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Novel Excitation-Contraction Uncoupled RYR1 Mutations in Patients With Central Core Disease

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Abstract

Central core disease, one of the most common congenital myopathies in humans, has been linked to mutations in the RYR1 gene encoding the Ca²⁺ release channel of the sarcoplasmic reticulum (RyR1). Functional analyses showed that disease-associated RYR1 mutations led to impairment of skeletal muscle Ca²⁺ homeostasis, however, thorough understanding of the molecular mechanisms underlying central core disease and other RyR1-related conditions is still lacking. We screened by sequencing the complete RYR1 transcripts in ten unrelated patients with central core disease and identified five novel, p.M4640R, p.L4647P, p.F4808L, p.D4918N and p.F4941C, and four recurrent mutations. Four of the novel mutations involved amino acid residues that were positioned within putative transmembrane segments of the RyR1. The pathogenic character of the identified mutations was demonstrated by bioinformatic analyses and by the *in vitro* functional studies in HEK293 cells and RYR1-null (dyspedic) myotubes.

Characterization of Ca²⁺ channel properties of RyR1s carrying one recurrent and two novel mutations upholds the view that diminished intracellular Ca²⁺ release caused by impaired Ca²⁺ channel gating and/or Ca²⁺ permeability is an important component of central core disease etiology. This study expands the list of functionally characterized disease-associated RyR1 mutations, increasing the value of genetic diagnosis for RyR1-related disorders.

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Keywords

central core disease; congenital myopathies; ryanodine receptor type 1; calcium transport; excitation-contraction coupling

Introduction

Central core disease (CCD; MIM# 117,000) is one of the most common congenital myopathies. The disease typically presents with hypotonia and delayed attainment of motor skills, skeletal abnormalities, a life-long difficulty in performing anti-gravity movements such as climbing stairs and running and chronically elevated serum CK levels in some cases. The clinical phenotype of affected individuals varies widely from mild to severe. CCD symptoms show considerable overlap with other congenital myopathies and diagnosis is based on the detection of characteristic histological abnormalities, “cores”, areas devoid of mitochondrial enzyme activity and exhibiting myofibrillar disorganization which are found predominantly in type I skeletal muscle fibers [1, 2]. CCD exhibits an autosomal dominant pattern of inheritance, although cases with autosomal recessive inheritance and sporadic cases due to *de novo* mutations have also been reported [3-5].

CCD is closely associated with malignant hyperthermia susceptibility (MHS; MIM# 145600), a life-threatening pharmacogenetic disorder triggered by exposure of susceptible individuals to inhalational anesthetics and succinylcholine. Genetic research has shown that the major causal gene for both CCD and MH is the skeletal muscle ryanodine receptor gene (RYR1, MIM# 180901) that encodes the Ca^{2+} release channel (RyR1) of the sarcoplasmic reticulum (SR). RyR1 plays an essential role in maintenance of Ca^{2+} homeostasis and in excitation-contraction (EC) coupling in skeletal muscle cells [6]. Beside CCD and MH, several other skeletal muscle conditions and congenital myopathies have been linked to mutations in the RYR1 gene, namely, multiminicore disease (MmD; MIM# 255320) [7], congenital myopathy with cores and rods [8], central nuclear myopathy [9, 10], neuromuscular disease with uniform type 1 fibers (CNMDU1; MIM# 117000) [11], heat/exercise-induced exertional rhabdomyolysis [12] and atypical periodic paralysis [13]. A common RYR1-related etiology suggests that impaired skeletal muscle Ca^{2+} homeostasis and EC coupling caused by specific RYR1 mutations underlie the wide spectrum of clinico-pathologic conditions [14].

On the basis of functional studies four distinct molecular mechanisms were proposed to explain how altered Ca^{2+} release channel function caused by specific RyR1 mutations could result either in congenital myopathy or lead to the MHS [15-19].

However, a practically useful conclusion regarding the prevalent mechanisms of pathogenesis is only tentative, since the number of thoroughly characterized CCD mutations is still rather small. Thus, continuing search for novel CCD mutations and analysis of their structural and functional consequences on RyR1 function is crucial for development of highly sensitive and specific genetic diagnosis and effective therapy. The aim of this study was to screen a cohort of unrelated CCD patients for the presence of RYR1 variants and to validate the pathogenicity of each identified mutation through genetic and functional characterization.

Methods

Patients

Malignant Hyperthermia Investigation Unit in Toronto, Canada is one of the referral centers in the world for central core disease studies. Following Research Ethics Board approval, ten unrelated individuals, referred to our center, were selected and consented for genetic screening of their entire RYR1 transcripts on the basis that they displayed both clinical symptoms of congenital myopathy and the presence of cores in type I fibers in muscle biopsy. Patient C-2 was included because her mother who was diagnosed with CCD was unavailable for the study (Table 1a). To analyze phenotype-genotype correlation, available relatives of the index patients (a total of 23 individuals) were subsequently enrolled and consented for genetic analyses.

