

**Functional properties of ryanodine receptors carrying 3 amino acid substitutions identified in patients affected by multi-minicore disease and central core disease, expressed in immortalised lymphocytes.**

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**Running title:** Functional impact of three novel ryanodine receptor 1 substitutions

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## **ABSTRACT**

More than 80 mutations in the skeletal muscle ryanodine receptor gene have been found to be associated with autosomal dominant forms of malignant hyperthermia and central core disease and recessive forms of multi-minicore disease. Studies on the functional effect of pathogenic dominant mutations have shown that they mostly affect intracellular calcium homeostasis, either by rendering the channel hypersensitive to activation (malignant hyperthermia) or by altering the amount of calcium released subsequent to physiological or pharmacological activation (central core disease). In this report we present for the first time data on the functional effect of 2 recently identified recessive ryanodine receptor 1 amino acid substitutions, P3527S and V4849I as well as that of R999H, another substitution identified in two siblings affected by multi-minicore disease. We studied the intracellular calcium homeostasis of EBV-transformed lymphoblastoid cells from the affected patients, their healthy relatives and control individuals. Our results show that the P3527S substitution at the homozygous state affects the amount of calcium released after pharmacological activation with 4-chloro-m-cresol and caffeine but did not affect the size of the thapsigargin sensitive  $\text{Ca}^{2+}$  stores. The other substitutions had no effect on either the size of the intracellular calcium stores, or on the amount of calcium released after ryanodine receptor activation, however both the P3527S and V4849I had a small but significant effect on the resting  $[\text{Ca}^{2+}]$ .

## INTRODUCTION

In skeletal muscle, calcium is a key second messenger of the excitation-contraction mechanism and the sarcoplasmic reticulum (SR) is the intracellular organelle involved in its regulation. The SR is endowed with numerous proteins involved in calcium handling, such as calsequestrin, a low affinity  $\text{Ca}^{2+}$  binding protein which functions as an intracellular  $\text{Ca}^{2+}$  pool, the ryanodine receptor (RYR) which functions as the  $\text{Ca}^{2+}$  release channel and the ER/SR  $\text{Ca}^{2+}$  pump (SERCA), involved in pumping the released  $\text{Ca}^{2+}$  back into the lumen of the SR [1-3]. RYRs play a key role in  $\text{Ca}^{2+}$  homeostasis since they function as calcium release channels through which luminal calcium is released thereby contributing actively to the elevation of the myoplasmic  $[\text{Ca}^{2+}]$ , which is necessary for muscle contraction [4-6]. In view of its important role as a second messenger, the intracellular  $[\text{Ca}^{2+}]$  is finely regulated and any alteration in the proteins involved in  $\text{Ca}^{2+}$  handling can potentially lead to pathological conditions. Examples of diseases linked to a dysregulation of  $\text{Ca}^{2+}$  homeostasis include Malignant Hyperthermia (MH, OMIM 145600), Central Core Disease (CCD, OMIM 117000), Multi-minicore Disease (MmD OMIM 602771) and Brody's disease (OMIM 601003)[7-9]. The precise mechanism by which genetic alterations of these proteins trigger different pathophysiological patterns remains to be elucidated.

MmD is an autosomal recessive congenital myopathy characterized histologically by the presence of multiple cores, which are areas devoid of mitochondria, thus lacking oxidative enzymes and with highly disorganized sarcomeric structures. Multiple small cores can occur in both type 1 and type 2 fibres [10,11] and do not run the entire length of the muscle fibre. In contrast, the histological pattern observed in biopsies from CCD patients is characterised by fibre type uniformity, with strong predominance of type 1 fibres, core lesions with clearly defined borders, occurring exclusively in type 1 fibres and running the entire length of the muscle fibre [12-14]. Both MmD and CCD are characterized by hypotonia during infancy, muscle weakness, delayed motor development; however, they differ in their pattern of muscle weakness and modes of inheritance. Clinical and histopathological overlap between CCD and MmD can occur [15,16], and were recently reported in two families where patients carried recessive homozygous *RYR1* mutations [17,18].

The ryanodine receptor type 1 gene (*RYR1*), composed of 106 exons, maps to chromosome 19q13.1 and encodes for a protein of 5038 amino acids [19,20]. More than 80

mutations in *RYR1* have been genetically linked to MH and CCD [21,22]. Disease-causing mutations appear to cluster in 3 defined regions of the *RYR1*: the cytoplasmic N-terminal domain 1 (Cys35-Arg614; region 1), the cytoplasmic central domain (Asp2129-Arg2458; region 2) and the C-terminal hydrophobic domain (Ile3916-Ala4942; region 3). In Malignant hyperthermia, a pharmacological disorder triggered by exposure to volatile anaesthetics and/or muscle relaxants in genetically predisposed individuals [23-25], disease-linked mutations predominantly cluster in regions 1 and 2, whereas in CCD most *RYR1* substitutions occur in the hydrophobic pore forming region 3. MmD is genetically heterogeneous; more than 50% of the cases presenting with the “classical” MmD phenotype harbour causative mutations in the selenoprotein N gene (*SEPN1*) [26]; however, a significant proportion of the remaining cases carry recessive *RYR1* mutations, distributed over all the gene.

Previous studies have demonstrated that EBV-immortalized human B-lymphocytes express the skeletal muscle isoform of the RYR [27,28] and that they could be used as a model to test the effect of mutations on RYR function. In order to shed light on the functional impact of *RYR1* mutations linked to MmD, in the present report we investigated the influence of naturally occurring *RYR1* mutations identified in patients with MmD and/or CCD, on intracellular Ca<sup>2+</sup> homeostasis and pharmacological RYR activation, of EBV-immortalized B-lymphocytes. The clinical genotypes and phenotypes of the two MmD cases have been described previously [17, 18] and are linked to the *RYR1* mutations P3527S and V4849I. We also included in this study the functional effect of the R999H *RYR1* substitution, which was identified at the heterozygous state, in a family in which two siblings were affected by MmD/CCD.

