Recessive ryanodine receptor 1 mutation in a CCD patient affects RyR/Cav1.1 functional coupling

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SUMMARY

The ryanodine receptor plays a crucial role in skeletal muscle excitation-contraction coupling by releasing calcium 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 effect of the c.7304G>T *RYR1* substitution (p.Arg2434Leu) identified in a patient affected by central core disease. Both parents were heterozygous for the substitution while the affected proband was homozygous. We characterized Ca^{2+} homeostasis in myoD transduced myotubes from controls, the heterozygous parents and the homozygous proband expressing the endogenous mutation. We also expressed the recombinant mutated channel in heterologous cells and characterized its [^{3}H]ryanodine binding and single channel properties. Our results show that the presence of the p.Arg2434Leu substitution either at the homozygous or heterozygous state, affects neither the resting [Ca^{2+}], nor the sensitivity of the ryanodine receptor to pharmacological activators, but rather reduces KCl-induced Ca^{2+} release via the voltage sensing dihydropyridine receptor.

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 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 [1,2]. 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 [3-6]. 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) [3,4], 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 [7-10]. 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 NH_2 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: (i) one class of mutations (mostly associated with MHS phenotype) cause Ca^{2+} channels to become hypersensitive to membrane depolarisation and pharmacological activation; (ii) a second class of mutations render the Ca^{2+} channel unable to conduct Ca^{2+} [11], (iii) a third class of mutations uncouples RyR1 from the voltage sensor (mutation class 2 and 3 are mostly associated to CCD

and have not been clearly distinguished); (iv) the forth class of mutations (found in hemyzygous MmD patients) results in protein Ca^{2+} channel instability which ultimately leads to a decrease of the expression level within muscle [9, 12]. 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 [13]. This substitution was also identified at the heterozygous level in some patients who were classified as MHS by the *in vitro contracture test* [14-16]. 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 [17|. A similar p.Arg2435Leu substitution was also identified at the heterozygous state in some MHS and CCD families [14, 15,18].

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 [19]. Because in some patients affected by core myopathies, monoallelic expression of the mutation has been found in muscles [9, 19, 20], 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 Ca^{2+} homeostasis in MyoD transduced myotubes from controls, heterozygous parents and the homozygous proband expressing the endogenous mutation and (ii) expressed the recombinant mutated channel in heterologous cells and characterized its [^{3}H]Ryanodine binding characteristics and single channel properties. Our results show that the presence of the p.Arg2434Leu substitution either at the homozygous or heterozygous state, affects neither resting [Ca^{2+}], nor the sensitivity of the ryanodine receptor to pharmacological activators, but rather reduces Ca^{2+} release induced by activation of the voltage sensor by KCl membrane depolarization.

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 toewalking 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 [19] 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 [12].

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 [12,21]. 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 [22]. Briefly, a fragment of RyR1 cDNA (*XhoI-BbrPI* (6598-7692)), subcloned into cloning vector, served as a template for PCR. The mutated 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 [23].

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% CO_2 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 [22].

[^{3}H]Ryanodine Binding

[^{3}H]Ryanodine binding experiments were performed with crude membrane fractions prepared from HEK 293 cells as previously described [22]. Unless otherwise indicated, membranes were incubated with 3 nM [^{3}H]ryanodine in 20 mM imidazole, pH 7.0, 0.15 M sucrose, 250 mM KCl, 5 mM Mg^{2+}, 5mM AMPPCP (a nonhydrolyzable ATP analogue), 5 mM reduced glutathione, protease inhibitors, and indicated Ca^{2+} and caffeine concentrations. 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 [^{3}H]ryanodine.

B_{max} values of [^{3}H]ryanodine binding were determined by incubating membranes for 4 h at 24^{o}C with a saturating concentration of [^{3}H]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 100 μM Ca^{2+}. 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 [11]. 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, 2 μM Ca^{2+}). After the appearance of channel activity, trans

(SR lumenal side) KCl concentration was increased to 250 mM to prevent further fusion of proteoliposomes. To determine K^+ and Ca^{2+} conductances and permeability ratios, single channel activities were recorded in symLeurical 250 mM KCl solution before and after addition of 10 mM Ca^{2+} to the trans side, and analyzed as described [11].