Molecular genetic studies

Muscle samples for RNA isolation were available for index cases C-1, C-5, C-9. For the remaining individuals blood samples were used as a source of RNA. RNA isolation from blood leukocytes and cDNA synthesis and PCR amplification of the RYR1 transcript were performed as described previously [20] with minor modifications. Sequence analysis of the exons 9, 31, 66, 70, 83 and 94 skipped in some RNA samples was done using patients' genomic DNA samples as described [20, 21]. The entire coding regions of ACTA (MIM# 102610) and SEPN1 (MIM# 606210) genes were sequenced at the genomic level in a RYR1 mutation-negative patient C-10. Sequencing reactions were run at the DNA Sequencing and Synthesis Facility, The Centre for Applied Genomics (TCAG), Toronto, Canada. Raw sequence data analysis, i.e. contig building and sequence comparison to the reference RYR1 sequences GenBank: NM_000540.2 and GenBank: NC_000019 were done using Sequencher 4.10.1 (Gene Codes, Ann Arbor, MA). Sequence analysis was done using various bioinformatics tools via HTTP interface as described [21]. Each novel missense DNA variant was confirmed by sequencing of the corresponding region from the patient's genomic DNA. A mutation-specific genotyping assay was designed for each novel variant and a panel of 200-300 normal control chromosomes was screened to assess the mutation frequency in the general population. The segregation analysis was done using DNA samples of available relatives. Three software tools were used for prediction of the functional impact of each of the amino acid substitutions identified: PolyPhen-2 [22], SIFT [23] and PMut [24].

RYR1 *in vitro* mutagenesis

For heterologous expression the constructs of rabbit RYR1 cDNAs carrying nucleotide substitutions for mutants p.L4646P, p.F4807P, p.D4917N and p.R4892Q equivalent to p.L4647P, p.F4808L, p.D4918N and p.R4893Q mutations in humans were generated as described previously [15]. Constructs for expression of RyR1 proteins carrying polymorphisms p.P1787L (NCBI dbSNP: rs34934920) and p.S2060C (human p.G2060C, NCBI dbSNP: rs35364374) as well as of mutant RyR1 proteins carrying the p.R4892Q and p.P1787L present in patient C-6, and p.D4917N and p.S2060C present in patient C-7 were generated using the same method [15].

Expression in HEK293 cells

Wild-type (WT) RyR1 and the RyR1 mutants were expressed transiently in HEK293 cells. Culturing of HEK293 cells and cDNA transfection using the calcium phosphate precipitation method were performed as described previously [26].

Immunoblotting—Western blotting was performed to verify the expression of mutant RyR1 protein from cDNA constructs in HEK293 cells to be used for Ca^{2+} imaging experiments. HEK293 cells transfected with pcDNA 3.1 vector without a foreign DNA insert and HEK293 cells transfected with WT RyR1 cDNA were used as negative and positive controls, respectively. For each construct, the HEK293 cells grown on a 60-mm culture dish were harvested 36–48 hours post-transfection into 0.5 ml ice-cold homogenization buffer containing 50 mM Tris-HCl, pH 7.4, 10mM EDTA, 2 mM EGTA, 5 mM DTT, 0.1 mM PMSF and a cocktail of proteinase inhibitors (Roche Diagnostics, Germany). Cells were disrupted by two cycles of freezing and thawing followed by passing through 20 and 23 gauge needles. Protein concentration in homogenates was determined using Bio-Rad Bradford reagent and bovine serum albumin as a standard. Whole cell homogenate proteins (50 μg total protein loaded per lane) were diluted 1:1 in 2 $\times$ sample buffer, containing 0.2M Tris-HCl, pH 6.8, 10% glycerol, 3% (w/v) SDS, 10 mM DTT and 0.04% bromophenol blue, heated for 3 minutes in a boiling water bath and separated on 4–15% gradient polyacrylamide gels (Bio-Rad, USA). Transfer onto polyvinylidene difluoride membrane (Millipore, USA) was performed at room temperature overnight at 25 mA in 1 $\times$ Tris/Glycine transfer buffer (Bio-Rad, USA), containing 0.2% SDS and 20% (w/v) methanol. The blots were probed with anti-ryanodine receptor monoclonal 34C antibody (1:500, Affinity BioReagents, USA) followed by horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1:1000, Sigma, USA) in phosphate-buffered saline, containing 1% milk and 0.1% BSA. The immune complexes were revealed using Luminata™ Forte Western HRP substrate (Millipore, USA) and the images were generated using a FluoS™ Max MultiImager and quantified using Quantity One software (Bio-Rad, USA). For loading control, the blots were probed with anti- α -tubulin mouse monoclonal antibody (1:2000, Sigma, USA).

Fluorescence Measurements—The functional effect of the mutations p.L4646P, p.F4807P, p.D4917N and p.R4892Q as well as the polymorphisms p.P1787L and p.S2060C was studied by comparing the effect of caffeine on calcium release of WT and mutant RyR1 proteins transiently expressed in HEK293 cells. Changes in intracellular Ca^{2+} concentration were measured by calcium fluorescence photometry at 340/380 nm with Fura 2AM indicator using a microfluorimetry system (Photon Technology International, Inc., Birmingham, NJ, USA) as described [27]. Dose response curves were constructed by measuring peak amplitudes of the fluorescence ratio (340/380), which, after background subtraction, were normalized against a maximal response produced by 30 mM caffeine. Data normalization was necessary to compare individual samples to the response to caffeine due to different samples expressing ryanodine receptor proteins at different levels. For each drug concentration, data from six independent experiments were averaged and expressed as mean $\pm$ SEM. The resultant datasets were used to fit sigmoidal dose-response curves for caffeine using functions included in GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). The same software package was used to apply unpaired Student t- test or one-way ANOVA (for multiple comparisons) for data comparison, with statistical significance accepted at $P < 0.05$.