## EXPERIMENTAL PROCEDURES

### MATERIALS

Thapsigargin and fura-2/AM were from Calbiochem. Caffeine was from Merck (Darmstadt, Germany), 4-chloro-*m*-cresol was from Fluka Chemicals (Buchs, Switzerland). ULTRASPEC RNA isolation system was from Biotecx laboratories (Houston, TX, U.S.A.). cDNA synthesis kit and *Taq* polymerase were from Roche Molecular Biochemicals. Tissue culture media and reagents were from Invitrogen. All other chemicals were reagent or the highest available grade.

### METHODS

#### Patients

As *SEPN1*-mutations have been identified in 50% of classical severe MmD, haplotyping studies were first performed for every family and *SEPN1* was clearly excluded as a candidate gene (data not shown).

Family 1: the P3527S substitution resulting from a C>T transition at position 10579 in exon 71 of the *RYR1*, was identified in 3 siblings from an Algerian consanguineous family [17]. In the present study, we immortalized lymphoblastoid cells from 2 of the affected daughters carrying the homozygous mutation and their healthy mother, heterozygous for this mutation. This mutation therefore, behaved as a recessive trait. In early childhood, minicores were observed in the biopsies of the siblings but when the probands were biopsied again during adult life, their muscle showed a typical CCD pattern. Patients were classified as recessive CCD with transient morphological presentation as MmD.

Family 2: the V4849I substitution resulting from a G>A transition at position 14545 in exon 101 of the *RYR1*, was identified in the homozygous state in the affected daughter and in the heterozygous state in her healthy parents [18]. A muscle biopsy in the proband revealed marked type 1 predominance with presence of few minicores and central cores. A muscle MRI showed the typical skeletal muscle involvement that has been associated in patients with CCD. A diagnosis of CCD secondary to a recessive *RYR1* mutation was made.

Family 3: the R999H substitution resulting from a G>A transition at position 2996 in exon 24 of the *RYR1* gene, was identified in the heterozygous state in a patient classified as MmD, with short cores present only in type 1 fibres. Her brother was mildly affected, also carried the mutation in the heterozygous state and presented short cores in both fibre types. The mother

had a normal CT scan, no signs of any neuromuscular disorder, but was later found to also harbour the R999H substitution at the heterozygous state.

### **Lymphoblastoid cell lines**

Heparinized peripheral blood was obtained from healthy control individuals, family members of patients and patients with core myopathy carrying proven *RYR1* mutations. Mononuclear cells were isolated and transformed with Epstein Barr Virus according to the protocol of Neitzel [29]. Cells were cultured in RPMI medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate and 100 Units of penicillin and streptomycin.

### **Mutation screening**

Total RNA was isolated using an RNA isolation kit. RNA was converted into cDNA using a commercially available kit following the manufacturers instructions. Approximately 100 ng of cDNA were used for each PCR amplification using a 2720 Thermal cycler (Applied Biosystem). The following primes were used to amplify cDNA: 24F 5'-TGGACCGTCTGGCAGAAAATG-3', 24R 5'GGTCAGGAGGCTCGATGTTGTA-3'; 71F 5'-TCCGGTGGCTCGGACCAGGAA-3', 71R 5'-TTGGCCAGCGTGATGAGGTCTT-3'; 101F 5'-ACCTGGCCCCATCCTG-3', 101R 5'-GCTAGGGGAGGGGCTCAC-3'. Amplification conditions were: 5 min 95 °C followed by 40 cycles: 30 s annealing at 56 °C (24F/R), 71 °C (71F/R) and 60 °C (101 F/R), 60 s extension at 72 °C and 30 sec denaturation at 94 °C, followed by a final extension for 3 min at 72 °C. The presence of the nucleotide substitutions was detected by restriction enzyme digestion using *Bst* *UI*, *Hha* *I* or *Acc* *I*. In order to demonstrate that the cDNA preparations used for PCR amplification were not contaminated by genomic DNA, a PCR reaction spanning exons 39-40 was carried out as previously described [28].

### **Intracellular calcium measurements**

Changes in the intracellular  $\text{Ca}^{2+}$  concentration,  $[\text{Ca}^{2+}]_i$ , of the lymphoblastoid cells were monitored with the fluorescent  $\text{Ca}^{2+}$  indicator fura-2. Experiments were carried out on populations of cells, in an LS-50 Perkin Elmer spectrofluorimeter as previously described [28,30,31] or at the single cell level by digital imaging microscopy as previously described [32]. In the latter case, lymphoblastoid cells loaded with 5  $\mu\text{M}$  fura-2 were allowed to attach to poly-L-lysine treated glass coverslips for 10 minutes prior to the experiments. Individual cells were stimulated with a 12-way 100 mm diameter quartz micromanifold computer controlled

microperfuser (ALA Scientific) as described [32]. 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 40x oil-immersion Plan-NEOFLUAR objective (0.17 NA), filters (BP 340/380, FT 425, BP 500/530). The 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.

### **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. The EC<sub>50</sub> and Rmax values were calculated using the Origin program from sigmoidal curve fitting of all the data points.

## RESULTS

### *RYR1-mutation analysis*

The EBV-immortalised lymphoblastoid cells were analysed by RT-PCR to verify the presence of the different mutations at the transcriptional level. Figure 1 shows the results after digestion of the PCR amplified cDNA obtained from the lymphoblastoid cell lines from controls or 3 probands, each carrying one of the identified mutations. The primers used to amplify the cDNA fragment depicted in figure 1A span exons 39-40 and the size of the amplified band (338 bp) corresponds to that of the cDNA, since the corresponding genomic sequence would encompass intron 39 and yield a fragment of 1400 bp. The G<sub>2996</sub>>A substitution in exon 24 abolishes a Bst UI restriction site, resulting in the presence of four bands of 127, 93, 47 and 46 bp at the heterozygous state and of three bands of 127, 47 and 46 bp in controls (figure 1B, lanes 1-4). The C<sub>10579</sub>>T substitution in exon 71 abolishes a Hha I restriction site, resulting in the presence of two bands of 118 and 38 bp after digestion of the amplified cDNA from the homozygous proband with Hha I and of three bands of 86, 38 and 32 bp after digestion of the amplified cDNA from controls (fig.1 B, lanes 5-8). The G<sub>14545</sub>>A substitution in exon 101 abolishes an Acc I restriction site. After digestion, the amplified cDNA from the homozygous carrier remains uncut (176 bp fragment), whereas digestion of the amplified cDNA from controls yields two bands of 118 and 58 bp (figure 1B, lanes 9-12).