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 [12]. We determined the resting [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 laded myotubes, since we have previously demonstrated that most pathogenic *RYR1* mutations can alter any of these parameters [12,21]. Figures 1A and B show that the presence of the p.Ar2435Leu substitution at the heterozygous or homozygous state did not increase the sensitivity of Ca^{2+} release either to caffeine or to 4-chloro-m-cresol. The presence of the mutation at the homozygous state did however significantly diminish the peak of Ca^{2+} release by caffeine.

To verify whether the functional coupling of the RyR1 with the dihydropyridine receptor (DHPR) voltage sensor was affected by the mutation, we activated the RyR1 via the DHPR, using the KCl-induced membrane depolarisation protocol. As shown in Fig.1C the response of myotubes carrying the p.Arg2435Leu substitution to a maximally activating KCl concentration (100 mM) was reduced by more than 50% in cells harbouring the mutation, compared to cells from control individuals. Our results 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 [24]. However, at variance with their data, we found that the presence of the p.Arg2435Leu mutation does not affect the resting [Ca^{2+}].

To more directly analyse the functional consequences of the p.Arg2435Leu mutation, HEK293 cells were transfected with the wild type or mutated *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 [^{3}H]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 [^{3}H]ryanodine binding at low Ca^{2+} concentrations, mutant channels exhibited an increased level of [^{3}H]ryanodine binding in the presence of 1-5 mM caffeine compared to WT (Fig. 2B). Similar [^{3}H]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 mutated RyR1s were compared. Figures 3A and C show that mutant channels exhibited some small (not significant) increases in channel activity at low (0.1 μM) and high (10 mM) free Ca^{2+}; however, none of these were statistically significant. 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 this 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 [13-16]. 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 [25]. Thus the substitution of His or Leu for Arg, most likely has a different effect on the channel's 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 skeletal muscle, the DHPR is present on the transverse tubular membrane where it serves as a voltage sensor; the II-III loop of the $\alpha 1$ subunit (Cav1.1) of the DHPR physically interacts with RyR1s which are located on the terminal cisternae of SR [26-28]. After sensing an action potential, the DHPR undergoes a conformational change whereby bi-directional signalling with the RyR is activated, leading to release of Ca^{2+} from the SR [29-31]. The domains involved in the DHPR-RyR1 interaction have been identified; one of these (R10) lies between residues 1635 and 2636 of the RyR1 and is important for skeletal type excitation-contraction coupling [30, 32]. While on the DHPR, the region lying between residues 720-765 of the Cav1.1 is critical for skeletal excitation contraction-coupling [30,33]; the latter region contains a stretch of charged residues and has a net negative charge (13 negatively charged residues and 3 positively charged residues). If residue p.Arg2435 is part of the interacting domain between RyR1 and Cav1.1, it is plausible that replacement of a positively charged residue within the RyR1/Cav1.1 interaction site may affect the electrostatic interaction between the two proteins, leading to a less efficient functional coupling and reduced Ca^{2+} release. Our hypothesis is that the pathophysiological

mechanism of the p.Arg2435 substitution may be associated with attenuated orthograde coupling between Cav1.1 and RyR1.

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 [17] or an increased resting [Ca^{2+}], typically observed in many cells expressing MHS-linked mutations [21, 34]. In this context, our results are at variance with those of Dirksen and Avila [24] 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, whereas Dirksen and Avila over-expressed the mutated RYR1 cDNA in dyspedic myotubes [24]. Interestingly Du et al. [35] showed that some *RYR1* mutations within malignant hyperthermia domain 2 actually decrease the caffeine sensitivity of the skeletal muscle RyR1, and postulate that amino acids within residue 2163-2458 form a regulatory domain. Similarly, Jones et al. [36] have shown that insertion of GFP after residues Thr2030 of the cardiac ryanodine receptor isoform, leads to reduced activation by caffeine. In agreement with these reports, we found that the p.Arg2435Leu substitution in RyR1 resulted in a reduced caffeine response compared to that obtained by 4-chloro-m-cresol.

In conclusion this study shows that the homozygous p.Arg2435Leu mutation found in the CCD patient is consistent with a channel defect of class 3, which leads to the functional uncoupling of the RyR1 from the DHPR.