Preparation and nuclear cDNA microinjection of dyspedic myotubes

Primary cultures of dyspedic myotubes were cultured from skeletal myoblasts isolated from newborn dyspedic mice [29, 30]. After allowing myoblasts to differentiate into multinucleated myotubes for 4–7 days, nuclei of individual myotubes were microinjected with cDNAs encoding CD8 (0.1 $\mu\text{g}/\mu\text{l}$) and RYR1 constructs at a final concentration of 0.5 $\mu\text{g}/\mu\text{l}$. In co-expression experiments, nuclei of dyspedic myotubes were microinjected with a 1:1 cDNA mixture (0.25 $\mu\text{g}/\mu\text{l}$ each) of two plasmids (WT+p.P1787L, WT+p.R4892Q, WT+p.P1787L-p.R4892Q). Expressing myotubes were identified 2–4 days post-injection by

incubation with CD8 antibody-coated beads (Dynabeads, Dynal ASA, Oslo, Norway). All animals were anaesthetized and humanely euthanized following procedures that were reviewed and approved by the University Committee on Animals Resources at the University of Rochester School of Medicine and Dentistry.

Measurements of electrically-evoked and 4-chloro-m-cresol-induced Ca^{2+} transients in myotubes

Intracellular Ca^{2+} measurements in intact myotubes were obtained from indo-1 AM (TefLabs Inc., Austin, TX, USA) loaded myotubes. Cytosolic dye within a rectangular region of the cell was excited at 350 nm and fluorescence emission at 405 and 485 nm was measured at 100 Hz sampling frequency using a 40 $\times$ oil objective, a photomultiplier detection system (Photon Technology International, Birmingham, NJ). Results are presented as the ratio of 405 nm and 485 nm (F_{405}/F_{485}). All indo-1 measurements in intact myotubes were conducted 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. Electrically-evoked Ca^{2+} transients were elicited using field stimulation applied every 10s. Peak responses to 500 μM 4-chloro-m-cresol (4-CMC), a RyR1 agonist, were obtained during application of the drug using a rapid local perfusion system (Warner Instruments, Hamden, CT). Peak responses were expressed as ΔRatio ($R_{\text{stimulation}} - R_{\text{baseline}}$). Data were collected and analyzed using pClamp 10 software suite (Molecular Devices, Sunnyvale, CA). Statistical significance ($p < 0.05$) was determined using a Student's two-tailed t-test.

Simultaneous measurements of macroscopic Ca^{2+} currents and transients in myotubes

The whole-cell patch-clamp technique in conjunction with a Ca^{2+} -sensitive dye (fluo-4) was used to measure voltage-gated L-type Ca^{2+} currents (L-currents) and intracellular Ca^{2+} transients simultaneously in expressing myotubes, as described previously [29, 31]. All patch-clamp experiments were carried out after an approximately 5-min period of dialysis after establishment of the whole-cell configuration. The external solution consisted of (in mM): 145 TEA-Cl, 10 CaCl_2 and 10 HEPES (pH 7.4). The internal patch pipette solution consisted of (in mM): 145 Cs-Aspartate, 10 CsCl, 0.1 $\text{Cs}_2\text{-EGTA}$, 1.2 MgCl_2 , 5 Mg-ATP, 0.2 $\text{K}_5\text{-Fluo-4}$ and 10 HEPES (pH 7.4). A 1-s prepulse to -20 mV delivered immediately before each test pulse was used to inactivate sodium and T-type Ca^{2+} channels without producing significant L-channel inactivation. L-currents were subsequently elicited by 200 ms test depolarizations applied from a holding potential of -80 mV. Capacitative currents were minimized to ~10% using the capacitance cancellation feature of the patch-clamp amplifier. Remaining linear components were leak-subtracted using P/3 protocol delivered from the holding potential before each test pulse. Peak L-current magnitude was normalized to cell capacitance (pA/pF) and plotted as a function of membrane potential (V_m) and fitted according to: $I = G_{\text{max}} \cdot (V_m - V_{\text{rev}}) / (1 + \exp[(V_{G1/2} - V_m)/k_G])$ (Eq. 1), where G_{max} is the maximal L-channel conductance, V_m is test potential, V_{rev} is extrapolated reversal potential, $V_{G1/2}$ is the voltage for half-maximal activation of G_{max} , and k_G is a slope factor. Relative changes in intracellular Ca^{2+} in these experiments were measured following dialysis with $\text{K}_5\text{-Fluo-4}$ salt. Fluo-4 dialyzed myotubes were excited at 480 nm and fluorescence emission measured at 535 nm was digitized at 10 kHz. A computer-controlled shutter was used to eliminate dye illumination during intervals between test pulses. Relative changes in Ca^{2+} were expressed as $\Delta F/F$ ($[F_{\text{peak}} - F_{\text{base}}]/F_{\text{base}}$) at the end of each test pulse, plotted as a function of V_m , and fitted according to: $\Delta F/F = (\Delta F/F_{\text{max}}) / \{1 + \exp[(V_{F1/2} - V_m)/k_F]\}$ (Eq. 2), where $(\Delta F/F)_{\text{max}}$ is the calculated maximal change in fluorescence, $V_{F1/2}$ is the voltage for half-maximal activation of $(\Delta F/F)_{\text{max}}$, and k_F is a slope factor. Pooled current voltage (I-V) and fluorescence-voltage ($\Delta F/F$ -V) data were expressed as mean $\pm$ SE. Statistical significance ($p < 0.05$) was determined using a two-tailed Student's t-test.

Results

Mutations in RYR1 transcripts

By sequencing entire RYR1 transcripts from muscle or blood RNA samples of ten index patients, we identified nine missense nucleotide sequence variants in 9 patients. All variants were single-nucleotide changes and were present in the heterozygous state (Table 1b). Three resultant amino acid changes, p.R2435H, p.R2435L and p.A4940T have been reported in association with CCD and MH [32-35], the p.R4893Q mutation has been reported in association with CCD only [32] and five, p.M4640R, p.L4647P, p.F4808L, p.D4918N and p.F4941C, were novel mutations (Figure 1, A). None of the novel mutations was found in at least 100 control individuals. The mutations p.R2435L, p.F4808L, p.R4893Q and p.A4940T segregated perfectly with the disease phenotype within corresponding families, consistent with a dominant mode of disease inheritance (Figure 1, B). In the remaining cases, the mode of inheritance could not be determined conclusively, as relatives were not available for the study.