### *Resting $[Ca^{2+}]_i$ and status of the intracellular $Ca^{2+}$ stores in lymphoblastoid cells carrying RYR1 substitutions*

The aim of this work was to establish the characteristics of the intracellular  $Ca^{2+}$  pools of lymphoblastoid cells carrying the RYR1 substitutions R999H, P3527S and V4849I. To this end, we first analysed the resting  $[Ca^{2+}]_i$  of lymphoblastoid cells from mutation carriers and compared it to that observed in cells from control individuals (fig. 2). The resting fluorescence intensity observed in cells from patients harbouring the R999H substitution was not significantly different from that observed in control cells, while the presence of the other substitutions caused a small but significant increase in fluorescence intensity ratio ( $P < 0.04$ ; ANOVA). In terms of  $[Ca^{2+}]_i$ , the observed increases are of the order of 10-30 nM.

We next examined the status of the intracellular  $Ca^{2+}$  pools by comparing the peak  $[Ca^{2+}]_i$  obtained after addition of the SERCA inhibitor thapsigargin, in the absence of external



calcium (fig. 3A). This treatment causes the release of the  $\text{Ca}^{2+}$  present in the intracellular pool into the cytoplasm; since  $\text{Ca}^{2+}$  cannot be pumped back into the ER and no  $\text{Ca}^{2+}$  is present in the extracellular medium, the peak fluorescence obtained reflects the size of the rapidly releasable intracellular  $\text{Ca}^{2+}$  stores [27,30,31]. When cells from controls or mutation-bearing individuals were treated with 400 nM thapsigargin, no significant differences were observed in the amount of  $\text{Ca}^{2+}$  released, suggesting that none of the mutations affect the size of the thapsigargin-sensitive intracellular stores (fig. 3 B).

#### *Sensitivity of lymphoblastoid cell lines to pharmacological activation with RYR agonists*

In the next set of experiments, we tested the sensitivities of lymphoblastoid cells to the RYR1 agonists 4-chloro-m-cresol (4-cmc) and caffeine. We first performed dose-response curves to 4-cmc by studying changes in  $[\text{Ca}^{2+}]_i$  in cell populations, in  $\text{Ca}^{2+}$ -free Krebs Ringer and expressed these as % release with respect to the thapsigargin-sensitive pool (set as 100 %). 4-cmc is a specific activator of the ryanodine receptor and can be used pharmacologically to discriminate between MHN and MHS [33-35]; when *RYR1*-mutations are associated with the MHS phenotype, the sensitivity of the receptor to 4-cmc is increased [28,34]. Figure 4 shows the dose response curves to 4-cmc in lymphoblastoid cells from control individuals, from individuals harbouring the R999H substitution and from individuals harbouring the P3527S and V4849I substitutions at the homozygous and heterozygous states. None of the substitutions caused a significant shift in the dose response curve to lower agonist concentration, while the P3527S substitution at the homozygous state, caused a small but significant reduction in the amount of  $\text{Ca}^{2+}$  released by 4-cmc (the % maximal releases were  $77.6 \pm 3.0$  and  $89.3 \pm 3.3$  for homozygous P3527S and controls, respectively. Students' *t* test  $P < 0.02$ ).

In order to gain more information on how different *RYR1* mutations may affect the response to different pharmacological activators and since under our experimental conditions in cell populations we were unable to detect caffeine-induced changes in  $[\text{Ca}^{2+}]_i$ , we next examined the  $\text{Ca}^{2+}$  response of lymphoblastoid cells at the single cell level. Individual cells were stimulated by a pressure pulse of either 4-cmc or caffeine and the release of  $\text{Ca}^{2+}$  was assessed by calcium imaging. This technique is more sensitive and allowed us to detect changes in the  $[\text{Ca}^{2+}]_i$  even after addition of caffeine (fig. 5); it also helped us monitor the proportion of cells responding to a particular concentration of agonist. Figures 6 and 7 show

the 4-cmc and caffeine dose-response curves obtained from control cells and from cells from mutation-bearing individuals. These curves were generated taking into consideration only cells responding to the added agonist: using these values, only the P3527S homozygous mutation caused a reduction in the amount of  $\text{Ca}^{2+}$  released by 4-cmc while none of the substitutions caused a significant change in the maximal amount of  $\text{Ca}^{2+}$  released by caffeine (figure 6 and 7, compare curve in panel A with those in panels B-F). The sensitivities to 4-cmc and caffeine and maximal change in  $[\text{Ca}^{2+}]_i$  induced in lymphoblastoid cells bearing the different substitutions are shown in Table I. The maximal fluorescence change ( $R_{\text{max}}$ ) and  $\text{EC}_{50}$  were calculated from the data shown in figures 6 and 7 and the given values were calculated using the Origin program for sigmoidal curve generation. The results show: (i) that none of the substitutions significantly reduced the sensitivity of the RYR1 to activation to both caffeine and 4-cmc, while in some cases there was a shift to higher agonist concentrations; (ii) the  $R_{\text{max}}$  value for 4-cmc induced  $\text{Ca}^{2+}$  release was only different for the P3527S homozygous carriers and (iii) the  $R_{\text{max}}$  values obtained for caffeine and 4-cmc were of comparable magnitude. Table II compares the % responding cells at each concentration of 4-cmc and caffeine in lymphoblastoid cells carrying different substitutions. It is apparent (i) that the % cells responding to 4-cmc was always higher than that responding to caffeine; (ii) that the number of cells harbouring the homozygous substitution P3527S responding to any concentration of caffeine was always lower than that from controls or from carriers of the two other substitutions. If the cumulative change in fluorescence of all analysed cells was taken into account (i.e. responding and non-responding cells), only the P3527S cells bearing the homozygous substitutions showed a significantly lower peak fluorescence change in response to 4-cmc and caffeine (mean values  $\pm$  s.e.m. were  $0.11 \pm 0.03$  and  $0.05 \pm 0.02$  for P3527S homozygous carriers vs  $0.23 \pm 0.04$  and  $0.12 \pm 0.03$  for controls, respectively.  $P < 0.05$  Student's  $t$  test).