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Figure legends

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 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 μM La^{3+}, thus the increase in $[Ca^{2+}]_i$ 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 (±S.E.) of the Δ fluorescence of measurements performed on 5-20 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 resting $[Ca^{2+}]$ (as fluorescent ratio) or peak Ca^{2+} release after addition of KCl, caffeine and 4-chloro-m-cresol. Values represent the mean± 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 resting $[Ca^{2+}]$ n= 149,147 and 145; for 100 mM KCl n= 13, 9 and 7; for 10 mM caffeine n= 16,18 and 21; for 600 μM 4-cmc n= 23, 28 and 30 in control, heterozygous and homozygous mutation bearing cells, respectively.

Figure 2: Calcium and caffeine dependence of [^{3}H]ryanodine binding to recombinant WT-RyR1 and RyR1-Arg2435Leu mutant. ***(A)*** Specific [^{3}H]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 [^{3}H]ryanodine and the indicated concentrations of calcium. ***(B)*** Caffeine

dependence was determined at 0.15 μM free Ca^{2+}. Data represent the mean± 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 mMKCl, 2 mMMgATPcis 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 μM 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 (Po) in symmetrical 250 mM KCl, 2 mM MgATP cis and indicated concentrations of free cis (cytosolic) Ca^{2+}. Data are the mean ± S.E. of 3-7 experiments.

