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A recessive ryanodine receptor 1 mutation in a CCD patient increases channel activity

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SUMMARY

Ryanodine receptors plays a crucial role in skeletal muscle excitation-contraction coupling by releasing calcium ions required for muscle contraction from the sarcoplasmic reticulum. At least three phenotypes associated with more than 100 *RYR1* mutations have been identified; in order to elucidate possible pathophysiological mechanisms of *RYR1* mutations linked to neuromuscular disorders, it is essential to define the mutation class by studying the functional properties of channels harboring clinically relevant amino acid substitutions. In the present report we investigated the functional effects of the c.7304G>T *RYR1* substitution (p.Arg2435Leu) found in a patient affected by central core disease. Both parents were heterozygous for the substitution while the proband was homozygous. We characterized Ca²⁺ homeostasis in myoD transduced myotubes from controls, the heterozygous parents and the homozygous proband expressing the endogenous mutation. We also expressed the recombinant mutant channels in heterologous cells and characterized their [³H] ryanodine binding and single channel properties. Our results show that the p.Arg2435Leu substitution affects neither the resting [Ca²⁺], nor the sensitivity of the ryanodine receptor to pharmacological activators, but rather reduces the release of Ca²⁺ from intracellular stores induced by pharmacological activators as well as by KCl via the voltage sensing dihydropyridine receptor.

Keywords

Central core disease; ryanodine receptor; dihydropyridine receptor; mutation; functional coupling

INTRODUCTION

The ryanodine receptor (RyR) ion channels are large protein complexes composed of four RyR 560 kDa peptides and various associated proteins with a total molecular weight of greater than

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2,500 kDa. They play a crucial role in skeletal muscle excitation-contraction coupling by releasing calcium ions required for muscle contraction from the sarcoplasmic reticulum [1–3]. Mutations in the skeletal muscle isoform of the ryanodine receptor, which is encoded by a gene located on human chromosome 19, are associated with at least three neuromuscular disorders and may be linked to certain forms of heat stroke [4–7]. Four phenotypes associated with more than 100 *RYR1* mutations have been identified to date; most commonly, dominant mutations have been found to associate with typical central core disease (CCD; OMIM # 117000) and the malignant hyperthermia susceptibility trait (MHS) (MH; OMIM #145600) [4,5], whereas few recessive mutations have been identified in patients affected by clinically distinct forms of multi-minicore disease (MmD) (MmD; OMIM # 602771) and forms of CCD with unusual or more severe presentations [8–11]. In most cases, dominant *RYR1* mutations linked to CCD localize to the COOH-terminal domain of the channel, containing the transmembrane and pore-forming domains of the RyR1 channel, whereas MHS-linked mutations were initially mapped to hot spot areas within the NH₂ and central RyR domains; however, more recently identified MHS-linked mutations seem to be distributed throughout the entire *RYR1* coding region.

It appears that *RYR1* mutations result in 4 different channel defects [6]: (i) one class of mutations (mostly associated with MHS phenotype) cause Ca²⁺ channels to become hypersensitive to membrane depolarisation and pharmacological activation; (ii) a second class of mutations causes the RyR1 channels to become leaky (iii) a third class of mutations renders the Ca²⁺ channel unable to conduct Ca²⁺ and/or uncouples RyR1 from the voltage sensor [12], (mutation class 2 and 3 are mostly associated to CCD); (iv) the forth class of mutations (found in hemizygous MmD patients) results in protein Ca²⁺ channel instability which ultimately leads to a decrease of the expression level within muscle [10,13]. In order to elucidate possible pathophysiological mechanisms of neuromuscular disorders linked to *RYR1* mutation, it is essential to define the mutation class by studying the functional properties of channels harbouring clinically relevant amino acid substitutions.

One of the first mutations identified in *RYR1* associated with the CCD phenotype, was the heterozygous A>G7302 substitution, leading to the p.Arg2434His mutation [14]. This substitution was also identified at the heterozygous level in some patients who were classified as MHS by the *in vitro* contracture test [15–17]. Functional analysis indeed confirmed that when expressed as a recombinant channel in heterologous HEK293 cells, the presence of this mutation led to a shift in sensitivity to both caffeine and halothane [18]. A similar p.Arg2435Leu substitution was also identified at the heterozygous state in some MHS and CCD families [15,16,19].