## DISCUSSION

During the past decade a number of reports dealing with mutations in the skeletal muscle *RYR1*  $\text{Ca}^{2+}$  channel, their genetic association to neuromuscular disorders and impact on protein function have appeared [36-39]. While it is well established that most MH-causing mutations affect the RYR1 by making it hypersensitive to activating substances, and CCD-causing mutations affect the amount of  $\text{Ca}^{2+}$  released after activation, not much is known about the functional effect of *RYR1* mutations leading to MmD. In the present report, we investigated the functional effect of three amino acid substitutions linked to a mixed MmD/CCD phenotype [17,18] by studying the  $\text{Ca}^{2+}$  homeostasis of lymphoblastoid cells established from the patients. Two of these were clearly inherited as recessive mutations. Under our experimental conditions, only the P3527S substitution at the homozygous state significantly affected intracellular  $\text{Ca}^{2+}$  homeostasis. Our results show that unlike many CCD-linked mutations, the presence of this mutation did not affect the size of the intracellular  $\text{Ca}^{2+}$ -pools, but rather affected the total amount of  $\text{Ca}^{2+}$  released by pharmacological activation of the RYR. This finding implies that (i) the presence of the P3527S substitution on one allele alone is not sufficient to significantly alter the functional properties of the RYR; (ii) the homozygous substitution does not diminish the size of the  $\text{Ca}^{2+}$  stores and therefore does not cause the channel to become leaky; (iii) its presence causes a decrease in the maximal amount of  $\text{Ca}^{2+}$  released by 4-chloro-m-cresol and caffeine. In view of the fact that the substituted residue lies in the vicinity of binding sites for calmodulin and S100 [40,41], two proteins which enhance channel opening and promote  $\text{Ca}^{2+}$  release [42,43], and based on the fact that proline, an amino acid known to disrupt  $\alpha$ -helices, is substituted by a residue having a polar uncharged side chain, one may hypothesise that the P3527S substitution interferes with the binding of accessory proteins involved in stabilizing the RYR in the open state in the native tetrameric (in cells heterozygous for the mutation) conformation and that the presence of wild type channels within the tetramer are sufficient to bind regulatory proteins and therefore allow “normal“  $\text{Ca}^{2+}$  release after activation. Alternatively, the decrease of calcium released after the addition of 4-chloro-m-cresol, may reflect the fact that the mutation lies in proximity of the binding site for this agonist [44].

As to the other two substitutions, under our experimental conditions we found that channels carrying the R999H substitution behave like their wild type counterpart both in terms of resting  $[\text{Ca}^{2+}]$ , and total amount of  $\text{Ca}^{2+}$  released after RYR1 activation. We would like to

point out that genetic investigation into the family carrying the R999H substitution revealed that also the unaffected mother (with normal clinical examination and muscle CT scan) was a carrier of this substitution. Since the entire *RYR1* of the proband was sequenced and found to contain no other amino acid substitutions, it is unlikely that this substitution alone is pathological at the heterozygous state and substitutions in other genes must be responsible for the MmD/CCD phenotype. As far as the V4849I mutation is concerned, lymphoblastoid cells carrying this substitution at the homozygous and heterozygous states behaved like wild type cells in terms of  $\text{Ca}^{2+}$  store content and total amount of  $\text{Ca}^{2+}$  released after pharmacological RYR activation; however they exhibited a small but significant increase in their resting  $[\text{Ca}^{2+}]$ . Sambuughin et al. [45] recently reported that the V4849I substitution may be causative of MH since it was found in one proband with a very strong IVCT. On the other hand, Monnier et al. reported that this same substitution was also found in a control individual [46]. Our data show that cells carrying the V4849I substitution do not show an increased sensitivity to either 4-chloro-m-cresol, or caffeine, thus it is unlikely to be causative of MH when present (alone) either at the heterozygous and homozygous states. We are aware that lymphoblastoid cells do not express all the proteins which interact with the RYR1 in the sarcoplasmic reticulum, and we do not think that the conserved V4849I residue is involved in protein-protein interaction since it is predicted to be within a hydrophobic transmembrane domain (TM8) [19,47]. However, since the presence of this substitution affects the resting  $[\text{Ca}^{2+}]$  it may influence channel function in a way that could not be detected by our system.

An interesting observation emerging from the present report concerns the response of the lymphoblastoid cells to caffeine. We had previously attempted to obtain changes in  $[\text{Ca}^{2+}]$  in response to caffeine in populations of lymphoblastoid cells however, the results were not reproducible. In light of the results obtained on single cell measurements, we suggest that only a proportion of lymphoblastoid cells respond to caffeine; since spectrofluorimetric measurements average changes in  $[\text{Ca}^{2+}]$  occurring in millions of cells, if fewer than 50% of the cells respond synchronously, no increase in fluorescence can be observed. Imaging analysis revealed that the number of cells responding to different concentrations of caffeine was <50%, while that responding to 4-chloro-m-cresol was always higher, even though the cells were viable and fluorescent. Furthermore, both agonists induced comparable global increases in  $[\text{Ca}^{2+}]$  in responding cells. We do not know the reason for this but it may reflect the quenching

effect of caffeine on fura-2 fluorescence as well as the fact that the lymphoblastoid cell lines are of a polyclonal nature. The results obtained averaging all cells stimulated with 4-chloro-m-cresol and caffeine are consistent, and demonstrate that only the P3527S mutation causes significant alterations in  $\text{Ca}^{2+}$  homeostasis. The decrease in maximal amount of  $\text{Ca}^{2+}$  released after pharmacological activation was observed in cell lines established from two individuals carrying the same homozygous substitution and in none of the other cell lines thus we think that it is unlikely to reflect the selection of particular clones of cells.

One of the questions remaining to be answered is how *RYR1* mutations differently modify intracellular  $\text{Ca}^{2+}$  dynamics and contribute to the pathophysiology of MH, CCD and MmD. In MH-susceptible individuals, most mutations shift the sensitivity of the receptor to lower agonist concentration and the clinical signs translate to a hypermetabolic state triggered by a hyperactive RYR. In CCD, the function of the RYR calcium channel is altered either because mutations cause the channels to become excessively leaky or because they become unable to transport  $\text{Ca}^{2+}$  efficiently [36,37, 39]. The present results suggest that at least as far as the MmD-linked P3527S recessive mutation is concerned, the RYR channels do not become leakier but rather transport less  $\text{Ca}^{2+}$  upon activation. Single channel recording experiments will be important in order to confirm whether this mutation renders the channel unstable in the open state.