In one CCD patient (C-10) from our cohort no RYR1 mutations were found. The patient, however, was heterozygous for two common polymorphisms p.P1787L [NCBI dbSNP: rs34934920] and p.G2060C [NCBI dbSNP: rs35364374] as well as for several synonymous variants. Sequence analysis of the coding regions of ACTA1 and SEPN1, known to be associated with congenital myopathies [36] also gave negative results in this patient.

Localization and predicted functional effect of the RyR1 mutations

All mutations identified in this study involved amino acid residues that were positioned within RyR1 regions of high evolutionary conservation, within either the central (p.R2435) or C-terminal (p.M4640, p.L4647, p.F4808, p.R4893, p.D4918, p.A4940, p.F4941) hotspot regions. Moreover, p.M4640, p.L4647, p.F4808, p.R4893, p.D4918 and p.A4940 were positioned within putative transmembrane segments of the RyR1 (Figure 2). Bioinformatic analyses of the 9 mutations identified using online software tools PolyPhen, SIFT, and PMut predicted that each amino acid alteration is potentially damaging to the protein function and, thus, likely to be pathogenic (Table 2).

RYR1 polymorphisms and splicing variants

Three previously reported common RyR1 polymorphisms, p.P1787L (rs34934920), p.G2060C (rs35364374) and p.Q3756E (rs4802584) were detected in index cases C-6, C-7 and C-1, respectively (Table 1b). Additionally, in all of our patients we identified silent polymorphisms that were already recorded in NCBI SNP database for RYR1 gene. In line with previous reports [32, 37], we observed RYR1 transcript variants with skipped exons 70 and 83 in all mRNA, and skipped exons 9, 31, 66 and 94 in blood RNA samples of the patients. In all cases, missing exons, together with their adjacent intronic regions, were sequenced directly from genomic DNA samples to search for possible mutations in the regions and to confirm that exon skipping was not due to aberrant splicing that might have been caused by a contiguous mutation.

Functional characterization of the identified RyR1 variants by expression in HEK293 cells and dyspedic myotubes

In order to determine the functional effects of the identified RyR1 mutations on muscle Ca^{2+} homeostasis and EC coupling, mutations p.L4646P, p.F4807P, p.D4917N and p.R4892Q, equivalent to the novel mutations p.L4647P, p.F4808L and p.D4918N and the recurrent mutation p.R4893Q in humans were introduced into the rabbit RYR1 cDNA construct. Since prior studies suggested that missense RyR1 variants might affect release channel function [38], two additional cDNA constructs were generated, one construct carrying

p.R4892Q in combination with p.P1787L, both variants found in index case C-6, and another construct carrying p.D4917N along with p.S2060C, both variants found in index case C-7.

First, to evaluate the caffeine sensitivity of the mutant RyR1 channels, wild-type and mutant RyR1 proteins were expressed in HEK293 cells, and intracellular Ca^{2+} release induced by caffeine was measured by photometry (Supplementary Figure 1, A, B). Caffeine-induced calcium release was observed in HEK293 cells expressing WT RyR1 and in HEK293 cells expressing RyR1 channels carrying p.P1787L or p.S2060C, and comparison of normalized caffeine responses showed that the caffeine sensitivity of cells expressing either of the polymorphisms was not significantly different from that of cells expressing WT RyR1. In contrast, no measurable caffeine-induced Ca^{2+} release was observed in cells expressing any of the four mutated channels, even at maximal caffeine concentration of 10 mM. Caffeine-induced Ca^{2+} release was also absent in HEK293 cells co-expressing constructs containing either p.R4892Q and p.P1787L or p.D4917N and p.S2060C. Western blotting of whole cell homogenates showed that wild type and mutant proteins were expressed in HEK293 cells at comparable levels; there was no difference in apparent molecular mass between wild type and mutant RyR1 proteins, indicating absence of increased degradation of the expressed mutant proteins (Supplementary Figure 1, C). Thus, the absence of caffeine responses in the mutants was not due to the lack of RyR1 expression, but rather was the result of impaired RyR1 function.

The p.L4646P, p.R4892Q and p.D4917N mutations as well as the polymorphic variant p.P1787L were further assessed by expression in myotubes derived from RyR1-null (dyspedic) mice (Fig. 3 and 4, Suppl. Table 1). Unlike intact WT RyR1-expressing dyspedic myotubes, p.L4646P- and p.D4917N-expressing myotubes lacked both electrically-evoked and 4-CMC-induced Ca^{2+} release (Fig. 3, A, B).

Excitation-contraction (EC) coupling in skeletal muscle is controlled by a bidirectional interaction between sarcolemmal dihydropyridine receptors (DHPRs) and RyR1 proteins of the SR [30, 38]. Specifically, sarcolemmal depolarization induces voltage-driven conformational changes in the DHPR that open nearby SR Ca^{2+} release channels to trigger voltage-gated SR Ca^{2+} release (orthograde coupling). In addition, the presence of RyR1 proteins regulates the Ca^{2+} channel activity of the DHPR (retrograde coupling), demonstrating the reciprocal nature of the DHPR RyR1 gating interaction. In whole cell voltage clamp experiments, expression of the p.L4646P and p.D4917N variants in dyspedic myotubes restored L-type Ca^{2+} channel activity (retrograde coupling) to a similar degree as that observed for WT RyR1 (Figure 3, C and D *left*). These results indicate that the RyR1 variants were expressed, targeted properly to SR-sarcolemmal junctions, and interacted functionally with DHPRs present within these junctions. On the other hand, unlike WT RyR1, voltage-gated Ca^{2+} release (orthograde coupling) was not restored by either p.L4646P or p.D4917N (Figure 3, C and D, *right*), consistent with the results of the functional assessment of mutant RyR1s in HEK293 cells (above).