Recently, we characterized a large cohort of patients with clinical, histological and muscle imaging features suggestive of *RYR1* involvement and identified nine novel mutations, including the homozygous substitution c.7304G>T leading to a p.Arg2435Leu mutation in a patient affected by CCD [20]. Because in some patients affected by core myopathies, monoallelic expression of the mutation has been found in muscles [10,20,21], we undertook a more detailed investigation of the patient and her family members. Both parents were heterozygous for the p.Arg2435Leu substitution while the affected proband was homozygous, confirming the classical Mendelian inheritance of this mutation. Since both parents were unaffected, we reasoned that the genotype-phenotype correlation might be linked to the presence of the mutation at the homozygous state. In the present study we (i) characterized Ca²⁺ homeostasis in MyoD transduced myotubes from controls, heterozygous parents and the homozygous proband expressing the endogenous mutation and (ii) expressed the recombinant mutant channel in heterologous cells and characterized its [³H]ryanodine binding characteristics and single channel properties. Our results show that the presence of the p.Arg2435Leu substitution either at the homozygous or heterozygous state, affects neither

resting $[Ca^{2+}]_i$, nor the sensitivity of the ryanodine receptor to pharmacological activators, but rather increases ryanodine receptor ion channel activity, leading to reduced Ca^{2+} release after activation by pharmacological agents.

METHODS

Patient phenotype and family history

The patient is currently a 15 years old girl who presented, following an unremarkable perinatal history and normal early normal development, at the age of 3–4 years with toe walking and a hyperlordotic posture. At age 6 years she was operated for contractures of the Achilles tendons and the muscle biopsy performed at the time, was consistent with central core disease. Subsequently, in addition to her hyperlordosis, she developed a moderate scoliosis and bilateral scapular winging; on examination she had a mild Gowers' sign but was able to jump. Her CK was 700 IU/l. There was no known consanguinity although both parents originate from the same town in Italy. There was no history of MH in the parents (the mother has undergone anaesthesia on two separate occasions) or in other family members; no MH test has ever been done in her family but both parents are aware that they both have moderately increased CK (300 IU/l). Genetic investigation [20] showed that the parents carried the heterozygous substitution c.7304G>T in exon 45 and that the patient was homozygous for the substitution. Screening of the entire RYR1 cDNA did not reveal the presence of any other nucleotide changes.

MyoD transduced fibroblasts

It was not possible to obtain myotubes from the muscle biopsies but primary skin fibroblast cultures were established from the proband and unaffected parents. Functional studies were performed on fibroblasts transduced with myoD-encoding adenovirus in order to achieve myogenic conversion, as previously described [13].

Intracellular calcium measurements

Changes in the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, of myotubes were monitored with the fluorescent Ca^{2+} indicator fura-2 at the single cell level by digital imaging microscopy as previously described [13,22]. Individual cells were stimulated with a 12-way 100 mm diameter quartz micromanifold computer controlled microperfuser (ALA Scientific). On-line (340 nm, 380 nm and ratio) measurements were recorded using a fluorescent Axiovert S100 TV inverted microscope (Carl Zeiss GmbH, Jena, Germany) equipped with a 20x water-immersion FLUAR objective (0.17 NA), filters (BP 340/380, FT 425, BP 500/530). Cells were analyzed using an Openlab imaging system and the average pixel value for each cell was measured at excitation wavelengths of 340 and 380 nm.