In conclusion, the present data strongly support a causative role for the P3527S *RYR1* mutation at the homozygous level for the CCD/MmD phenotype; on the other hand our results show that the functional properties of *RYR1* carrying the R999H substitution are not different from controls and are most likely not causative of CCD/MmD. As to the V4849I substitution, our results show that its presence does not cause a shift in the sensitivity to pharmacologic activation of the ryanodine receptor, or cause alterations in the amount of thapsigargin, 4-chloro-m-cresol and caffeine-induced calcium release. However, since its presence affected the resting calcium concentration, the V4849I substitution may perturb the function of the ryanodine receptor channel in a way which was not discernable in the present study. Finally, these results confirm that lymphoblastoid cells can be used as a tool to study the effects of causative mutations vs polymorphisms among *RYR1* substitutions, however one must keep in mind that these cells do not express all the proteins of the skeletal muscle sarcoplasmic reticulum involved in calcium homeostasis.

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**Abbreviations:**

[Ca<sup>2+</sup>]<sub>i</sub>, intracellular calcium concentration; CCD, central core disease; MH, malignant hyperthermia; MmD, multiminicore disease; RYR, ryanodine receptor;

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**Table I:**  $R_{max}$  and  $EC_{50}$  for 4-chloro-m-cresol and caffeine activation of calcium release from control and *RYR1* carrying the indicated amino acid substitutions.

Cell type	$R_{max}$ 4cmc	$EC_{50}$ 4cmc ( $\mu$ M)	$R_{max}$ caffeine	$EC_{50}$ Caffeine (mM)
WT	0.36 $\pm$ 0.03	136 $\pm$ 33	0.33 $\pm$ 0.03	1.08 $\pm$ 0.33
Heterozygous R999H	0.37 $\pm$ 0.14	191 $\pm$ 27	0.34 $\pm$ 0.02	1.40 $\pm$ 0.27
Homozygous P3527S	0.23 $\pm$ 0.04*	75 $\pm$ 18	0.43 $\pm$ 0.04	1.21 $\pm$ 0.18
Heterozygous P3527S	0.34 $\pm$ 0.04	157 $\pm$ 21	0.40 $\pm$ 0.03	1.56 $\pm$ 0.47
Homozygous V4849I	0.36 $\pm$ 0.05	184 $\pm$ 23	0.36 $\pm$ 0.03	1.39 $\pm$ 0.31
Heterozygous V4849I	0.47 $\pm$ 0.05	204 $\pm$ 36	0.43 $\pm$ 0.03	1.94 $\pm$ 0.29

Values represent the mean $\pm$ s.e.m. (the number of cells analysed is given in the legend to figures 6 and 7).

\*  $P < 0.009$  Student's *t* test.

**Table II:** Number of lymphoblastoid cells carrying *RYR1* substitutions, responding to different concentrations of caffeine and 4-chloro-m-cresol.

	<b>Responding cells (% of total)</b>				
<b>[Caffeine] (mM)</b>	<b>0.5 mM</b>	<b>2.5 mM</b>	<b>5 mM</b>	<b>10 mM</b>	<b>20mM</b>
<b>Control</b>	21.6%	21.3%	25%	29.2%	22.9%
<b>R999H hetero</b>	18.1%	21.7%	25%	14.3%	17%
<b>P3527S homo</b>	11.6%	14.9%	10.6%	10%	17.6%
<b>P3527S hetero</b>	20.1%	33%	22.4%	20%	18.4%
<b>V4849I homo</b>	26.3%	43.8%	40%	18.3%	17.8%
<b>V4849I hetero</b>	20.2%	35.6%	14.6%	18.9%	19.2%

	<b>Responding cells (% of total)</b>				
<b>[4cmc] (µM)</b>	<b>75µM</b>	<b>150µM</b>	<b>300µM</b>	<b>600µM</b>	<b>1000µM</b>
<b>Control</b>	31.6%	40%	39.3%	61.3%	29.7%
<b>R999H hetero</b>	20.7%	26.7%	47.4%	30.3%	32%
<b>P3527S homo</b>	41.7%	42.9%	38.5%	50%	39.3%
<b>P3527S hetero</b>	29.4%	29.1%	53.8%	50%	22.5%
<b>V4849I homo</b>	35.7%	40%	35%	26.1%	34.8%
<b>V4849I hetero</b>	38.9%	47.6%	60%	57.9%	60%

The number of cells analysed ranged between 15-302.

## FIGURE LEGENDS

### **Figure 1: EBV-immortalised B-cells from patients express a mutated ryanodine receptor.**

Polyacrylamide gels showing PCR-amplification of cDNA using the primer pairs indicated in the Methods section. **Panel A-** amplification of cDNA spanning *RYR1* exons 38 and 40 from control (lane 1), R999H (lane 2), P3527S (lane 3) and V4849I (lane 4) yields a band of 338 bp corresponding to the expected cDNA product, and none from genomic DNA amplification. **Panel B-** Lanes 1-4: amplification of exon 24 of the *RYR1* gene for the R999H mutation. Lanes 1 and 3, undigested cDNA, lanes 2 and 4, cDNA after digestion with BstU I. Lanes 1, 2 control, lanes 3,4 patient harbouring the heterozygous R999H substitution. Lanes 5-8: amplification of exon 71 of the *RYR1* gene for the P3527S mutation. Lanes 5 and 7 undigested cDNA, lanes 6 and 8, cDNA after digestion with Hha I. Lanes 5, 6 control, lanes 7, 8 patient harbouring the homozygous P3527S substitution. Lanes 9- 12: amplification of exon 101 for V4849I mutation. Lanes 9 and 11, undigested cDNA; lanes 10 and 12 cDNA after digestion with Acc I. Lanes 9 and 10 control, lanes 11 and 12, patient harbouring the homozygous V4849I substitution.

