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Identification of four novel mutations in the C-terminal membrane spanning domain of the ryanodine receptor 1: association with central core disease and alteration of calcium homeostasis

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The skeletal muscle ryanodine receptor gene (*RYR1*; OMIM 180901) on chromosome 19q13.1 encodes the skeletal muscle calcium release channel. To date, more than 25 missense mutations have been identified in *RYR1* and are associated with central core disease (CCD; OMIM 117000) and/or the malignant hyperthermia susceptibility phenotype (MHS1; OMIM 145600). The majority of *RYR1* mutations are clustered in the N-terminal hydrophilic domain of the protein. Only four mutations have been identified so far in the highly conserved C-terminal region encoding the luminal/transmembrane domain of the protein which forms the ion pore. Three of these mutations have been found to segregate with pure or mixed forms of CCD. We have screened the C-terminal domain of the *RYR1* gene for mutations in 50 European patients, diagnosed clinically and/or histologically as having CCD. We have identified five missense mutations (four of them novel) in 13 index patients. The mutations cluster in exons 101 and 102 and replace amino acids which are conserved in all known vertebrate *RYR* genes. In order to study the functional effect of these mutations, we have immortalized B-lymphocytes from some of the patients and studied their $[Ca^{2+}]_i$ homeostasis. We show that lymphoblasts carrying the newly identified RYR1 mutations exhibit: (i) a release of calcium from intracellular stores in the absence of any pharmacological activators of RYR; (ii) significantly smaller thapsigargin-sensitive intracellular calcium stores, compared to lymphoblasts from control individuals; and (iii) a normal sensitivity of the calcium release to the RYR inhibitor dantrolene. Our data suggest the C-terminal domain of RYR1 as a hot spot for mutations leading to the CCD phenotype. If the functional alterations of mutated RYR channels observed in lymphoblastoid cells are also present in skeletal muscles this could explain the predominant symptom of CCD, i.e. chronic muscle weakness. Finally, the study of calcium homeostasis in lymphoblastoid cells naturally expressing RYR1 mutations offers a novel non-invasive approach to gain insights into the pathogenesis of MH and CCD.

INTRODUCTION

Central core disease (CCD) of muscle (OMIM 117000) is a rare congenital myopathy of autosomal dominant inheritance (1,2). Affected individuals present with infantile hypotonia (floppy infant syndrome) and a delay in achieving motor milestones. Later in life, the predominant symptom is a generalized muscle weakness affecting the proximal muscle groups more than the distal ones. The clinical severity is highly variable but the course is usually slow or non-progressive. Additional clinical features may include congenital hip dislocation, kyphoscoliosis, foot deformities and joint contractures (3). The diagnosis is difficult on the basis of clinical findings alone and a histological examination of muscle tissue is essential. Typically, type I fibres predominate and, on cross sections, contain single, well demarcated and centrally located cores, which do not stain

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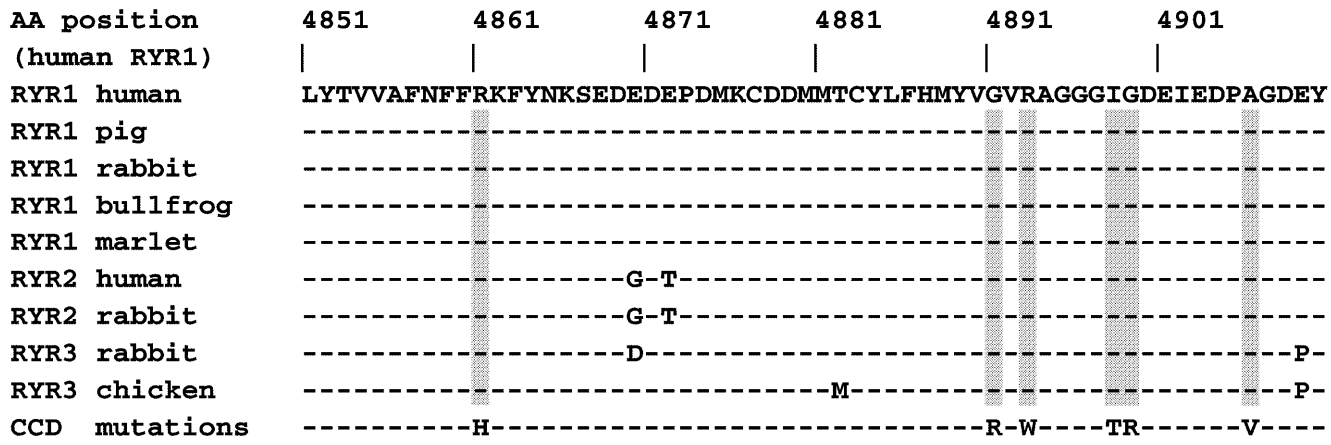

Figure 1. Alignment of the C-terminal amino acid sequences of nine vertebrate RYR genes. Identical amino acids are indicated by a horizontal dash. The last line shows the substitutions found in CCD patients.

with oxidative and phosphorylase histochemical stains. In longitudinal sections the amorphous-looking areas run along the length of the fibres (4,5).