Construction of mutant cDNAs and expression of wild-type and mutant RyR1 proteins in HEK293 cells

Base changes of rabbit RyR1 cDNA were performed by *Pfu*-turbo polymerase-based chain reaction using mutagenic oligonucleotides and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described previously [23]. Briefly, a fragment of RyR1 cDNA (*XhoI*-*BbrPI* (6598–7692)), subcloned into cloning vector, served as a template for PCR. The mutant fragment amplified by PCR, was confirmed by DNA sequencing and cloned back into the original position by standard cloning techniques. Finally, expression plasmids for full-length RyR1 with the mutation were constructed by ligating three RyR1 fragments (*ClaI*/*XhoI* (polylinker-6598), *XhoI*/*EcoRI* (6598–11767), *EcoRI*/*XbaI* (11767-polylinker)) and pCMV5 (*ClaI*/*XbaI*) expression vector. Nucleotide numbering is as described previously [24].

Wild-type RyR1 and mutant cDNAs were transiently expressed in HEK293 cells transfected with FuGENE 6 according to the manufacturer's instructions. Cells were maintained at 37°C and 5% CO₂ in high glucose Dulbecco's modified eagle medium containing 10% fetal bovine serum and plated the day before transfection. For each 10 cm tissue culture dish, 3.5 µg of cDNA was used. Cells were harvested 48 h after transfection. Crude membrane fractions and proteoliposomes containing the purified RyR1s were prepared in presence of protease inhibitors as described [23].

[³H]PN200-110 and [³H]Ryanodine Binding

[³H]PN200-110 binding experiments were carried out on the 10K supernatant of MyoD transduced cells from controls or the homozygous p.Arg2435Leu mutation bearing patient as previously described [25].

[³H]Ryanodine binding experiments were performed with crude membrane fractions prepared from HEK 293 cells as previously described [23]. Unless otherwise indicated, membranes were incubated with 3 nM [³H]ryanodine in 20 mM imidazole, pH 7.0, 0.15 M sucrose, 250 mM KCl, 5 mM Mg²⁺, 5mM AMPPCP (a non-hydrolyzable ATP analogue), 5 mM reduced glutathione, protease inhibitors, 1 mM EGTA and [Ca²⁺] to yield the indicated concentrations of free Ca²⁺. Free [Ca²⁺] were adjusted with the use of a Ca²⁺ selective electrode. Nonspecific binding was determined using a 1000–2000 fold excess of unlabeled ryanodine. After 20 h, samples were diluted with 8 volumes of ice-cold water and placed on Whatman GF/B filters preincubated with 2% polyethyleneimine in water. Filters were washed with three 5 ml ice-cold 100 mM KCl, 1 mM KPipes, pH 7.0 solution. The radioactivity remaining with the filters was determined by liquid scintillation counting to obtain bound [³H]ryanodine.

B_{max} values of [³H]ryanodine binding were determined by incubating membranes for 4 h at 24°C with a saturating concentration of [³H]ryanodine (30 nM) in 20 mM imidazole, pH 7.0, 0.6 M KCl, 0.15 M sucrose, 1 mM glutathione (oxidized), protease inhibitors, and 200 µM Ca²⁺. Specific binding was determined as described above.

Single channel recordings

Single channel measurements were performed using planar lipid bilayers containing a 5:3:2 mixture of bovine brain phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (25 mg of total phospholipid/ml *n*-decane as previously described [12]. Proteoliposomes containing the purified recombinant RyR1s were added to the *cis* (SR cytosolic side) chamber of a bilayer apparatus and fused in the presence of an osmotic gradient (250 mM *cis* KCl/20 mM *trans* KCl in 20 mM KHepes, pH 7.4, 0.23 mM EGTA and 0.21 mM Ca²⁺). After the appearance of channel activity, *trans* (SR luminal side) KCl concentration was increased to 250 mM to prevent further fusion of proteoliposomes. To determine K⁺ and Ca²⁺ conductances and permeability ratios, single channel activities were recorded in symmetrical 250 mM KCl solution before and after addition of 10 mM Ca²⁺ to the *trans* side, and analyzed as described [12].

Statistical analysis

Statistical analysis was performed using the Student's *t* test for paired samples or using ANOVA when more than two groups were compared. Origin computer program (Microcal Software, Inc., Northampton, MA, USA) was used for statistical analysis and dose response curve generation.