**Figure 2: Resting calcium concentration of EBV-immortalised lymphoblastoid cells from control individuals and individuals bearing different *RYR1*-mutations.** Average resting myoplasmic  $[Ca^{2+}]_i$  of cells from controls and individuals bearing the indicated *RYR1* mutations. Values are the mean ( $\pm$ s.e.m. of n= 43-98). Data were determined in fura-2-loaded lymphoblastoid cells ( $1 \times 10^6$  cells/ml) in nominally  $Ca^{2+}$ -free Krebs Ringer supplemented with 0.5 mM EGTA. The  $[Ca^{2+}]_i$  was measured using the fluorescent  $Ca^{2+}$  indicator fura-2. (\*P<0.04).

**Figure 3: Thapsigargin-sensitive  $[Ca^{2+}]_i$  stores of EBV-immortalised lymphoblastoid cells from control individuals and *RYR1*-mutated patients are not significantly different.** (A) Representative trace of the effect of 400 nM thapsigargin on the  $[Ca^{2+}]_i$  of lymphoblastoid cells from a control individual. Conditions as described in fig. 2. Once the steady state was obtained, 400 nM thapsigargin were added where indicated by the arrow. (B) The mean increase in

$[Ca^{2+}]_i$  (=peak ratio 340/380 nm-resting ratio 340/380 nm) induced by the addition of 400 nM thapsigargin was calculated. Results are the mean  $\pm$ s.e.m. of 6-23 experiments.

**Figure 4: Dose dependent 4-chloro-m-cresol induced changes in  $[Ca^{2+}]_i$  in EBV-immortalized lymphoblastoid cells from control individuals and patients carrying different *RYRI* mutations.** The increase in  $[Ca^{2+}]_i$  induced by the indicated concentrations of 4-chloro-m-cresol were calculated as a percentage of the maximal amount which could be released by 400 nM thapsigargin (which was set at 100%). Each point represents the mean ( $\pm$ s.e.m) of the  $\Delta$  fluorescence of 4-13 measurements. Sigmoidal dose response curves were generated using the Origin software. **A:** Control; **B:** heterozygous R999H *RYRI*-substitution; **C and D** P3527S substitution, C: 2 homozygous daughters and D heterozygous mother; **E and F** V4849I mutation, E homozygous daughter, F, heterozygous parents.

**Figure 5: Calcium release stimulated by caffeine in individual EBV-immortalized lymphoblastoid cells from a control individual.** **A,** phase contrast; **B-E,** single cell intracellular  $[Ca^{2+}]_i$  measurements of fura-2-loaded lymphoblastoid cells from control individuals; B, resting  $[Ca^{2+}]_i$ , C, 36 sec, D, 50 sec and E, 77 sec after the application of 10mM caffeine. Cells were individually stimulated by addition of caffeine in Krebs-Ringer medium containing 1 mM  $CaCl_2$ . The trace in the right hand panel is a representative trace obtained after stimulation of a single cell with 5mM caffeine (arrow).

**Figure 6: Changes in  $[Ca^{2+}]_i$  induced by 4-chloro-m-cresol in EBV-immortalized lymphoblastoid cells from control individuals and from patients harbouring different *RYRI* mutations.** Single cell intracellular  $Ca^{2+}$  measurements of fura-2 loaded cells were measured before and after the addition of the indicated concentration of 4-chloro-m-cresol. The curves show the 4-chloro-m-cresol dependent change in  $[Ca^{2+}]_i$ , expressed as  $\Delta$  in fluorescence ratio (peak ratio 340/380 nm-resting ratio 340/380 nm). Each point represents the mean ( $\pm$ s.e.m) of the  $\Delta$  fluorescence of 4-19 measurements. The curve was generated using a sigmoidal dose response curve function included in the Origin software.

**Figure 7: Changes in  $[Ca^{2+}]_i$  induced by caffeine in EBV-immortalized lymphoblastoid cells from control individuals and from patients harbouring different *RYR1* mutations.** Single cell intracellular  $Ca^{2+}$  measurements of fura-2 loaded cells were measured before and after the addition of the indicated concentration of caffeine. The curves show the caffeine dependent change in  $[Ca^{2+}]_i$ , expressed as  $\Delta$  in fluorescence ratio (peak ratio 340/380 nm-resting ratio 340/380 nm). Each point represents the mean ( $\pm$ s.e.m) of the  $\Delta$  fluorescence of 4-15 measurements. The curve was generated using a sigmoidal dose response curve function included in the Origin software.