CCD is closely associated with malignant hyperthermia susceptibility (MHS1; OMIM 145600), a pharmacogenetic disease inherited as an autosomal dominant trait. MH susceptible individuals can suffer from adverse reactions to commonly used inhalative anaesthetics and depolarizing muscle relaxants with an acute hypermetabolic crisis which can include skeletal muscle rigidity, tachycardia, hyperthermia, acidosis, cardiac arrhythmias and rhabdomyolysis (6–8). Biochemical studies have demonstrated that MHS is due to abnormalities in skeletal muscle calcium homeostasis (9). Genetic linkage studies have mapped the primary locus of MH on chromosome 19q13.1, to the gene encoding the ryanodine receptor *RYR1* (10,11). However, only ~50 % of MH families have mutations in the *RYR1* gene and linkage to other loci has been reported (12–15).

The ryanodine receptor (RYR1), the calcium release channel of skeletal muscle sarcoplasmic reticulum (SR), is encoded by a gene comprised of 106 exons (16) producing one of the largest known proteins (5038 amino acids) (17,18). The functional channel is composed of four identical subunits of 565 kDa each and has been shown to interact with a number of regulatory proteins. The first 4000 amino acids comprise the hydrophilic cytoplasmic domain which bridges the gap between the transverse tubules and the SR whereas the last 1000 amino acids form the hydrophobic membrane-spanning plate, containing the pore (7,19). To date, more than 25 missense mutations in the *RYR1* gene have been associated with susceptibility to MHS and/or CCD (20). The mutated codons giving rise to CCD and MHS are clustered in three regions of the *RYR1* gene: CCD/MHS region 1 extends from Met1 to Arg614, CCD/MHS region 2 lies between Arg2162 and Arg2458. Both regions are predicted to reside in the myoplasmic foot domain of the protein. CCD/MHS region 3 is located in the transmembrane/luminal region of the highly conserved C-terminal domain. The majority of *RYR1* mutations appear to be clustered in regions 1 and 2 and so far only four mutations have been found in region 3 (21–24).

Recently, Sei *et al.* (25) demonstrated that circulating B-lymphocytes as well as the human B-cell line DAKIKI express a functional type 1 RYR calcium release channel. Following this observation, we have studied the calcium homeostasis in lymphoblastoid cell lines from MHS patients carrying the Val2168Met mutation. Our data indicate that their sensitivity to calcium mobilizing RYR activators were different from those of control individuals (T.Girard, D.Cavagna, E.Padovan, G.Spagnoli, A.Urwyler, F.Zorzato and S.Treves, manuscript submitted for publication). Thus, measurements of the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$ in EBV-immortalized lymphoblastoid cells can be used to assay the function of RYR1 mutations *ex vivo.*

In the present work, we describe five mutations (four of them novel) identified in 13 unrelated individuals by screening the C-terminal domain of *RYR1* in a cohort of 50 patients with clinical CCD. In addition, we demonstrate that four of these mutations lead to a marked disturbance of calcium homeostasis in patients' lymphoblastoid cells: Ca^{2+} is released from intracellular stores in the absence of pharmacological activators of RYR1 leading to smaller thapsigargin-sensitive Ca^{2+} stores whereas the sensitivity of RYR to dantrolene is not impaired. Our results suggest the C-terminal, transmembrane domain of RYR1 as a hot spot for CCD mutations and give evidence for the usefulness of Ca^{2+} release measurements in B-lymphocytes as a novel, non-invasive approach to test RYR1 function *ex vivo*.

RESULTS

RYR1 mutation analysis

We have screened by single strand conformation analysis (SSCA) exons 98–103 of the *RYR1* gene in 50 unrelated patients being diagnosed clinically and/or histologically as having CCD. We concentrated on this region because it is thought to be part of the highly conserved hydrophobic C-terminal domain of the RYR1 protein and was previously found to harbour three CCD-related mutations (21–23). Aberrant fragments were observed in the DNA from seven patients in exon

Table 1. Clinical and molecular data of 25 patients with well documented CCD

Patient identifier	Infantile hypotonia	Delayed motor milestones	Muscle weakness	Progression slow or nil	Skeletal deformity	Central cores	Type I fibre pre-dominance	Nucleotide change	Amino acid change	Familial segregation (no. of affected)	Ref. for pedigree
1	+	+	+			+	+	G14582A	Arg4861His	Yes (3)	36
3	+	+	+	+		+	+	G14582A	Arg4861His	Yes (3)	36
8			+			+	+	G14582A	Arg4861His	Yes (3)	36
19			+			+	+	G14582A	Arg4861His	Yes (2)	This work
22			+		+	+	+	G14582A	Arg4861His	Not testable	This work
26	+		+			+	+	G14582A	Arg4861His	Not testable	This work
31	+	+	+	+		+		G14582A	Arg4861His	Yes (3)	This work
29			+			+		G14671C	Gly4891Arg	Not testable	This work
4			+			+		T14693C	Ile4898Thr	Yes (3)	36
5			+			+		T14693C	Ile4898Thr	Yes (4)	36
49		+	+	+		+	+	T14693C	Ile4898Thr	Yes (2)	This work
13			+			+		G14695A	Gly4899Arg	Yes (3)	This work
36	+	+	+			+		C14717T	Ala4906Val	Not testable	This work
2	+	+	+			+	+	?	?	?	
9			+		+	+	+	?	?	?	
10			+			+		?	?	?	
23			+	+		+	+	?	?	?	
30			+			+		?	?	?	
32			+			+	+	?	?	?	
33		+	+			+		?	?	?	
35			+	+		+	+	?	?	?	
45			+	+	+	+		?	?	?	
47			+		+	+		?	?	?	
50	+		+			+	+	?	?	?	
51		+	+			+	+	?	?	?	

