Grubbs' test, and was discarded from the final statistical analyses. All results are given as means ± SEM with statistical significance ($P < 0.05$, two-tailed) determined using either Student *t* test for comparison between two groups or one-way analysis of variance (ANOVA) and *post hoc* Duncan's test for multiple comparisons (identical results were also obtained using Student–Newman–Keuls and Fisher's least significant difference tests). All curve-fitting and statistical analyses were conducted using SigmaPlot10 and SigmaStat software suites (Systat Software Inc., San Jose, CA).

Results

Identification of RYR1 Variants of Highly Conserved Residues in Patients

A heterozygous *RYR1* nucleotide change c.11947C>T (NM_ 000540.2) in exon 87 resulting in a p.Arg3983Cys (R3983C) amino acid substitution was identified in both cases (see figure, Supplemental Digital Content 3, http://links.lww.com/ALN/A772). The R3983C substitution was further screened in 98 previously reported MHS individuals from North America and in 150 controls (the Caucasian population in the United States) with negative results. Identification of two index cases with R3983C mutation in 100 North American individuals with MHS provides an estimated carrier frequency of ~2%. No additional non synonymous *RYR1* variants were identified in case 1 (see table, Supplemental Digital Content 4, http://links.lww.com/ALN/A773). However, a second heterozygous *RYR1* nucleotide variation c.13513G>C resulting in a p.Asp4505His (D4505H) substitution in exon 92 was identified in case 2 (Supplemental Digital Content 2, http://links.lww.com/ALN/A771). The D4505H variant was not found in the 100 previously reported MHS individuals from North America or in the 100 controls representing the Caucasian population in the United States.

The R3983C variant was absent in the parents of both children (fig. 1A). Four microsatellite markers tightly linked to the *RYR1* region confirmed paternity and maternity and the inheritance of these markers in the two families (fig. 1A). In addition, genotyping showed segregation of different haplotypes in each family, indicating that the two families are unrelated. These data demonstrate that the R3983C variant occurred *de novo* in both probands. In the second family, a novel D4505H variant was identified on a separate haplotype from the R3983C variant (Supplemental Digital Content 2, http://links.lww.com/ALN/A771) and was present in both the patient's mother and brother. Segregation analysis of microsatellite markers and six single-nucleotide polymorphisms within the *RYR1* gene (data not shown) in the second family showed association of the 4505H variant with the maternal 10–1–7–1 haplotype (fig. 1A). The two identified *RYR1* residues are highly conserved across a wide range of species and are also conserved in the type 2 and 3 ryanodine receptor isoforms (*RYR2* and *RYR3*, respectively) (fig. 1B).

Effects of R3983C and D4505H Mutations on the Sensitivity of Caffeine-induced Ca^{2+} Release after Expression in RYR1-null Myotubes

Increased sensitivity to activation of the *RYR1* by caffeine is used as a primary diagnostic determinant of MHS. Moreover, an increase in caffeine sensitivity of Ca^{2+} release is recapitulated for MH mutations in *RYR1* after expression in either human embryonic kidney 293 cells[16] or skeletal myotubes derived from *RYR1*-knockout mice.[17] Because the proband in the family of case 1 was heterozygous for the R3983C variant and the mother and sibling of case 2 were heterozygous for the D4505H variant, we determined whether the sensitivity of sarcoplasmic reticulum Ca^{2+} release to activation by caffeine was increased by coexpression of WT *RYR1* with either the R3983C or D4505H variants (fig. 2). Dyspedic myotubes expressing WT *RYR1* (fig. 2A), WT *RYR1* + R3983C (fig. 2B), or WT *RYR1* + D4505H (fig. 2C) were exposed to sequential 30-s applications of