Figure 1

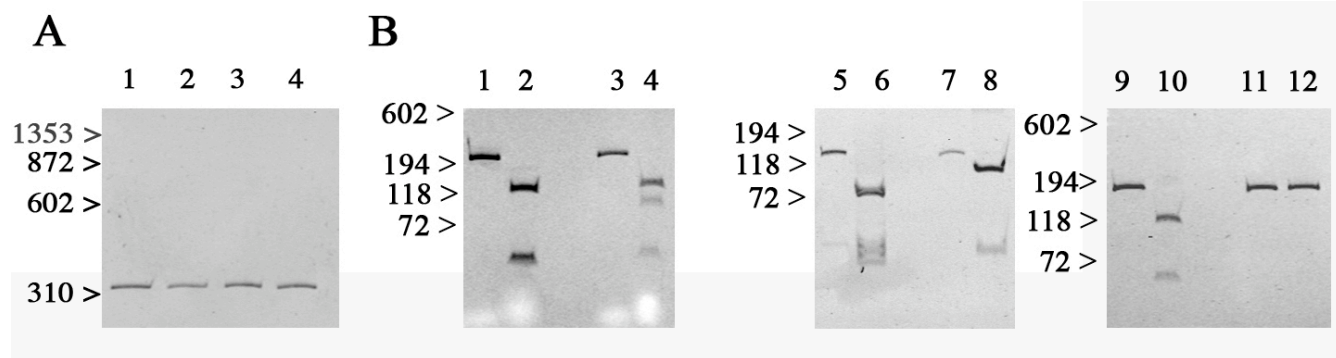


Figure 2

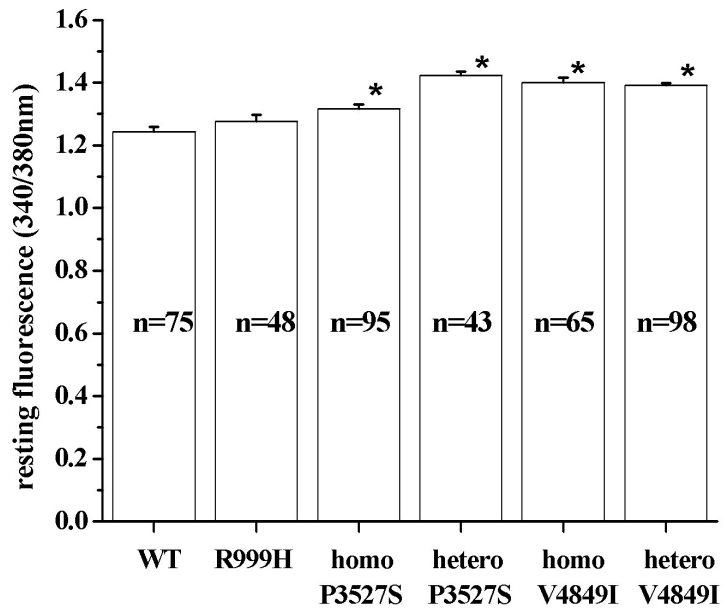


Figure 3

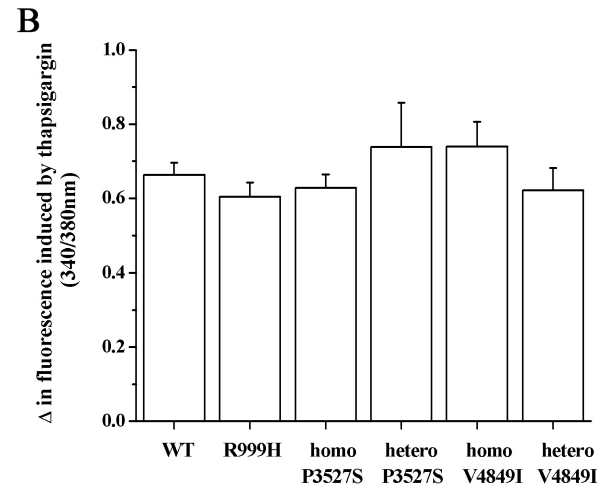
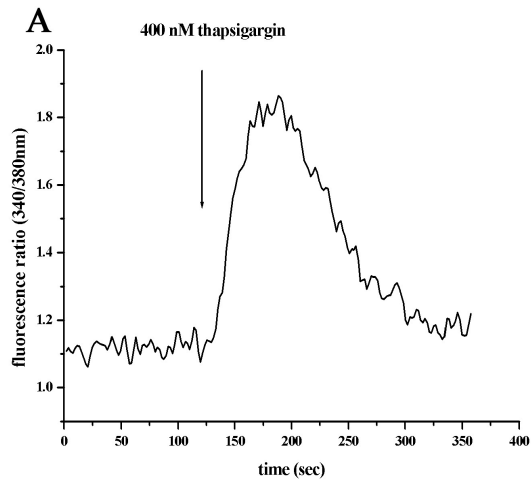