101 and from six patients in exon 102. No variant SSCA patterns were found in exons 98–100 and 103, respectively. Direct sequencing of aberrant PCR fragments revealed a total of five heterozygous sequence variants, all leading to the substitution of single amino acids which are conserved in all known vertebrate *RYR* genes (Fig. 1). The novel mutation Arg4861His was found in five multiplex pedigrees and two simplex cases of German and Austrian origin, respectively. The mutation Ile4898Thr (21) was observed in two Italian and one Swiss–Italian pedigree. The remaining three novel mutations were found only once: Gly4891Arg in one German patient, Gly4899Arg in an Italian multiplex pedigree and Ala4906Val in another German patient. The candidate mutations were shown to be absent in 50 control individuals. In nine pedigrees, affected and unaffected family members of the index cases were available and were screened for the respective mutation by restriction analysis or sequencing. In all instances, the familial mutation segregated perfectly with the disease (Table 1). No relatives of the remaining four index cases were available for study. Thus, in our cohort, there is no case of a proven *de novo* RYR1 mutation.

Functional studies of cells expressing the mutated RYR1 calcium channel

Since circulating B-lymphocytes and EBV-immortalized B-cell lines express a functional type 1 RYR calcium release channel (25), we established cell lines from patients carrying three of the above mutations (Arg4861His, Ile4898Thr and Gly4899Arg) and studied their intracellular Ca^{2+} homeostasis. We were also able to include a cell line from another CCD patient carrying the Arg4893Trp mutation (C.Sewry, unpublished data). The presence of the *RYR1* gene mutation in the cell lines was checked by PCR amplification and restriction enzyme digestion (not shown). We next loaded the cells with the fluorescent calcium indicator fura-2 and studied their $[Ca^{2+}]_i$ in nominally Ca^{2+}-free EGTA-containing Krebs-Ringer buffer. Figure 2 shows that cells harbouring the RYR1 mutations Arg4893Trp (Fig. 2B), Arg4861His (Fig. 2C), Ile4898Thr (Fig. 2D) and Gly4899Arg (Fig. 2E) released calcium from intracellular stores when placed in Krebs-Ringer medium containing 0.5 mM EGTA. This increase in $[Ca^{2+}]_i$ was not an artefact since: (i) changes in fluorescence occurred at both wavelengths (at 340 nm the fluorescence increased whereas at