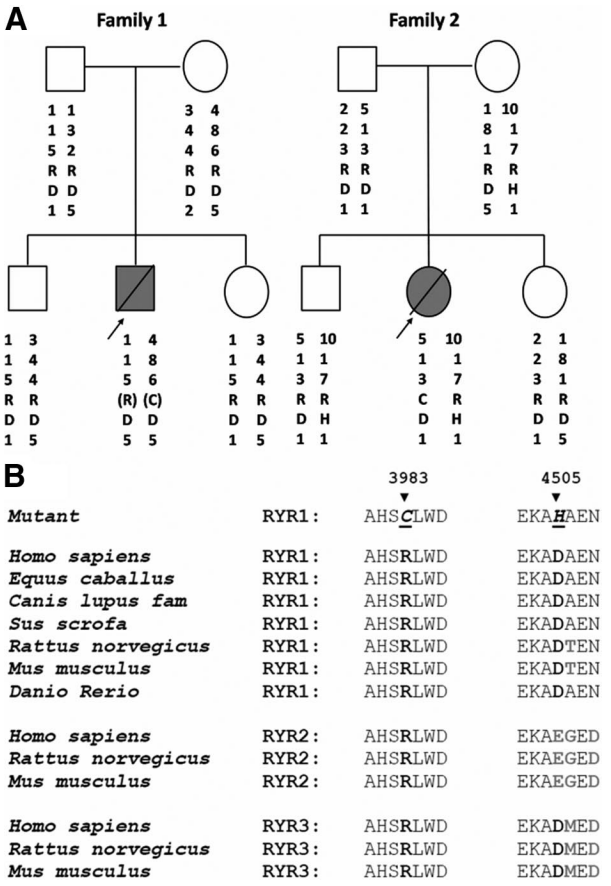

Fig. 1. Pedigrees of two unrelated families with a child who experienced fatal, nonanesthetic episodes. The haplotype was constructed on the basis of genotyping with microsatellite markers D19S191, D19S224, D19S220, and D19S47. The marker order is from centromere to telomere. The parentheses for the proband in family 1 indicate that the relative location of the 3983C haplotype is unknown. In family 2, the 3983C and 4505H variants were located on different alleles, and the R4505H variant was associated with the maternal 10–1–7–1 haplotype. *Filled symbols with a bisecting line* indicate individuals who died of a nonanesthetic episode; *empty symbols* represent clinically healthy family members. R or C = arginine or cysteine residue at position 3983; D or H = an aspartic acid or histidine residue at position 4505 *(A)*. Alignment of the region of ryanodine receptor (RYR) variants and flanking residues across species and RYR isoforms. The mutated residues are shown in bold. Nonconserved residues across RYR isoforms are shaded *(B)*.

increasing concentrations of caffeine (0.1, 0.3, 0.7, 1.0, 2.0, 3.0, 10, and 30 mM), with each application followed by a 30-s wash with control Ringer's solution. Because naïve dyspedic myotubes lack robust caffeine-induced Ca^{2+} release,[14] functional *RYR1* expression was confirmed in each experiment by the presence of robust Ca^{2+} release when challenged with a high concentration of caffeine (30 mM). Average (± SEM) caffeine concentration-response curves are presented in figure 2D. The caffeine sensitivity of *RYR1* Ca^{2+} release in WT *RYR1*-expressing myotubes (EC_{50} = 2.85 ± 0.49 mM) was modestly enhanced after coexpression of