We previously reported that a CCD mutation in the pore-lining region of rabbit RyR1 (p.R4892W) resulted in a partial (~85%) reduction in voltage-gated SR Ca^{2+} release following expression in dyspedic myotubes that occurred in the absence of a change in the voltage sensitivity of RyR1 Ca^{2+} release [17]. In the current study, the index case C-6 exhibited a different amino acid alteration of the same residue (p.R4893Q) that occurred together with the p.P1787L RyR1 polymorphism. We evaluated the impact of the p.R4892Q variant on electrically-evoked and 4-CMC-induced Ca^{2+} release in intact myotubes (Fig. 4, A and B) and bidirectional DHPR-RyR1 coupling in voltage clamped myotubes (Fig. 4, C and D) in the presence and absence of p.P1787L. Similar to results obtained previously for

p.R4892W, expression of the p.R4892Q variant in dyspedic myotubes resulted in a partial reduction (~60%) in electrically-evoked, 4-CMC-induced and sigmoidal voltage-gated Ca^{2+} release in the absence of a change in the voltage sensitivity of RyR1 Ca^{2+} release ($V_{F1/2}$ was not significantly altered by the p.R4892Q mutation; Supp. Table 1). In addition, the impact of the p.R4892Q mutation on electrical - or 4-CMC-induced Ca^{2+} release in intact myotubes and bidirectional DHPR-RyR1 coupling in voltage clamped myotubes was not significantly altered by p.P1787L. On the other hand, heterotypic expression of the p.R4892Q mutant, either with or without P1787L (i.e. WT vs. either WT+RQ or WT+PL/RQ), fully restored the magnitude ($F_{\max}$) and voltage dependence ($V_{F1/2}$) of RyR1 Ca^{2+} release to a level similar to that of WT RyR1 (Fig. 4, D). Specifically, $F_{\max}$ was not significantly different ($p=0.321$) between WT+PL/RQ and WT+RQ (2.1 ± 0.3 and 2.5 ± 0.3 , respectively) (Suppl. Table 1). Together, the results presented in Fig. 3 and Fig. 4 indicate that the p.L4646P, p.D4917N and p.R4892Q mutants exhibit an EC uncoupled phenotype, although the effect is less pronounced for p.R4892Q.

Discussion

In the present study we analyzed complete RYR1 transcripts in 10 patients with clinical and histological features of central core myopathy and identified 9 different RYR1 mutations in 9 patients. Five of the identified mutations were novel and four were recurrent (Table 1b). In line with the reports that CCD is mainly inherited as an autosomal dominant trait [3], the identified mutations were putative dominant mutations: for four of the mutations p.R2435L, p.F4808L, p.R4893Q and p.A4940T, dominant inheritance was established by segregation analysis, while for the remaining mutations, it was inferred from the fact that the affected patients were heterozygous for a single missense RYR1 mutation.

Genetic analysis supported a causal role for those mutations that were recurrent, and indicated that all 5 novel variants, p.M4640R, p.L4647P, p.F4808L, p.D4918N, and p.F4941C, are strong candidates as CCD causative mutations. Each of the affected amino acid residues is highly conserved throughout evolution and maps to the functionally important C-terminal domain of the RyR1 protein. Four of the novel mutations involved amino acid residues were positioned within putative transmembrane segments of the RyR1 (Figure 2). Bioinformatic analyses performed for each of the mutations using three independent software tools predicted that all mutations identified would have a damaging effect on RyR1 function (Table 2).

Since for seven of our patients we screened leukocyte RYR1 transcripts there was a possibility to miss a mutation due to the absence of expression or silencing of the mutated allele in lymphocytes [5, 7, 33, 39]. However, all the mutations identified in this study were present in a heterozygous state and all the patients were heterozygous for synonymous polymorphisms indicating the expression of both alleles in leukocytes and arguing against leukocyte-specific allele silencing. Therefore, it is not likely that a mutation was missed in this group of patients by screening their leukocyte RYR1 transcripts.

In a single case C-10 where no RYR1 mutations were found, leukocytes-specific monoallelic expression could be excluded because the patient was heterozygous for two nonsynonymous and several synonymous polymorphisms spread across the RYR1 transcript— indicating that both alleles were expressed in the patient's blood RNA. It is plausible that the set of SNPs identified in this patient may have an additive effect on RyR1 expression or stability. However, since our analysis was limited to the coding regions of the RYR1 gene, it would miss mutations in either intronic or regulatory regions of the gene that might contribute to the patient's disease phenotype. Alternatively, the absence of mutations

in the RYR1, ACTA1 and SEPN1 genes of this patient might indicate that defects in other genes are responsible for congenital core myopathy in this patient.