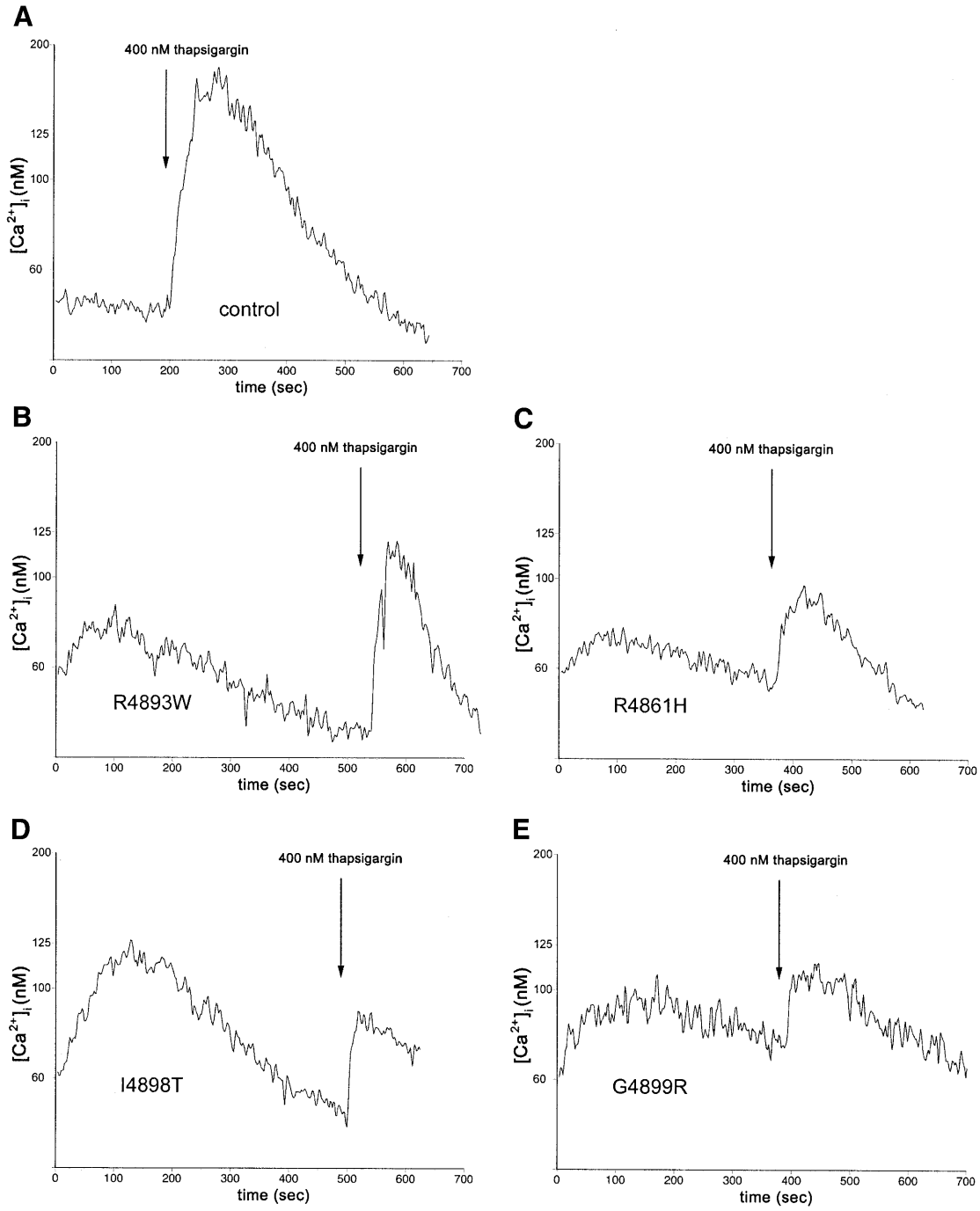

Figure 2. EBV-immortalized B cells from CCD patients carrying the Arg4893Trp, Arg4861His, Ile4898Thr and Gly4899Arg mutations 'spontaneously' release calcium from intracellular stores. Cells were loaded with the fluorescent calcium indicator fura-2/AM as described in Materials and Methods. Prior to the $[Ca^{2+}]_i$ measurements, 0.7×10^6 cells/ml were centrifuged, resuspended in nominally Ca^{2+}-free Krebs-Ringer buffer containing 0.5 mM EGTA, placed in the fluorimeter cuvette and the $[Ca^{2+}]_i$ was recorded. Lymphoblastoid cells from patients with the newly identified mutations exhibited an increase in their $[Ca^{2+}]_i$, in the absence of exogenous trigger agents (**B**–**E**), whereas cells from controls did not exhibit this Ca^{2+} release (**A**). Where indicated, 400 nM thapsigargin was added. Traces are representative of experiments carried out at least five times on different days.

380 nm the fluorescence decreased); (ii) the increase in fluorescence ratio varied among the different cells and did not occur in cells from control individuals (Fig. 2A) nor in cells carrying RYR1 mutations which have been linked to MH (not shown); (iii) the $[Ca^{2+}]_i$ returned to basal levels; and (iv) cells in which this release occurred had smaller thapsigargin-induced increases in $[Ca^{2+}]_i$. In fact, the intracellular calcium pool from which the calcium was being released was endowed with a SERCA type Ca-ATPase. When we compared the peak Ca^{2+} released by 400 nM thapsigargin, the cells harbouring the

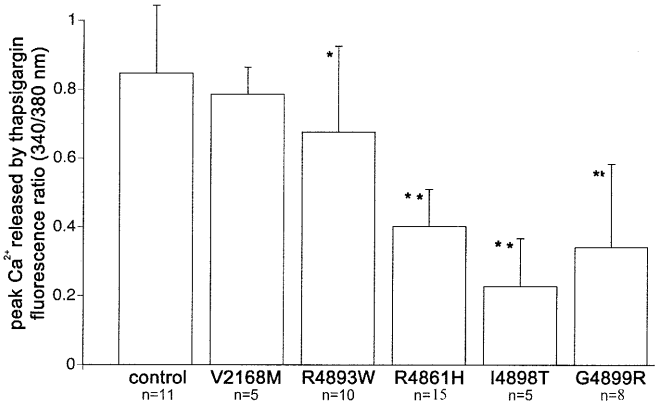

Figure 3. The thapsigargin-sensitive $[Ca^{2+}]_i$ stores of EBV-immortalized cells from CCD patients carrying the novel mutations are significantly smaller than those from control individuals and patients carrying the RYR1 Val2168Met mutation. Conditions as described in Figure 2. The peak $[Ca^{2+}]_i$ induced by the addition of 400 nM thapsigargin was calculated as increase in fluorescence ratio. Results are the means (± SD) of the indicated number of experiments. No difference in the thapsigargin-sensitive stores of cells from control individuals and from patients with the Val2168Met mutation was observed (ANOVA: $*P < 0.03$; $**P < 0.0001$).

four CCD-linked mutations released significantly less calcium (Fig. 3), indicating that their stores had previously been depleted. No difference was observed in the thapsigargin-sensitive stores of cells from controls or individuals carrying the MH-associated Val2168Met mutation (Fig. 3).

We next tested the sensitivity of lymphoblastoid cells carrying the newly identified RYR1-mutations to the RYR-activator 4-chloro-m-cresol. This compound has been shown to activate type 1 and 2 RYR but has no effect on type 3 RYR (26–28). When lymphoblastoid cells from CCD patients were stimulated with 300 µM 4-chloro-m-cresol in nominally Ca^{2+}-free EGTA-containing Krebs-Ringer buffer, almost no increase in the $[Ca^{2+}]_i$ was observed (Fig. 4A). In contrast, the addition of 300 µM 4-chloro-m-cresol to lymphoblastoid cells from control individuals (Fig. 4B) or from an individual harbouring the MHS mutation Val2168Met (Fig. 4C) caused a substantial increase in the $[Ca^{2+}]_i$. This result supports the view that the calcium released from lymphoblastoid cells of CCD patients in the absence of a pharmacological trigger, probably comes from a RYR1-endowed (4-chloro-m-cresol sensitive) intracellular pool.