RESULTS AND DISCUSSION

In order to assess the functional effect of the p.Arg2435Leu mutation, we used myotubes derived from MyoD transduced fibroblasts obtained from controls, the heterozygous parents and the homozygous patient. We have previously shown that such myotubes express a functional RyR1 ion channel and represent a valid model to assess the effects of RyR1 mutations on stored Ca^{2+} release [13]. We determined the resting $[\text{Ca}^{2+}]$, the dose dependent-sensitivity of the myotubes to caffeine and 4-chloro-m-cresol and the peak Ca^{2+} release from fura-2 loaded myotubes, since we have previously demonstrated that most pathogenic *RYR1* mutations can alter any of these parameters [13,22]. Figures 1A and B show that the presence of the p.Arg2435Leu substitution at the heterozygous or homozygous state did not increase the sensitivity of Ca^{2+} release either to caffeine or 4-chloro-m-cresol. No significant differences were observed in stored Ca^{2+} release when control cells and cells expressing p.Arg2435Leu heterozygously were exposed to caffeine or 4-chloro-m-cresol; however, cells carrying the mutation at the homozygous state, released significantly less Ca^{2+} after pharmacological activation (fig. 1C). Interestingly, the mutation at the heterozygous state diminished the peak of Ca^{2+} release by greater than 2-fold, when a maximally activating KCl concentration (100 mM) was applied (fig. 1C). No significant differences in PN200-110 binding between control and p.Arg2435Leu homozygous mutation carrying cells were found (the B_{max} for control, heterozygous and homozygous mutant cells was 60.9 ± 12.1 , 73.2 ± 9.0 and 69.8 ± 6.9 pmoles PN200-110 bound/mg protein, respectively) indicating that the patient harbouring the *RYR1* mutation does not have altered DHPR expression levels. Our results using myoD transduced skin fibroblasts are in partial support with the data of Dirksen and Avila, who showed that in dyspedic myotubes transduced with the *RYR1* cDNA encoding the p.Arg2435Leu mutation, the maximal voltage-activated Ca^{2+} release was reduced [26]. However, at variance with their data, we found that the presence of the p.Arg2435Leu mutation does not affect resting $[\text{Ca}^{2+}]$ (Figure 1 D).

To more directly analyse the functional consequences of the p.Arg2435Leu mutation, HEK293 cells were transfected with wild type or mutant *RYR1* cDNAs. Crude membrane fractions were prepared from the cells, and the sensitivity of recombinant channels to caffeine and Ca^{2+} was determined. To simulate *in vivo* conditions, experiments were done in presence of 5 mM Mg^{2+} , 5mM AMPPCP (a nonhydrolyzable ATP analogue), and 5 mM reduced glutathione. As shown in figure 2A, the p.Arg2435Leu substitution causes a small but significant increase in $[\text{Ca}^{2+}]$ ryanodine binding at low (μM) and high (mM) calcium concentrations. Caffeine sensitivity was determined at 0.15 μM free Ca^{2+} . In accordance with increased $[\text{Ca}^{2+}]$ ryanodine binding at low Ca^{2+} concentrations, mutant channels exhibited an increased level of $[\text{Ca}^{2+}]$ ryanodine binding in the presence of 1–5 mM caffeine compared to WT (Fig. 2B). Similar $[\text{Ca}^{2+}]$ ryanodine binding levels were obtained at 10 and 20 mM caffeine. We conclude that the p.Arg2435Leu substitution increases the sensitivity of RyR1 to low and high concentrations of Ca^{2+} compared to WT without a noticeable shift in sensitivity to caffeine.

In order to gain insight into the biophysical properties of the channels harbouring the p.Arg2435Leu substitution, the single channel properties of recombinant wild type and mutant RyR1s were compared. Figures 3A and C show that mutant channels exhibited some small but significant increases in channel activity at low (0.1 μM) and high (10 mM) free Ca^{2+} . Kinetic analysis indicates a significant increase in the number of channel events and mean open and closed (10 mM Ca^{2+} only) times of mutant channels compared to WT (Table 1). The p.Arg2435Leu substitution did not significantly alter the K^{+} conductance and Ca^{2+} over K^{+} permeability ratio of mutant channels compared to WT (Fig. 3B).