Functional characterization of RyR1 variants *in vitro*

Functional studies of mutant RyR1 proteins are an indispensable tool to elucidate possible functional effects of the RyR1 mutations and to validate their relevance to the disease phenotype. That is especially true for validation of CCD-associated mutations because many of them are private and often represent sporadic cases with no family history of neuromuscular disorders. Previous functional studies demonstrated that disease-associated mutations in RyR1 differ in their impact on intracellular Ca^{2+} homeostasis. Specifically, MHS and CCD mutations located in the N-terminal and central cytoplasmic region of RyR1 when expressed in HEK293 cells or dyspedic myotubes typically displayed a “leaky” phenotype with elevated resting myoplasmic Ca^{2+} and depleted SR Ca^{2+} stores [31, 40]. In contrast, CCD mutations located in the C-terminal transmembrane region of RyR1 often exhibit varying degrees of EC uncoupling with impaired Ca^{2+} permeation [16, 17]. However, functional consequences of specific RyR1 mutations cannot be predicted based solely on their relative location within the RyR1 linear sequence, since several mutations mapped to the C-terminal region of the channel were shown to enhance agonist sensitivity of the mutant RyR1s characteristic to MH-associated mutations [41]. Additionally, several mutations mapped to the cytoplasmic RyR1 region were shown to display dual functional characteristics: RyR1 channels harboring these mutations were hypersensitive to agonist and voltage activation, accounting for the MHS trait, and, at the same time, exhibited increased basal activity that enhances resting Ca^{2+} and reduces both SR Ca^{2+} content and voltage-gated Ca^{2+} release during EC coupling, accounting for the CCD phenotype [18].

In this study we functionally characterized CCD mutations located in the C-terminal transmembrane region of RyR1 using two different expression systems. We demonstrated that when expressed in HEK293 cells, the homotetrameric RyR1 mutants harbouring p.L4646P, p.F4807P, p.D4917N and p.R4892Q mutations abolished caffeine-induced Ca^{2+} release similar to some other functionally characterized CCD mutations located in the transmembrane region of the RyR1 [16, 17]. Our data are in line with the results of an earlier study on functional consequences of mutagenesis of conserved, polar amino acids, including p.D4917N, in the transmembrane region of rabbit RyR1 [28].

Analysis of p.L4646P, p.D4917N and p.R4892Q mutations by expression in dyspedic myotubes showed that RyR1 proteins carrying p.L4646P, p.D4917N and p.R4892Q mutations led to either complete (p.L4646P and p.D4917N) or partial (p.R4892Q) loss of orthograde coupling without an effect on retrograde DHPR coupling. Thus, functional analysis of homotypically expressed mutated channels in HEK293 cells and in dyspedic myotubes clearly demonstrates that mutations, p.L4647P, p.D4918N and p.R4893Q belong to EC-uncoupling mutations.

Unexpectedly, the results of co-expression of one of the mutations, p.R4892Q and WT RyR1 channels in dyspedic myotubes showed normal magnitude and sensitivity of voltage-gated SR Ca^{2+} release. This apparent conflict with strong clinical and genetic evidence indicating a dominant character of this mutation is likely due to the fact that transient *in vitro* expression experiments do not fully reflect the *in vivo* physiological situation present in adult human skeletal muscle fibers [42]. In such cases, the generation and analysis of an animal model carrying the corresponding mutation would likely provide a better approximation to the situation *in vivo*.

Our results regarding common polymorphic RyR1 variants p.P1787L, p.G2060C and p.Q3756E are of interest since their functional significance remains unknown although these

variants were detected in general population as well as in MH/CCD patients [21, 32, 43]. Bioinformatic analyses predicted p.Q3756E to be benign, whereas bioinformatic evaluation of possible functional impact of p.P1787L and p.G2060C variants using three software tools gave ambiguous results (Table 2). Functional studies demonstrated that caffeine responses of HEK293 cells expressing p.P1787L or p.S2060C RyR1 proteins were not significantly different from those of WT RyR1 expressing cells. Furthermore, p.P1787L RyR1 channels, when expressed in dyspedic myotubes, exhibited the same magnitude and sensitivity of voltage-gated SR Ca²⁺ release as WT RyR1 channels. Thus, our functional studies of the common polymorphic variants demonstrate the absence of the effect of p.P1787L and p.G2060C variants on Ca²⁺ release channel function. However, further studies may be necessary to detect possible subtle effects of these variants that may not be captured by transient expression.

Complex genotype-phenotype correlation in CCD

Our findings with regard to the inheritance of the mutations augment previous reports of the complexity of genotype-phenotype correlations in RyR1-related congenital myopathies (Table 1 a, b). Thus, the p.R2435L and p.R2435H mutations located in the central RYR1 domain were previously reported in several studies in association with MHS [32] and in association with dominantly inherited CCD [44, 45]. However, recently they were shown to cause CCD only when inherited in a homozygous or compound heterozygous state [33, 46]. In our study, the p.R2435H mutation was identified in a clinically unaffected daughter whose mother was diagnosed with CCD, both mother and daughter showing central cores on muscle biopsy. The absence of clinical symptoms in the daughter can be explained by low expressivity of the mutation, and, since congenital myopathies are slowly progressive, symptoms might develop in this individual later in life. Importantly, functional studies showed that p.R2435L and p.R2435H confer characteristics of both hypersensitivity to activation and enhanced 'leak' following expression in either HEK293 cells or dyspedic myotubes [18, 27], which could account for their potential to cause MHS alone, CCD alone, or both MHS and CCD.

All the mutations identified in the C-terminal domain - novel mutations p.M4640R and p.F4808L, as well as recurrent CCD mutations p.R4893Q and p.A4940T, displayed dominant mode of inheritance. This finding is consistent with the reports indicating that CCD mutations located in the C-terminal transmembrane domain of the RyR1 are dominant albeit with the variable clinical phenotype [14].

It is not clear yet why the same RyR1 mutation could result in different clinical phenotypes or be inherited as dominant or recessive. Unidentified factors from familial genetic background might influence expression of the RYR1 mutations and their pathogenic consequences. However, most recently insights into molecular processes leading to clinical variability in the RyR1-related disorders have been gained from the analyses of the first mouse model of CCD carrying an equivalent of the human uncoupling CCD mutation I4898T [47]. Since individual heterozygous RyR^{I4895T/+} (IT/+) mice developed variable myopathic features in spite of their uniform genetic background and based on the data of functional heterogeneity of heterozygous I4898T RyR1 channels composed of the random combination of WT and mutant RyR1 subunits [42], it was proposed that the random spatio-temporal fluctuations of Ca²⁺ release within myofibers is the main pathogenic mechanism leading to the formation of structural abnormalities and to clinical variability in the RyR1-related disorders (for a detailed discussion of this issue see [6]).