Finally, in order to confirm that the 'unprompted' calcium release was due to a leaky RYR channel, we carried out experiments in the presence of dantrolene, a specific inhibitor of the skeletal muscle ryanodine receptor (29,30). When cells carrying the newly identified RYR1 mutations were resuspended in Ca^{2+}-free, EGTA-containing medium plus 10 µM dantrolene, the transient increase in $[Ca^{2+}]_i$ was abolished (Fig. 5A, bottom). Furthermore, the addition of 400 nM thapsigargin to dantrolene pre-treated lymphoblastoid cells carrying the RYR1 mutation caused a larger increase in $[Ca^{2+}]_i$ than that observed in non-dantrolene-treated cells (Fig. 5A, top and bottom). Figure 5B shows that the pre-treatment of lymphoblastoid cells carrying the Arg4861His or the Gly4899Arg mutations with 10 µM dantrolene (hatched boxes) significantly increased the peak $[Ca^{2+}]_i$

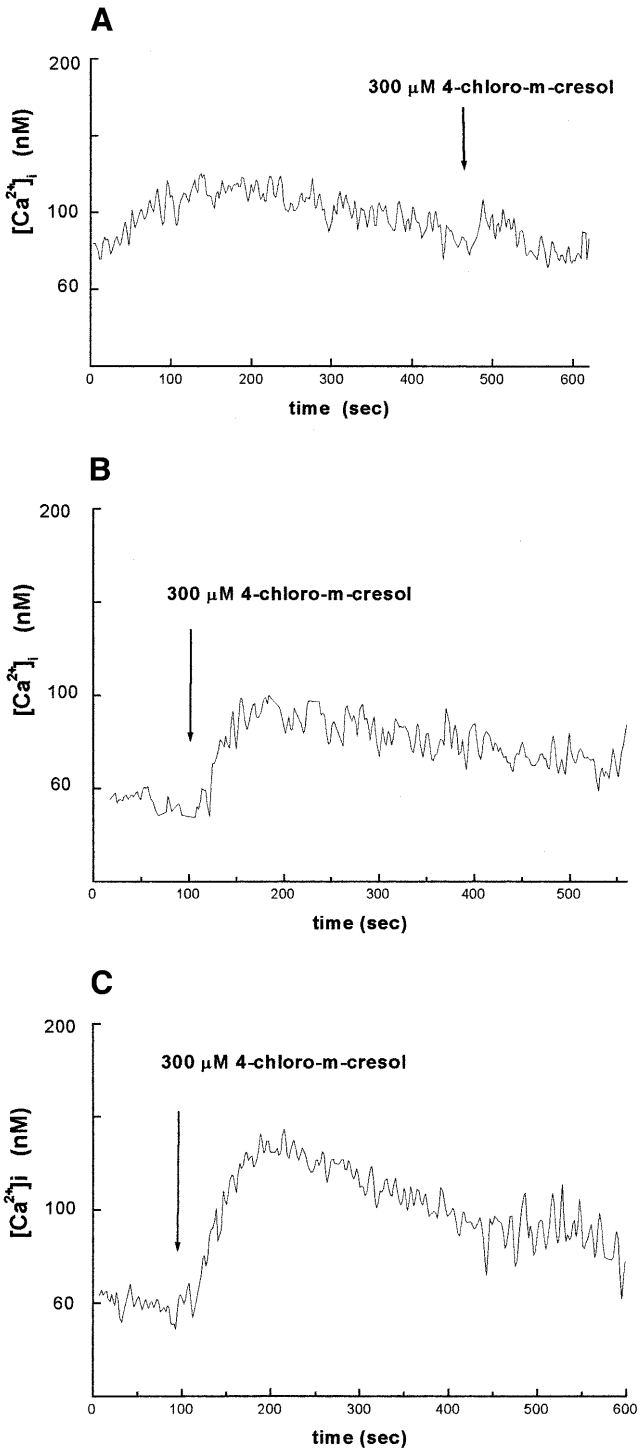

Figure 4. Addition of 4-chloro-m-cresol to EBV-immortalized B cells from a CCD patient carrying the Arg4861His results in a smaller release of calcium from intracellular stores compared to that observed in B cells from control groups. Conditions as described in Figure 2. (**A**) Representative trace from lymphoblastoid cells from an individual carrying the CCD associated Arg4861His RYR1 mutation. (**B**) Representative trace from lymphoblastoid cells from a control individual and (**C**) from an individual carrying the Val2168Met RYR1 gene mutation associated with the MHS phenotype. Traces are representative of at least three experiments.