From the results of the present and other investigations, it appears that the impact of the substitution of Arg2435 is quite complex and may relate to several variables, including the

genotype of the carrier (homozygous vs heterozygous) and the actual substituted residue. In fact, several groups have reported the presence of a similar but not identical dominant p.Arg2434His mutation in CCD/MH families [14–17]. The biochemical properties of amino acid residues Leu, His and Arg are quite diverse: the former is a non-polar, non-charged residue, the latter carries a positively charged polar side chain, while His is a polar residue whose charge is strongly influenced by pH, being approximately 50% positively charged at physiological pH [27]. Thus the substitution of His or Leu for Arg, most likely has a different effect on ryanodine receptor channel function and we would like to emphasize that the results of the present investigation can only be viewed in light of the p.Arg2435Leu substitution. In view of the results of the present study, it is plausible that the p.Arg2435Leu residue contributes to allosteric interactions that are important for transducing the E-C coupling signal to the pore region of RyR1; an altered signal leads to channels with an increased P_o particularly evident at low (nanomolar) and high (millimolar) $[Ca^{2+}]$.

As to the presence of the heterozygous mutation in MH individuals, our results do not show the typical increase in sensitivity to caffeine and 4-chloro-m-cresol observed in association with p.Arg2435His expression in HEK293 cells [18] or an increased resting $[Ca^{2+}]$, typically observed in many cells expressing MHS-linked mutations [22,28]. In this context, our results are at variance with those of Dirksen and Avila [26] as they observed an increase in resting $[Ca^{2+}]$, whereas we did not. This difference may be linked to differences in the experimental models. We measured the activity of endogenously expressed RyR1s in myoD transduced skin fibroblasts, whereas Dirksen and Avila over-expressed the mutant RYR1 cDNA in dyspedic myotubes [26]. In a recent paper, we showed that myoD transduced fibroblasts harbouring the compound S71Y+N2283H mutations (the latter being linked to the MHS phenotype) had a small but significantly higher resting $[Ca^{2+}]$ compared to cells from controls, or from cells bearing other *RYR1* substitutions linked to recessive core myopathies [13]. Thus, cells from the patients carrying the p.Arg2435His are apparently better equipped to handle the increased Ca^{2+} leak occurring through the RyR1 mutant channels either because of a smaller leak and/or more efficient re-uptake and extrusion mechanisms.

In conclusion, the present study shows that the homozygous p.Arg2435Leu mutation found in a CCD patient is complex but compatible with a class 2 channel defect. MyoD transduced fibroblasts from the homozygous patient and studies using the recombinant mutant construct, indicate a “leaky” channel with increased channel activity. However, myoD transduced fibroblasts from the heterozygous parents exhibited partial functional uncoupling of the RyR1 from the DHPR since they exhibited reduced depolarization-induced Ca^{2+} release (class 3 channel defect). Interestingly, the latter effect does not manifest itself in the heterozygous parents because they are both clinically unaffected.

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Figure 1. Comparison of intracellular calcium homeostasis in myotubes from controls and p.Arg2435Leu heterozygous and homozygous mutation-bearing individuals

Dose response curves of fura-2 loaded myotubes to (A) caffeine and (B) 4-chloro-m-cresol. Myotubes were individually stimulated by addition of the agonist in Krebs-Ringer buffer containing no added Ca^{2+} and $100 \mu\text{M}$ La^{3+} , thus the increase in $[\text{Ca}^{2+}]_i$ represents only release of calcium from intracellular stores. Curves show the caffeine, 4-chloro-m-cresol and caffeine dose dependent changes in calcium, expressed as Δ in fluorescence ratio (peak ratio 340/380 nm-resting ratio 340/380 nm). Each point represents the mean ($\pm$ S.E.) of the Δ fluorescence of measurements performed on 8–36 different cells (open circles, straight line, controls; half full box, dash-dot line, heterozygous; closed boxes, dotted line, homozygous). Dose response