Figure 1

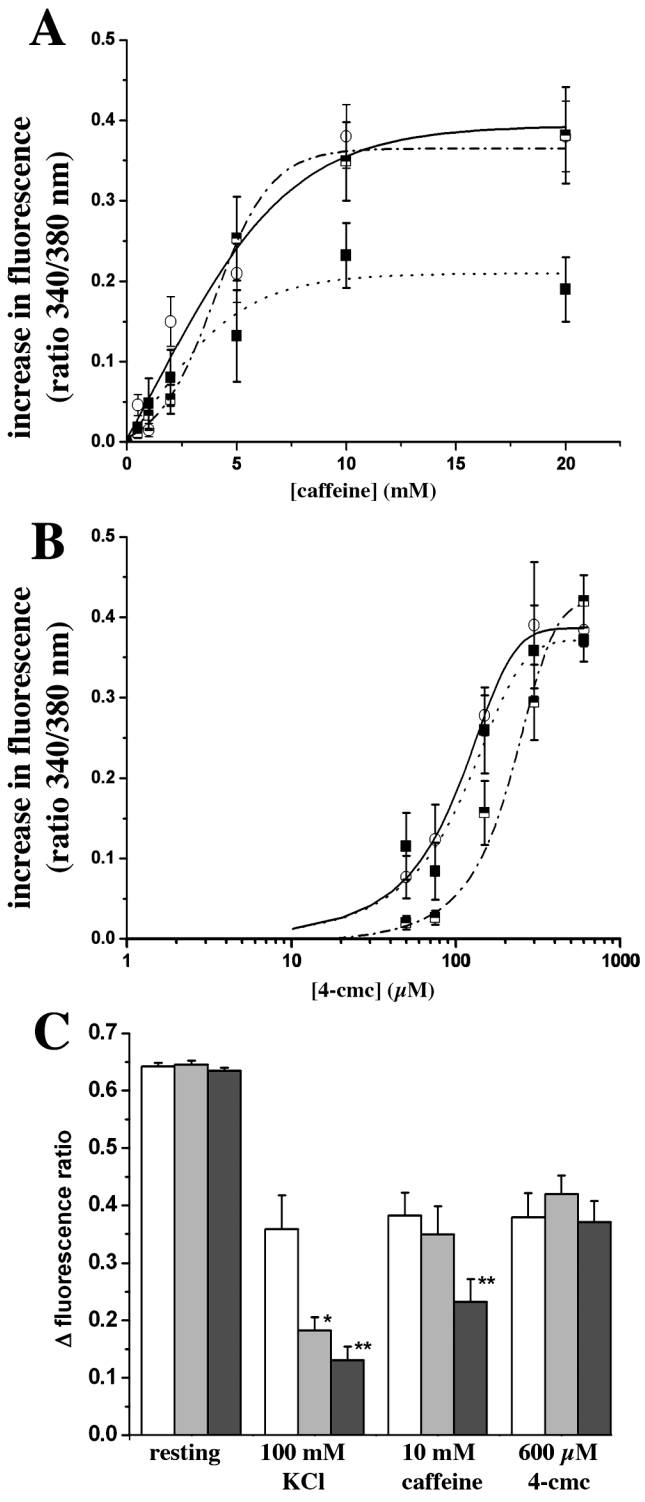

A
increase in fluorescence (ratio 340/380 nm)
0.0
0.1
0.2
0.3
0.4
0.5
0
5
10
15
20
[caffeine] (mM)
B
increase in fluorescence (ratio 340/380 nm)
0.0
0.1
0.2
0.3
0.4
0.5
1
10
100
1000
[4-cmc] (μM)
C
Δ fluorescence ratio
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
*
**
*
**
resting
100 mM KCl
10 mM caffeine
600 μM 4-cmc

Figure 2

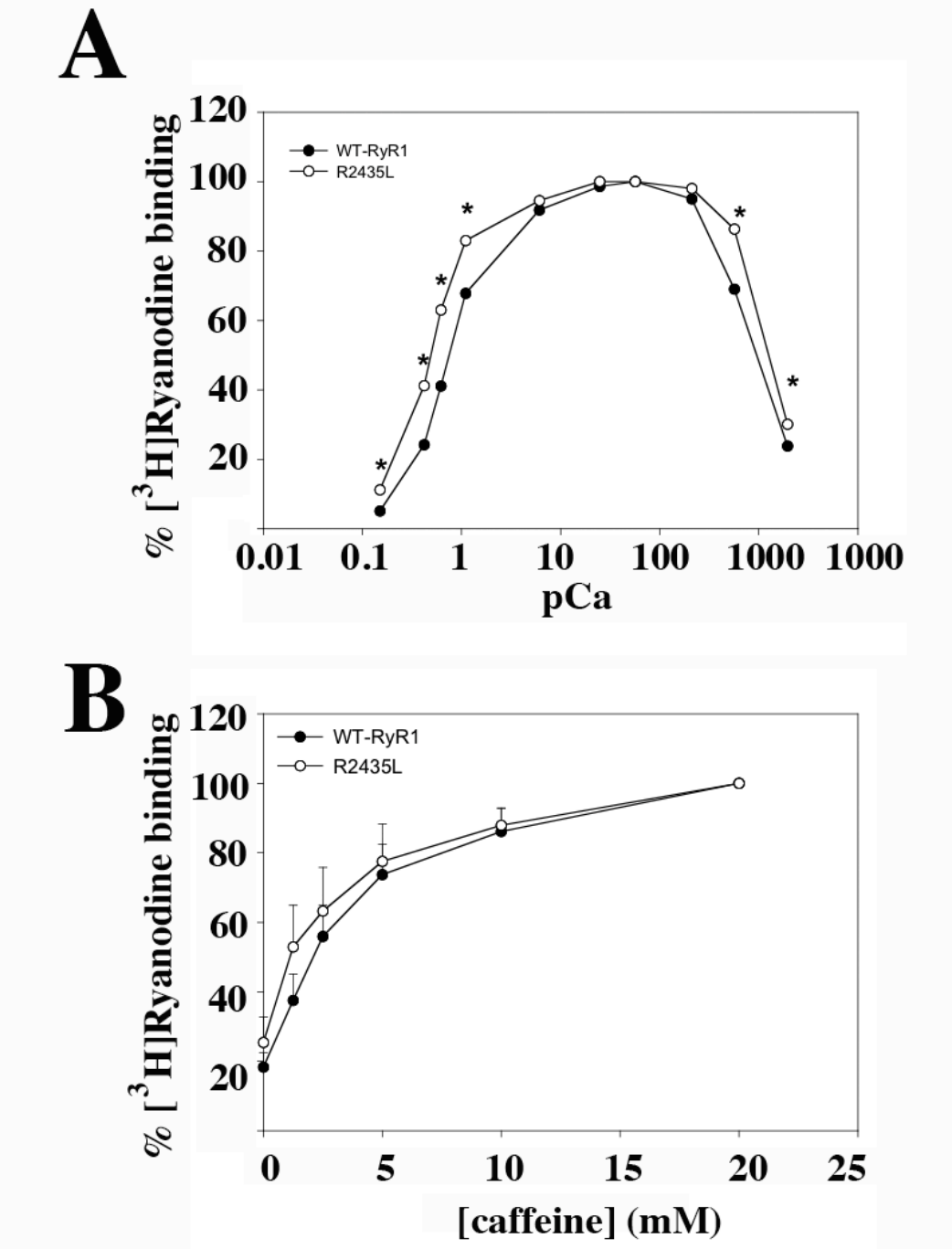