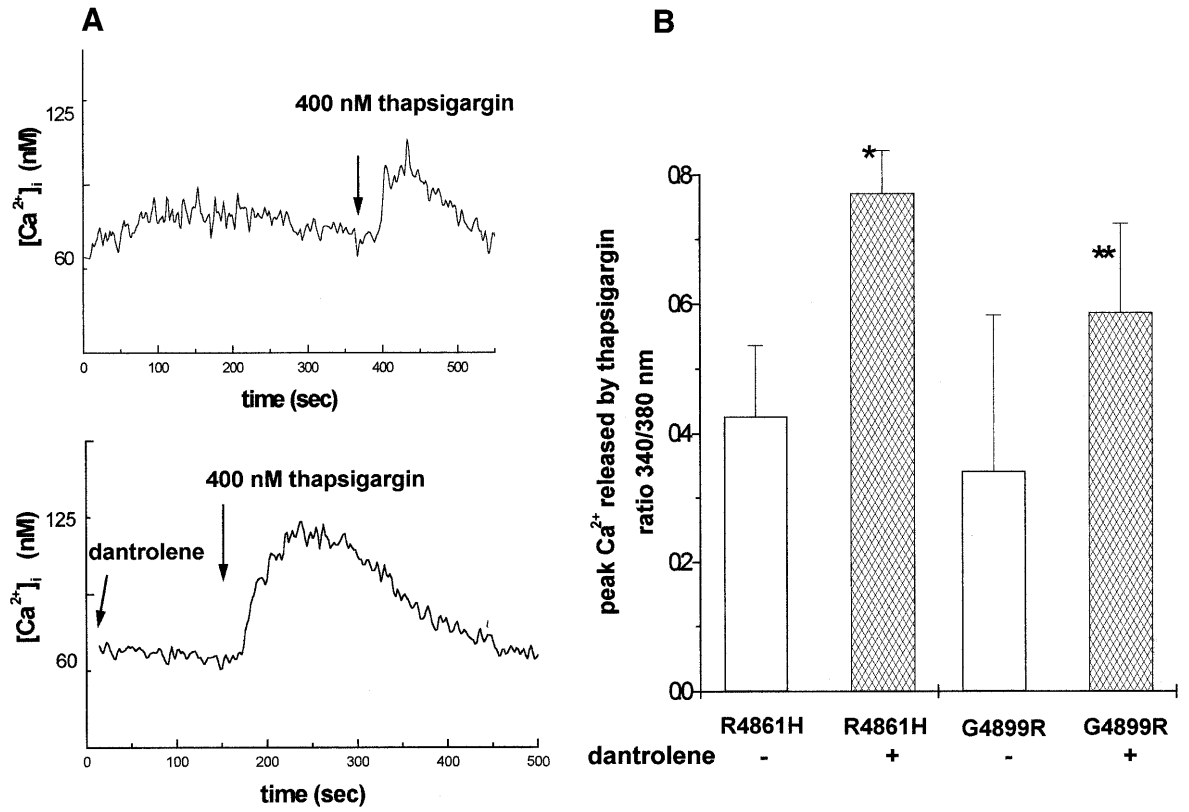

Figure 5. Dantrolene blocks the Ca^{2+} release from intracellular stores observed in the absence of pharmacological activators and increases the peak calcium released by thapsigargin. Conditions as described in Figure 2. (**A**) Representative traces from lymphoblastoid cells of an individual carrying the CCD associated Arg4861His RYR1 mutation. Top, the addition of thapsigargin (arrow) after the unprompted calcium release induces a small release of calcium from intracellular calcium stores. Bottom, pre-treatment of cells with 10 μM dantrolene (first arrow) blocks the unprompted calcium release and increases the thapsigargin-induced peak Ca^{2+} release. (**B**) Peak Ca^{2+} induced by thapsigargin in cells from patients carrying the Arg4861His and Gly4899Arg RYR1 mutations is significantly higher in cells pre-treated with 10 μM dantrolene (hatched bars) than in untreated cells. Results are expressed as means (± SD) of experiments carried out five and three times on cells + 10 μM dantrolene carrying the Arg4861His and Gly4899arg mutation, respectively (Student's *t*-test for paired samples: $*P < 7 \times 10^{-8}$; $**P <$

induced by thapsigargin, restoring the $[Ca^{2+}]_i$ to levels similar to those seen in cells from control individuals or individuals carrying the MHS1-associated Val2168Met mutation.

DISCUSSION

In the present work, we have screened 50 individuals of European origin diagnosed as having CCD and identified five different sequence changes in 13 individuals. The presumed mutations cluster in exons 101 and 102 of the *RYR1* gene. Arg4861His, Gly4891Arg, Gly4899Arg and Ala4906Val are described here for the first time. The fifth mutation, Ile4898Thr was originally identified in a large Mexican kindred with early onset and severe CCD but was thought to be a 'private mutation' restricted to this Mexican family (21). We have found this mutation in three apparently unrelated pedigrees of presumed Italian ancestry. Likewise, the Arg4861His mutation was observed in seven German and Austrian index cases. Therefore, these two mutations may represent founder mutations in the European CCD population. All other mutations described here have been detected in single patients or families. Recently, two other point mutations between amino acids 4637 and 4898 have been reported in CCD patients (22,23), making the C-terminal domain of the RYR1 a hot spot region for CCD causing mutations.