curves were generated using the Origin software. **(C)** Comparison of peak Ca^{2+} release after addition of KCl, caffeine and 4-chloro-m-cresol and **(D)** resting $[\text{Ca}^{2+}]$ (as fluorescent ratio) or. Values represent the mean $\pm$ SEM of n number of different cells; * $P < 0.028$, ** $P < 0.015$. Open bars= control, light grey bars= heterozygous, dark grey bars= homozygous. For 100 mM KCl n= 13, 21 and 8; for 10 mM caffeine n= 16, 18 and 29; for 600 μM 4-cmc n= 23, 38 and 27; for resting $[\text{Ca}^{2+}]$ n= 149, 147 and 182 in control, heterozygous and homozygous mutation bearing cells, respectively.

Figure 2. Calcium and caffeine dependence of $[\text{}^3\text{H}]\text{ryanodine}$ binding to recombinant WT-RyR1 and RyR1-Arg2435Leu mutant

(A) Specific $[\text{}^3\text{H}]\text{ryanodine}$ binding to crude membrane fractions containing WT or mutant RyR1 was carried out as described in Methods in 0.25 M KCl, 20 mM imidazole pH 7.0 containing, 5 mM Mg^{2+} , 5 mM AMPPCP, 5 mM glutathione (reduced), 1.5 nM $[\text{}^3\text{H}]\text{ryanodine}$ and the indicated free $[\text{Ca}^{2+}]$. (B) Caffeine dependence was determined at 0.15 μM free Ca^{2+} . Data represent the mean $\pm$ SD of 3 experiments.

Figure 3.

Single channel activities and conductances of WT and p.Arg2435Leu RyR1 ion channels. **(A)** Traces represent single channel currents (openings shown as downward deflections from the closed state, c) of WT RyR1 (left) and RyR1-Arg2435Leu (right) at -35 mV recorded in symmetrical 250 mM KCl, 2 mM MgATPcis and indicated concentrations of free cis (cytosolic) Ca^{2+} . **(B)** Current voltage relationships of WT (solid symbols) and RyR1-Arg2435Leu (open symbols) in symmetrical 250 mM KCl, 2 $\mu\text{M Ca}^{2+}$ cis (circles) and following addition of 10 mM trans Ca^{2+} (triangles). No significant differences were observed between WT (closed symbols) and R2435L (open symbols) RyR1. One of four similar experiments is shown. **(C)** Ca^{2+} dependence of WT RyR1 and RyR1-Arg2435Leu single channel open probabilities (P_o)

in symmetrical 250 mM KCl, 2 mM MgATP cis and indicated concentrations of free cis (cytosolic) Ca^{2+} . Data are the mean $\pm$ S.E. of 6–19 experiments.

Table 1
Single channel parameters of WT-RyR1 and RyR1-R2435L

	WT-RyR1	RyR1-R2435L
0.1 μM free Ca^{2+}		
Po	0.003 $\pm$ 0.002(8)	0.074 $\pm$ 0.037(5) *
Number of events/min	183 $\pm$ 74	786 $\pm$ 324 *
Mean open time (ms)	0.77 $\pm$ 0.15	4.01 $\pm$ 1.32 *
Mean closed time (ms)	1607 $\pm$ 697	184 $\pm$ 84
10 mM free Ca^{2+}		
Po	0.006 $\pm$ 0.001(8)	0.109 $\pm$ 0.047(8) *
Number of events/min	1374 $\pm$ 298	17394 $\pm$ 5243 *
Mean open time (ms)	0.24 $\pm$ 0.01	0.31 $\pm$ 0.03 *
Mean closed time (ms)	68.8 $\pm$ 18.7	7.01 $\pm$ 2.41 *

Single channel activities were determined as in Fig. 2 with 0.1 μ M and 10 mM free Ca^{2+} in the cis chamber. Data are the mean $\pm$ S.E. of number of experiments in parenthesis.

* P<0.05 compared with WT-RyR1.