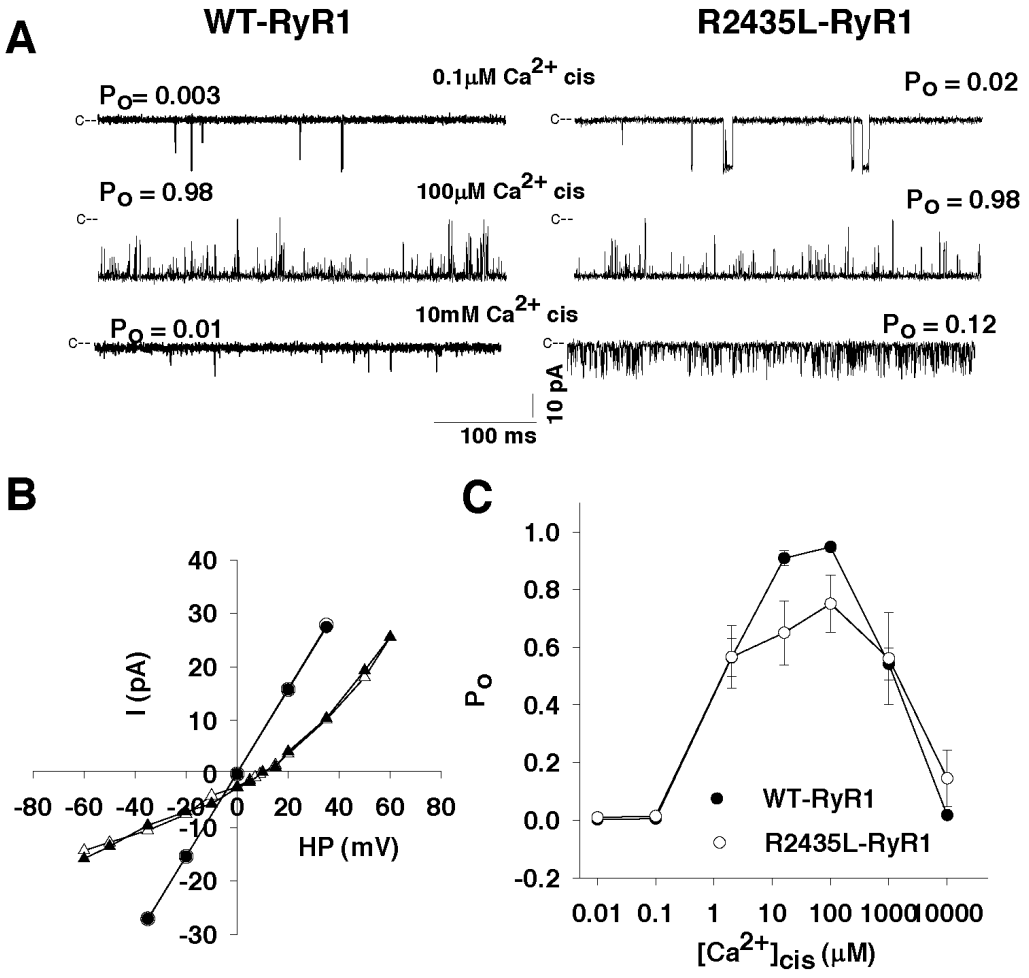

A
% [3H]Ryanodine binding
120
100
80
60
40
20
WT-RyR1
R2435L
*
0.01
0.1
1
10
100
1000
1000
pCa
B
% [3H]Ryanodine binding
120
100
80
60
40
20
WT-RyR1
R2435L
0
5
10
15
20
25
[caffeine] (mM)

Figure 3


A
WT-RyR1
R2435L-RyR1
0.1μM Ca2+ cis
Po= 0.003
Po = 0.02
100μM Ca2+ cis
Po = 0.98
Po = 0.98
10mM Ca2+ cis
Po = 0.01
Po = 0.12
10 pA
100 ms
B
I (pA)
HP (mV)
C
Po
WT-RyR1
R2435L-RyR1
[Ca2+]cis (μM)