Our evidence that the resulting amino acid substitutions are causal of CCD is as follows: (i) the substituted residues are conserved in all known vertebrate *RYR* genes of type 1, 2 and 3; (ii) the sequence changes co-segregate with the disease in nine multiplex pedigrees; (iii) they are absent from 100 control chromosomes; and (iv) the intracellular calcium homeostasis of lymphoblastoid cells derived from mutation carriers is significantly different from that of controls.

Three of the newly identified mutations result in loss or gain of a positively charged arginine residue, the most frequently observed pathological mutation in RYR1 (31). Two missense substitutions occurred in CpG dinucleotides (Arg4861His, Ala4906Val) of the coding strand, a finding which is in line with the observation that most mutations, but not polymorphisms, in the *RYR1* gene occur in CpG dinucleotide sequences (20).

So far, mutations found in the *RYR1* gene appear to cluster in specific domains: mutations in the N-terminal regions 1 and 2 are associated with MHS and/or CCD whereas mutations in the C-terminal region 3 seem to be mainly associated with CCD. The functional analysis of cells transfected with cDNA

encoding RYR1 mutations in the N-terminal domain have demonstrated that the Ca^{2+} release channel of such cells is significantly more sensitive to caffeine than that of cells expressing wild-type RYR1 channels (32,33). This hypersensitivity is in line with the clinical symptoms of MHS individuals whose muscles are hyper-reactive to activators of the ryanodine receptor, but is at odds with the clinical features of CCD whose predominant symptoms are chronic muscle weakness and hypotonia.

In this work, we used a novel *ex vivo* approach to study the effect of RYR1-linked mutations. We took advantage of the fact that B-lymphocytes express the skeletal muscle isoform of this Ca^{2+} channel (25; Girard *et al.*, manuscript submitted for publication) and were able to study the calcium homeostasis of lymphoblastoid cells from CCD patients with four C-terminal RYR1 mutations. Our results show that cells carrying the endogenous, heterozygous Ile4898Thr mutation have significantly smaller Ca^{2+} stores than cells carrying a wild-type RYR1 or the MHS-linked mutation Val2168Met. Our data support those of Lynch *et al.* (21) who transfected human embryonic kidney cells (HEK-293) and found that the expression of the Ile4898Thr cDNA resulted in reduction of thapsigargin-induced Ca^{2+} release. In contrast, Avila *et al.* (34) showed that dyspedic mouse skeletal muscle myotubes injected with the RYR1 cDNA carrying the same mutation were 'uncoupled', i.e. membrane depolarization was not accompanied by Ca^{2+} release from the SR, though the resting calcium concentration was not different from control myotubes. This apparent discrepancy may be due to the fact that in skeletal muscle cells, the SR Ca-ATPase is able to compensate for 'excessive' leakage through the mutated channel and other downstream effects. In the case of HEK-293 cells or EBV-immortalized B-lymphocytes on the other hand, the Ca^{2+} pump may not be able to compensate for the excessive leakage, resulting in a net Ca^{2+} efflux from the endoplasmic reticulum. It should be noted that the ryanodine receptor must assemble into a tetrameric structure in order to function as a calcium channel. Therefore, it is likely that the calcium channels in lymphoblastoid cells from CCD patients are composed of normal and mutated subunits.

Although the single channel properties of the RYR1 carrying the Ile4898Thr mutation are still not known, there is other evidence that substitution of this hydrophobic residue has a strong effect on the properties of the calcium release channel. Gao *et al.* (35) demonstrated that substitution of Ile4898 by Ala, Val or Leu, results in a channel which remains blocked in sub-conductance states and fails to close even in the presence of EGTA, i.e. at a free calcium concentration far below that of living cells. This would result in increased leakage of Ca^{2+} from intracellular stores. Likewise, substitutions of other amino acids in this region of the RYR1 cDNA, which is thought to make up the pore structure, should have similar effects and in fact our results show that lymphoblastoid cells from patients carrying the Gly4899Arg, Arg4893Trp and Arg4861His mutations all have reduced thapsigargin-sensitive Ca^{2+} stores. It should be noted that: (i) the $[Ca^{2+}]_i$ measurements were carried out in a population of cells; (ii) all the cell lines which were established from CCD patients exhibited a release of calcium from intracellular stores in the absence of pharmacological activators of the RYR; (iii) all experiments described in the present work were carried out in calcium-free/EGTA-containing medium, thus once the intracellular calcium stores of the cells had been depleted they could not be re-filled, leading to a single transient in the $[Ca^{2+}]_i$.

The addition of 4-chloro-m-cresol, a specific activator of type 1 and 2 RYR (26–28), failed to elicit an increase in the $[Ca^{2+}]_i$ of the CCD-derived lymphoblasts indicating that the RYR1-containing intracellular stores had already been depleted. Such unprompted calcium transients were never seen in cell lines of control individuals or individuals carrying the MHS-linked mutation Val2168Met (Girard *et al.*, manuscript submitted for publication). Transients could be blocked by the addition of dantrolene, a specific inhibitor of the skeletal muscle ryanodine receptor (29,30). Furthermore, dantrolene pre-treatment of CCD lymphoblasts restored the thapsigargin-induced Ca^{2+}-peak to control levels supporting the view that the smaller intracellular calcium stores observed in these cells are due to a leaky RYR calcium channel.