Conclusions

In summary, our comprehensive RYR1 mutation screening of 10 patients, diagnosed clinically and histologically as having CCD, resulted in the high mutation detection rate (90%), supporting a major role of the RYR1 gene in this congenital myopathy. Characterization of Ca²⁺ channel properties of RyRs carrying one recurrent and two novel mutations upholds the view that diminished intracellular Ca²⁺ release caused by impaired Ca²⁺ channel gating and/or Ca²⁺ permeability is a principal component of CCD etiology. This study expands the list of functionally characterized disease-associated RyR1 mutations, increasing the value of genetic diagnosis for RyR1-related disorders. Altogether, our results strongly suggest that the assessment of mutation causality should be based on a combination of all available data, including clinical observations, histopathological studies, genetic analyses and functional studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Genetic and molecular analysis of the RYR1 mutations identified

A, DNA sequencing chromatograms. **B**, Pedigrees of patients C-1, C-5, C-8. Filled symbols indicate family members diagnosed with CCD. The index case in each family is marked by an arrow. Symbols with a diagonal line indicate deceased individuals. Numbers from I to IV identify generations. Grey symbols in pedigree of index case C-1 denote individuals with elevated creatine phosphokinase. Circles and squares represent females and males, correspondingly; "+" denotes variant carriers and "-" denotes carriers of the WT allele.

Figure 2. Location of the mutations found in this study within the C-terminal RyR1 region

A two-dimensional plot of the accumulation (vertical axis) of MH- and core myopathy-associated mutations in RyR1 against amino acids 4500 to 5038 (horizontal axis) in the full-length human RyR1 protein was constructed from a portion of the same published mutation dataset as the one used in (6). In cases where several different substitutions at the same amino acid position have occurred, they are assigned accumulation values equal to 1, regardless of redundancy at the site. The slope of each interval was thus determined as the ratio of the number of mutations within the interval divided by the length of the interval (Interval) in amino acid residues. Transmembrane domain boundaries, shown as wavy lines, were superimposed on the mutation plot. Borders of the mutation clusters are shown by vertical arrows with the number of a borderline amino acid with a mutation. Locations of RyR1 transmembrane domains are shown by wavy lines. Assignment of the transmembrane domains M5 to M10 is according to a RyR1 topology model [48]. Transmembrane domain M9 includes the selectivity filter and the pore helix while M10 corresponds to the inner helix of the structural model derived from cryo-microscopy [49]. Boldface designates novel mutations.

Figure 3. Effects of p.L4646P and p.D4917N mutations on RyR1 Ca²⁺ release channel function following expression in dyspedic myotubes

A, Representative indo-1 responses from wild type (WT)- (left), p.L4646P- (middle), and p.D4917N-expressing dyspedic myotubes in response to three successive (0.1Hz) electrical stimulations (left traces) and a 30 s exposure to 500 μ M 4-CMC (right traces). Calibrations: vertical, 0.2 Δ Ratio; horizontal, 20 s. **B**, Average resting indo-1 ratio (left), peak response to electrical stimulation (middle), and peak response to 4-CMC exposure (right) in WT-, p.L4646P-, p.D-4917N-expressing dyspedic myotubes and na ve dyspedic myotubes. * $p < 0.01$ compared to WT. **C**, Representative depolarization-induced Ca²⁺ transients (upper traces) and L-type Ca²⁺ currents recorded from WT- (left), p.L4646P-(middle), and p.D4917N-expressing dyspedic myotubes during 200 ms depolarizing voltage steps to -50,

-20, 20, and 50 mV. Calibrations: vertical, 0.6 $\Delta F/F$ or 10 pA/pF; horizontal, 20 ms. **D**, Average voltage dependence of peak L-type Ca^{2+} current density (left) and intracellular Ca^{2+} transients (right) from WT- (circles), p.L4646P- (squares), and p.D4917N-expressing (triangles) dyspedic myotubes.

Figure 4. Effects of the p.P1787L (PL) polymorphism and p.R4892Q (RQ) mutation on RyR1 Ca²⁺ release channel function following expression in dyspedic myotubes

A, Representative indo-1 responses from wild type (WT)-, p.P1787L (PL)-, p.R4892Q (RQ)- and double mutant p.R4892Q plus p.P1787L (RQPL) - expressing dyspedic myotubes in response to five successive (0.2 Hz) electrical stimulations (left traces) and a 30 s exposure to 500 μ M 4-CMC. Calibrations: vertical, 0.2 Δ Ratio; horizontal, 10 s. **B**, Average resting indo-1 ratio (left), peak response to electrical stimulation (middle), and peak response to 4-CMC exposure (right) in from WT-, PL-, RQ- and RQPL- expressing dyspedic myotubes and na ve dyspedic myotubes. * $p < 0.01$ compared to WT. † $p < 0.01$ compared to PL. **C**, Representative depolarization-induced Ca²⁺ transients (upper traces) and L-type Ca²⁺ currents recorded from WT-, PL-, RQ- and RQPL- expressing dyspedic myotubes during 200 ms depolarizing voltage steps to -50, 20, and 50 mV. Calibrations:

vertical, 2 AF/F or 5 pA/pF; horizontal, 25 ms. **D**, Average voltage dependence of peak L-type Ca^{2+} current density (left) and intracellular Ca^{2+} transients (right) in dyspedic myotubes following homotypic expression of WT/WT (filled circles), PL/PL (filled triangles), RQ/RQ (filled squares), RQPL/RQPL double mutant (filled diamonds), and heterotypic expression of WT/PL (open circles), WT/RQ (open triangles), and WT/RQPL double mutant (open squares). Two mutations engineered into separate cDNAs is represented with a slash (e.g. WT/PL) and two mutations engineered into a single cDNA is denoted without a slash (e.g. WTPL).