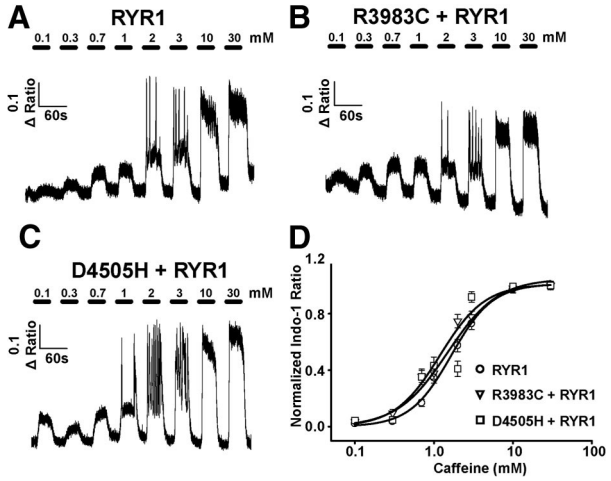

Fig. 2. Effect of coexpressing either the R3893C or D4505H variants with wild-type (WT) type 1 ryanodine receptor (*RYR1*) in dyspedic myotubes on the caffeine sensitivity of sarcoplasmic reticulum Ca^{2+} release. Representative caffeine concentration responses in dyspedic myotubes expressing WT *RYR1* alone (n = 34) *(A)*, WT *RYR1* +R3893C (n = 30) *(B)*, and WT *RYR1* +D4505H (n = 24) *(C)*. Average (±SEM) caffeine concentration-response curves for the conditions shown in A–C *(D)*.

R3983C (EC_{50} = 2.00 ± 0.39 mM) and significantly increased ~2-fold after coexpression of D4505H (EC_{50} = 1.64 ± 0.24 mM).

Genetic analysis revealed that the proband in the family of case 2 was compound heterozygous for both the R3983C and D4505H variants and that the two variants localized to different haplotypes (fig. 1A). We directly compared the effect of the two potential compound heterotypic expression conditions (*i.e.*, R3983C+D4505H and WT *RYR1* +R3983C-D4505H) on the sensitivity of sarcoplasmic reticulum Ca^{2+} release to activation by caffeine (fig. 3). For these experiments, dyspedic myotubes were injected with cDNAs encoding WT *RYR1* alone (fig. 3A) or a 1:1 mixture of either R3983C+D4505H (fig. 3B; the two variants on separate cDNAs) or WT *RYR1* +R3983C-D4505H (fig. 3C; WT *RYR1* plus the two variants engineered on the same cDNA). Average (± SEM) caffeine concentration-response curves are shown in figure 3D. The results indicate that coexpression of WT *RYR1* with the two variants on the same subunit exhibited a significantly ($P < 0.05$) greater sensitivity to caffeine-induced Ca^{2+} release (EC_{50} = 0.64 ± 0.17 mM) compared with that for expression of either WT *RYR1* alone (EC_{50} = 2.85 ± 0.49 mM) or the two variants on separate subunits (EC_{50} = 1.53 ± 0.18 mM). These results indicate a synergistic effect of the two variants on the sensitivity of caffeine-induced *RYR1* Ca^{2+} release when the variants are present on the same subunit but not when they are present on separate subunits.

Discussion

We report two cases in which novel *RYR1* variants are associated with fatal nonanesthetic awake episodes in children.

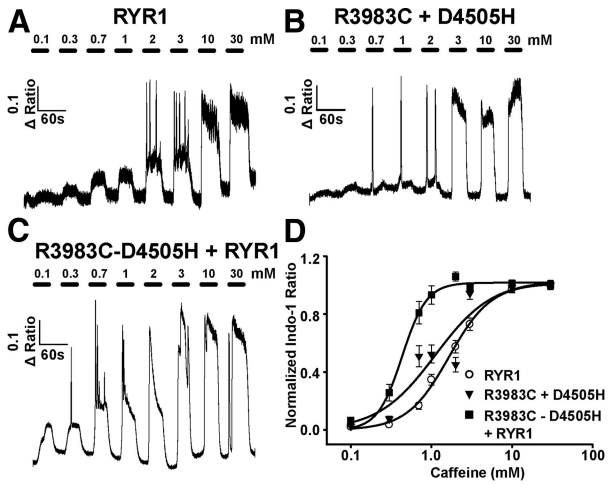

Fig. 3. Effect of coexpressing both the R3893C and D4505H variants together with wild-type (WT) type 1 ryanodine receptor (*RYR1*) in dyspedic myotubes on the caffeine sensitivity of sarcoplasmic reticulum Ca^{2+} release. Representative caffeine concentration responses in dyspedic myotubes expressing wild-type *RYR1* alone (n = 34) *(A)*, R3893C+D4505H (n = 25) *(B)*, and WT *RYR1* +R3893C-D4505H (n = 14) *(C)*. Average (± SEM) caffeine concentration-response curves for the conditions shown in A–C *(D)*.