Whatever mechanism is responsible for this increase in leakiness, we found that lymphoblastoid cells carrying CCD-associated mutations in domain 3 of the RYR1 exhibit release of $[Ca^{2+}]_i$ in the absence of pharmacological activators of a RYR1; this may be a distinguishing feature of cells with mutations in the pore region of the RYR1 calcium channel. If the situation were similar in CCD muscle, lower sarcoplasmic and higher cytosolic Ca^{2+} concentrations could readily explain the predominant symptom of chronic muscle weakness.

In conclusion, our data suggest a new mutational hot spot for CCD in the C-terminal domain of the RYR1 and confirm the importance of the transmembrane/luminal region (region 3) in channel function. Therefore, this region should be considered as the prime target for mutation screening in CCD patients. The 'ectopic' expression of RYR1 in lymphoblastoid cells from patients can be used as a non-invasive approach to test the function of presumed RYR1 mutations *ex vivo* and to further our understanding of the pathophysiology of CCD.

MATERIALS AND METHODS

Patient selection

The patients are a cohort of unselected persons from various European neuromuscular and genetic centres (who are not part of a study) diagnosed clinically and/or histologically as having CCD. DNA samples from 50 unrelated patients were collected after informed consent. Nine patients are members of multiplex pedigrees which had been used previously for linkage studies (36) (Table1). Detailed clinical and histopathological records which fulfilled the accepted criteria for CCD (37) were available from 21 patients. A summary of these data is given in Table 1. Medical documentation of the remaining cases was incomplete and is not detailed here. Mutations were found in four patients from the latter group and these are also included in Table 1.

Mutation screening

PCR amplification of exons 98, 99, 100, 101, 102 and 103 of *RYR1* was performed on 200 ng genomic DNA under standard PCR conditions (denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 30 s, annealing at primer-specific annealing temperatures for 30 s, extension at 72°C for 30 s and a final extension step of 72°C for 5 min). Primer sequences,

PCR product sizes and annealing temperatures were as follows: exon 98F, tgtgtctacacagcctgatgc/98R, ggggagagatgcttgagtgt/243 bp/59°C; exon 99F, ctggtgagcccaggacac/99R, agagtccctccccagtctgt/193 bp/59°C; exon 100F, agagtgctcctcgtgtgtcc/100R, tatcccttcaccacccactg/275 bp/61°C; exon 101F, ggtagagccacagggactga/101R, gagaaggaagggtcccagag/276 bp/63°C; exon 102F, aatgtcgaatgaatgcgtga/102R, ctgggcctgcattcttagc/268 bp/55°C; exon 103F, aagccctggaggtaggtagc/103R, tgaatcccgtaatccctctg/191 bp/59°C. Screening for mutations was performed by SSCA (38). Fragments were labelled by incorporation of ^{32}P-dCTP during PCR. After heat denaturation the single strands were separated on a 6% polyacrylamide gel with or without 10% glycerol in the monomer mixture. Electrophoresis was run at 6°C. PCR fragments with aberrant SSCA patterns were directly sequenced using the forward and reverse primers of the amplification reaction and a terminator cycle sequencing kit (Thermo Sequenase kit; Amersham Life Science, Freiburg, Germany) with ^{33}P-ddNTPs.

To test for the segregation of a mutation in the family, DNA from the available relatives (affected and unaffected) of the index patients was analysed by mutation-specific restriction enzyme digestion using *Aci*I for Arg4861His, *Bsr*I for Ile4898Thr and *Sac*II for Ala4906Val, and by direct sequencing for the remaining two mutations.

Lymphoblastoid cell lines

Mononuclear cells were isolated from peripheral blood leukocytes and EBV-transformed according to the protocol of Neitzel (39). Cells were cultured in RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and 100 Units of penicillin and streptomycin.

Intracellular Ca^{2+} measurements

Changes in the intracellular calcium concentration of the lymphoblastoid cells were monitored with the fluorescent calcium indicator fura-2/AM (Sigma, St Louis, MO) (final concentration 5 µM) as described by Zorzato *et al.* (26) and Grynkiewicz *et al.* (40). Fura-2 loaded cells (0.7×10^6/ml) were washed once by centrifugation, resuspended in Ca^{2+}-free Krebs-Ringer medium containing 0.5 mM EGTA and placed in a cuvette thermostated at 37°C. Fluorescence changes (ratio 340/380 nm) were measured in a Perkin-Elmer spectrofluorimeter equipped with a magnetic stirrer. All measurements were made in Ca^{2+}-free Krebs-Ringer buffer containing 0.5 mM EGTA. Experiments were performed at least four times on two different days. Where indicated 300 µM 4-chloro-m-cresol (Fluka Chemicals, Buchs, Switzerland), 400 nM thapsigargin (Sigma) or 10 µM dantrolene (Procter and Gamble Pharmaceuticals, Weiterstadt, Germany) were added.

Statistical methods

Data are expressed as means ± SD. The peak Ca^{2+} released by thapsigargin in cells from controls or individuals carrying the Val2168Met, Arg4893Trp, Arg4861His, Ile4898Thr and Gly4899Arg RYR1 mutations was compared using one-way ANOVA. Where significant, Fishers's protected least significant difference (PLSD) *post hoc* test was performed. Peak Ca^{2+} released by thapsigargin in the presence or absence of dantrolene were compared using the Student's *t*-test for paired samples. Significance was set to 5%.

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