Table 1

Table 1a - Summary of clinical features of the CCD patients

Patient	Sex/Ethnicity	Onset of symptoms (Age/Year)	Clinical information	Histomorphology	Family history
C-1	Male /Caucasian (Italian)	14 years-old	Muscle weakness; elevated CK	Myopathy with central cores	yes
C-2	Female / Caucasian (English)	32 years old	Asymptomatic- tested as mother has CCD	Myopathy with central cores	yes
C-3	Female / Caucasian (Irish)	one year old	Congenital hip dysplasia, delayed walking, muscle weakness, difficulty with climbing stairs, frequent falls; elevated CK	Myopathy with central cores	no
C-4	Male / Caucasian (Italian)	6 years old	Muscle weakness and cramps since age 6; after age 36 deterioration in strength of upper and lower extremity	Myopathy with central cores; Type I fiber dominance(60%); increased fiber size variability with central nuclei	no
C-5	Male / Caucasian (Italian)	2 years old	Muscle weakness; difficulty ambulating, elevated CK	Myopathy with central cores	yes
C-6	Male / Caucasian (German)	At birth	Congenital hip dysplasia and severe scoliosis; muscle weakness, lower limbs are the weakest, uses wheelchair	Myopathy with central cores	yes
C-7	Male / Caucasian (Irish)	At birth	Hypotonia at birth, strabismus, delayed walking; muscle weakness, walks with walker	Myopathy with central cores, Type I uniformity	yes
C-8	Female / Caucasian (English)	2 years old	Muscle weakness, difficulty walking; elevated CK	Myopathy with eccentric cores; affected uncle has fiber type I predominance with central cores, mutation carrier	yes
C-9	Male/ Caucasian (English)	4 years old	Hypotonia at age 4; proximal extremity muscle weakness	Complete fiber type I predominance; central cores in all fibers	no
C-10	Female /Caucasian (Israeli)	3 years old	Muscle weakness at age 3; asymmetric hypotonia; fever and rigidity during scoliosis repair, clinical grading scale score:30	Myopathy with eccentric cores	no

Table 1B - RYR1 mutations identified in this study*

Patient	Nucleotide change	Mutation	Common missense polymorphism	References
C-1	c.7304G>T	p.Arg2435Leu	p.Gln3756Glu (rs4802584); in <i>cis</i>	44, 50-51
C-2	c.7304G>A	p.Arg2435His	none detected	45, 46, 52
C-3	c.13919T>G	p.Met4640Arg	none detected	This study
C-4	c.13940T>C	p.Leu4647Pro	none detected	This study
C-5	c.14424C>A	p.Phe4808Leu	none detected	This study
C-6	c.14678G>A	p.Arg4893Gln	p.Pro1787Leu (rs34934920); in <i>trans</i>	9, 53
C-7	c.14752G>A	p.Asp4918Asn	p.Gly2060Cys (rs35364374); nd	This study
C-8	c.14818G>A	p.Ala4940Thr	none detected	35, 53
C-9	c.14822T>G	p.Phe4941Cys	none detected	This study
C-10		no RyR1 mutations	p.Pro1787Leu, p.Gly2060Cys; nd	

* Nucleotide and amino acid numbering is according to the reference sequences, GenBank: NP_000531.2, respectively, with +1 corresponding to A of the ATG translation start codon. Novel mutations are shown in boldface.

Table 2
Bioinformatic evaluation of causal potential of RyR1 mutations and polymorphisms identified

Aminoacid change	Causal potential [*]			Functional domain [†]	Positional conservation [‡]
	SIFT	PMut	Polyphen2		
p.R2435L	damaging	pathogenic	possibly damaging	Yes	1
p.R2435H	damaging	pathogenic	probably damaging	Yes	1
p.F4808L	damaging	pathogenic	possibly damaging	Yes	1
p.M4640R	damaging	pathogenic	unknown	Yes	1
p.L4647P	damaging	pathogenic	unknown	Yes	1
p.R4893Q	damaging	pathogenic	probably damaging	Yes	1
p.D4918N	damaging	pathogenic	unknown	Yes	1
p.A4940T	damaging	pathogenic	unknown	No [#]	1
p.F4941C	damaging	pathogenic	unknown	No [#]	1
p.P1787L	tolerated	pathogenic	possibly damaging	No	2
p.G2060C	tolerated	pathogenic	benign	No	2
p.Q3756E	tolerated	neutral	benign	No	3

^{*} Causal potential refers to prediction of mutation effect as made by the respective computer programs: substitutions damaging to the protein function are labeled as damaging, pathological or probably/possibly damaging; neutral substitutions are labeled as tolerated, neutral or benign. Substitutions labeled as unknown could not be classified by Polyphen 2.

[#] mutations positioned within "inner helix", an extended transmembrane segment M10 [40][41];

[†] mutation position within known RyR1 functional domains in InterPro database (<http://www.ebi.ac.uk/interpro/>);

[‡] Positional conservation of the amino acid residue affected by mutation:

¹ conserved across mammalian RyR1 and between human RyR1, RyR2 and RyR3 isoforms;

² conserved across mammalian RyR1 and between human RyR1 and RyR2 isoforms;

³ not conserved across any RyR isoforms.