Figure 4

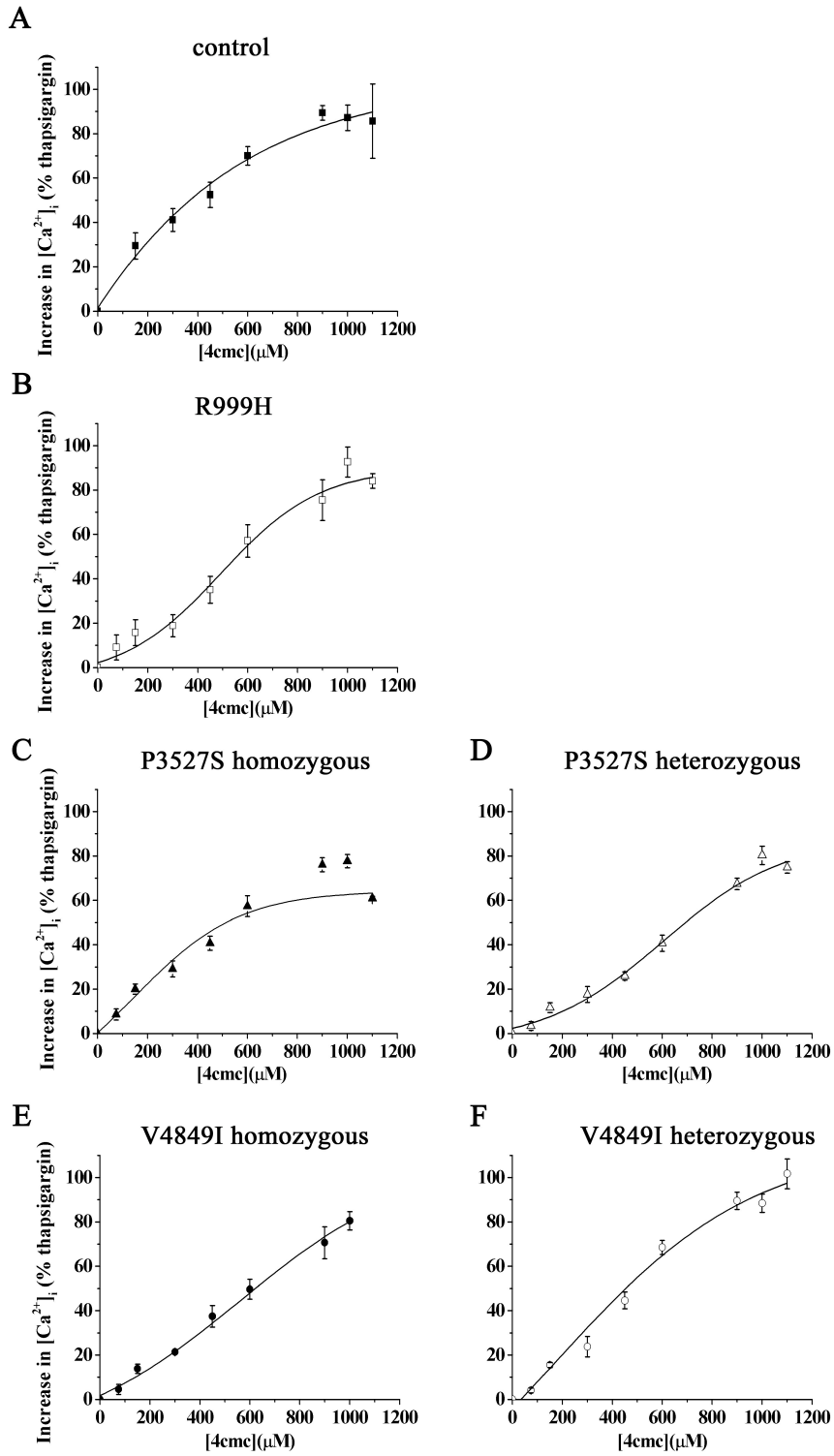


Figure 5

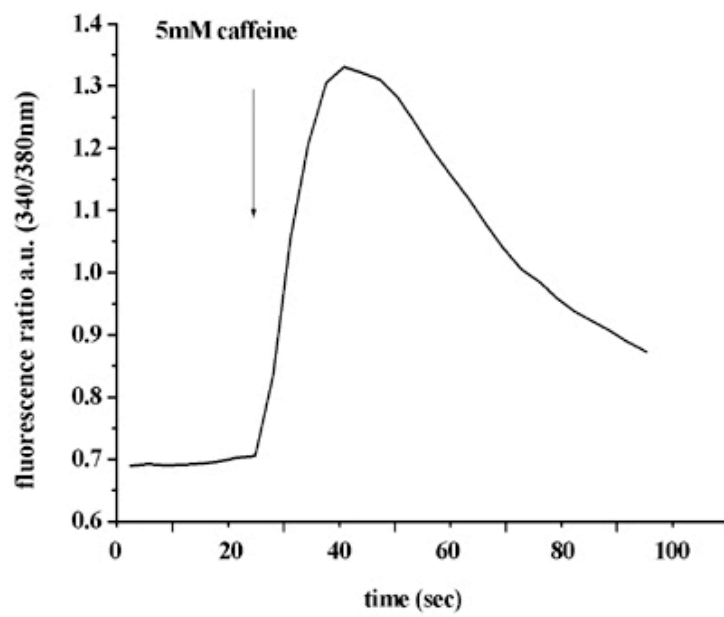
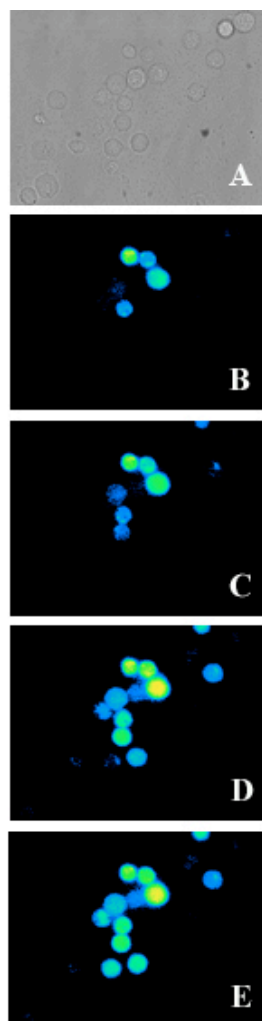
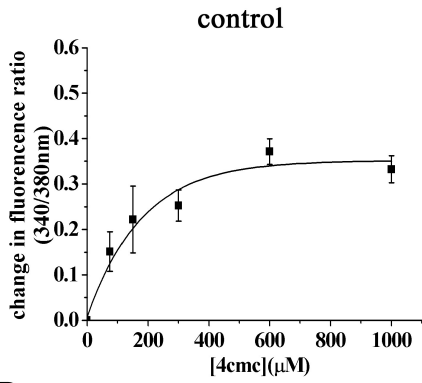
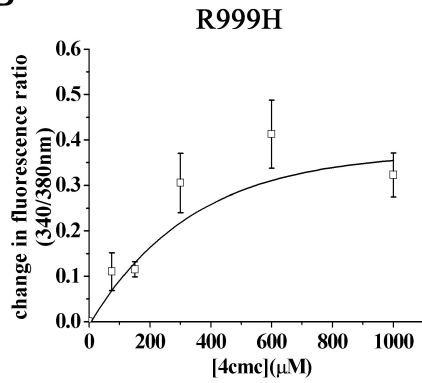


Figure 6

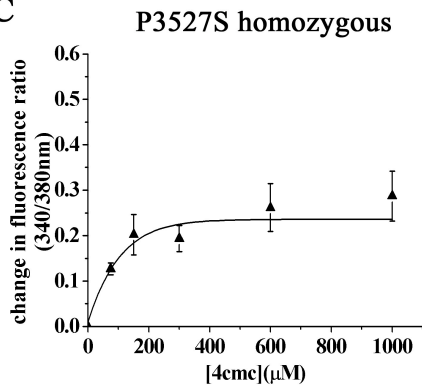
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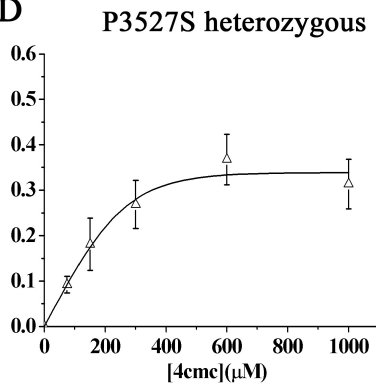
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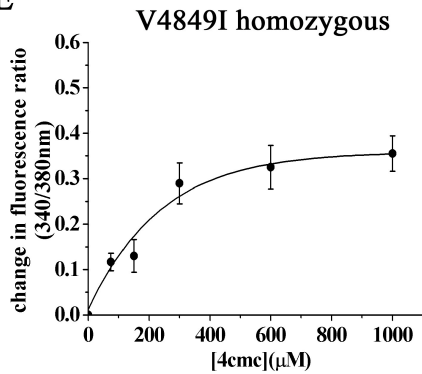
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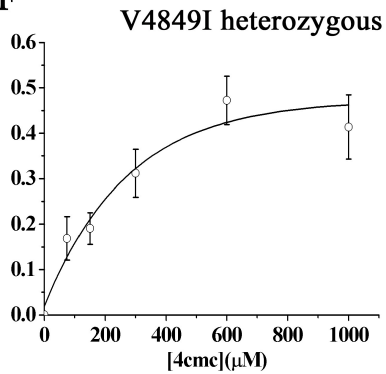


Figure 7