Like other confirmed MHS mutations in *RYR1*,[16,17] the D4505H variant resulted in a ~2-fold increase in the sensitivity to activation by caffeine. Importantly, this increase in caffeine sensitivity occurred when the D4505H variant was coexpressed with WT *RYR1*, demonstrating a gain-of-function effect of D4505H subunits on *RYR1*-release channel sensitivity, consistent with the known autosomal dominant pattern of inheritance of MH. Coexpression of the R3983C variant with WT *RYR1* produced a more modest enhancement in *RYR1* caffeine sensitivity. Remarkably, caffeine sensitivity was greatly potentiated when the two mutations were incorporated into the same subunit but not on separate subunits. Specifically, the EC_{50} for caffeine activation essentially was the same for the D4505H variant when coexpressed with either WT *RYR1* (EC_{50} = 1.64 ± 0.24 mM) or R3983C (EC_{50} = 1.53 ± 0.18 mM), whereas the R3983C-D4505H double mutant coexpressed with WT *RYR1* (EC_{50} = 0.64 ± 0.17 mM) resulted in a further 2.5-fold increase in caffeine sensitivity and a nearly 5-fold increase in sensitivity compared with WT *RYR1* alone (EC_{50} = 2.85 ± 0.49 mM). Thus, although the R3983C mutation only modestly altered *RYR1* sensitivity by itself, it is a potent enhancer of D4505H-induced sensitization when present on the same subunit. However, peak caffeine-induced responses were not significantly different between WT *RYR1* (ΔRatio = 0.52 ± 0.02) and any of the different coexpression conditions (ΔRatio was 0.54 ± 0.03, 0.55 ± 0.02, 0.52 ± 0.03, and 0.54 ± 0.03 for WT+R39833C, WT+D4505H, R3983C+D4505H, and WT+R3983C-D4505H, respectively). Nevertheless, we cannot exclude the possibility that minor changes in *RYR1* expression in our experiments might alter release channel caffeine sensitivity, but not efficacy.

Although the R3983C variant was the only *RYR1* alteration identified in case 1, susceptibility to nonanesthetic, stress-induced hyperthermic reactions appeared to be more pronounced in this patient than in the child in case 2, as evident from the clinical history of case 1. Discordance between susceptibility of the child in case 1 and the lower caffeine sensitivity of the R3893C variant after coexpression with *RYR1* in *RYR1*-null myotubes could reflect differences between effects of the mutation in human *versus* rabbit *RYR1*, effects in mature muscle fibers *versus* developing myotubes, or the influence of a modifying variable present only in the patient (*e.g.*, a mutation in regulatory or intronic regions of the *RYR1* gene, WT allele silencing, or a second mutation at another MH gene locus). However, extended genetic analyses for additional mutations, including three MH-associated mutations in the *CACNA1S* gene and the most common mutations in the *CPTII* gene, were negative. An *RYR1* mutation previously associated with King-Denborough syndrome also was not found.[18] Nevertheless, it remains possible that the nonanesthetic events observed in this individual could involve the R3983C variant-potentiating effects of a second, yet unidentified, mutation in either a noncoding region of *RYR1* or another MH susceptible loci.[1] Intronic mutations resulting in altered splicing of *RYR1* exons in core myopathies have been reported,[19,20] and epigenetic gene silencing of the normal *RYR1* allele in skeletal muscle has been demonstrated in families presenting with apparent recessively inherited core myopathies.[21,22] However, the only study of epigenetic *RYR1* allelic silencing in MH found no evidence in 14 discordant cases from 11 independent families.[23] Similar potentiating effects could explain why the proband in case 2 possessing both the R3983C and D4505H *RYR1* variants exhibited a more severe awake phenotype than did either her mother or brother, who carried only the D4505H variant.

The increase in internal nuclei observed in case 1 is consistent with recent studies showing an increase in nuclear internalization in *RYR1*-related myopathies.[20,24] Interestingly, Wilmshurst *et al.* identified a nonconservative substitution of a negatively charged *RYR1* residue (E4502G) in an individual with centronuclear myopathy that is only three amino acids upstream of a similar nonconservative substitution of a negatively charged *RYR1* residue (D4505H) identified in the proband in case 2.[24] Together, these findings are consistent with a histopathologic continuum between MH- and myopathy-related *RYR1* phenotypes.

It is important to note that a different *de novo* missense mutation of the same *RYR1* residue R3983 (R3983H) was described recently in a case report of a child with MH history who experienced a fatal nonanesthetic episode after ondansetron administration.[4] This child also presented with clinical and histopathologic signs consistent with multiminicore disease. Although multiminicore disease typically is inherited

as a recessive myopathy, no second *RYR1* variant or monoallelic *RYR1* expression was reported in this child. In addition, the report did not determine whether the identified R3893H variant was causative of increased MHS by assessing its impact on *RYR1* function. Nevertheless, the identification of *de novo* mutations to the identical *RYR1* residue (R3893) in three independent families indicates that sporadic cases of MH due to *de novo* mutations in the *RYR1* gene are likely to be more common than previously appreciated.

The R3983 residue is located within a putative ryanodine receptor and inositol 1,4,5-triphosphate receptor homology-associated domain that spans *RYR1* residues 3870–3992. Although the homology-associated domain is specific to RYR and IP3R, the function of this domain for this superfamily of intracellular Ca^{2+} release channels is unknown. The R3983 residue is conserved across species in all three RYR isoforms, whereas the D4505 residue is conserved only in *RYR1* (fig. 1B). In fact, the D4505 residue is located within *RYR1*-divergent region 1 (D1; residues 4254–4631), one of three evolutionarily divergent regions of RYR isoforms.[25] The D1 region maps to part of the "handle" domain on the RYR cryoelectron microscopy three-dimensional structure.[26] According to current RYR topologic models,[27] the D1 region includes at least one transmembrane domain and adjacent cytoplasmic and intraluminal sequences. Interestingly, deletion of the majority of the *RYR1* D1 region (Δ4274–4535) potentiates voltage-gated Ca^{2+} release and enhances release-channel sensitivity to activation by the dihydropyridine receptor.[28] Based on these results, the D1 region functions as a negative regulatory module that increases the energy barrier for Ca^{2+} release-channel opening. Thus, the D4505H mutation may enhance *RYR1* release-channel sensitivity to activation by disrupting the integrity of the D1-negative regulatory module.

Our results demonstrate that the functional impact of the two variants expressed in *RYR1*-null myotubes depends on whether the two variants are located on common or separate subunits. Genetic analysis of the second family revealed that the two variants are localized to separate subunits in case 2. Although the caffeine sensitivity with the variants on separate subunits is not as high as when they localize to the same subunit, the allelic relationship in case 2 does not necessarily indicate similar expression of the two proteins. In addition, coexpression of the two variants on separate cDNAs resulted in a 2-fold increase in caffeine sensitivity, consistent with the child's awake episodes and heat sensitivity. The unusually high caffeine sensitivity when the two variants localize to the same subunit demonstrates for the first time an allele-dependent synergism between two novel *RYR1* gene variants. Our results are consistent with the two residues contributing to a negative regulatory module within the D1 region of each monomer. As a result, variants of both residues within the same subunit may lead to a synergistic antagonism of D1 function that potentiates *RYR1* release-channel sensitivity to activation. Together, these findings indicate that allelic segregation can be a critical, and heretofore unappreciated, pathogenic factor in individuals with MH.

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