Am. J. Hum. Genet. 60:1316–1325, 1997

Malignant-Hyperthermia Susceptibility Is Associated with a Mutation of the α1-Subunit of the Human Dihydropyridine-Sensitive L-Type Voltage-Dependent Calcium-Channel Receptor in Skeletal Muscle

Nicole Monnier,[1] Vincent Procaccio,[1,3] Paul Stieglitz,[2] and Joël Lunardi[1,3]

[1]Laboratoire de Biochimie de l'ADN and [2]Département d'Anesthésie, CHU Grenoble, and [3]Laboratoire BECP, EA 2019 UJF, DBMS-CEA Grenoble, Grenoble

Summary

Malignant hyperthermia susceptibility (MHS) is characterized by genetic heterogeneity. However, except for the MHS1 locus, which corresponds to the skeletal muscle ryanodine receptor (RYR1) and for which several mutations have been described, no direct molecular evidence for a mutation in another gene has been reported so far. In this study we show that the CACNL1A3 gene encoding the α1-subunit of the human skeletal muscle dihydropyridine-sensitive L-type voltage-dependent calcium channel (VDCC) represents a new MHS locus and is responsible for the disease in a large French family. Linkage analysis performed with an intragenic polymorphic microsatellite marker of the CACLN1A3 gene generated a two-point LOD score of 4.38 at a recombinant fraction of 0. Sequence analysis of the coding region of the CACLN1A3 gene showed the presence of an Arg-His substitution at residue 1086, resulting from the transition of A for G3333, which segregates perfectly with the MHS phenotype in the family. The mutation is localized in a very different part of the α1-subunit of the human skeletal muscle VDCC, compared with previously reported mutations found in patients with hypokalemic periodic paralysis, and these two diseases might be discussed in terms of allelic diseases. This report is the first direct evidence that the skeletal muscle VDCC is involved in MHS, and it suggests a direct interaction between the skeletal muscle VDCC and the ryanodine receptor in the skeletal muscle sarcoplasmic reticulum.

Introduction

Malignant hyperthermia susceptibility (MHS), a skeletal muscle disorder mostly inherited as an autosomal dominant trait (Kalow 1987), is one the main causes of death due to anesthesia. In susceptible people, a malignant hyperthermia (MH) episode is triggered by exposure to commonly used volatile anesthetic agents such as halothane or depolarizing muscle relaxants such as succinyl choline. A fulminant MH crisis is characterized by any combination of hyperthermia, skeletal muscle rigidity, tachycardia or arythmia, respiratory and metabolic acidosis, and rhabdomyolysis. Except for this susceptibility to triggering agents, MHS patients are not clinically distinguishable from the general population. Presymptomatic assessment of MH risk is performed by use of the in vitro contracture test (IVCT). Protocols for this test have been standardized by the European Malignant Hyperthermia Group (1984) and the North American Study Group for Malignant Hyperthermia (Larach 1989). These protocols discriminate individuals as MH susceptible (MHS), MH normal (MHN), and MH equivocal (MHE). The latter category comprises individuals for whom the biopsy sample reacts positively to one, but not both, of the in vitro triggering agents, halothane (MHEh) and caffeine (MHEc).

Physiological and biochemical studies indicate that MHS is due to a malfunction in the mechanisms regulating sarcoplasmic calcium-ion fluxes (MacLennan and Phillips 1992). On the basis of linkage studies in Irish families, an MHS locus was localized on chromosome 19q12-13.2 (McCarthy et al. 1990). The gene for the human skeletal muscle ryanodine receptor (RYR1), a calcium-release channel of the sarcoplasmic reticulum, was mapped simultaneously to the same region and was shown to be tightly linked to MHS in Canadian families (MacLennan et al. 1990).

Biochemical and electrophysiological studies in swine and humans with MHS pointed to the ryanodine receptor as a likely candidate for the molecular defect (MacLennan and Phillips 1992). Base changes in the RYR1 gene, leading to amino acid substitutions in the gene product, cosegregate with the MHS phenotype in a subset of human pedigrees with MHS (Gillard et al. 1991; Quane et al. 1994*b*). More recently, mutations in the RYR1 gene have also been described in patients with central core disease (CCD), a rare myopathy that may predispose to MH (Quane et al. 1993,

Received January 27, 1997; accepted for publication March 28, 1997.

Address for correspondence and reprints: Dr. Joël Lunardi, Laboratoire BECP, EA 2019 UJF, DBMS-CEA Grenoble, 17 rue des Martyrs, 38054 Grenoble Cedex 09, France. E-mail: jlunardi@pilate.ceng.cea.fr

0002-9297/97/6006-0009$02.00

1994*a;* Zhang et al. 1993). However, a significant number of families have also been described in which no linkage exists between markers for the RYR1 region and MHS (Deufel et al. 1992; Iles et al. 1992; Levitt et al. 1992).

In the search for additional MHS genes, a region on chromosome 17q11-24 was proposed, on the basis of linkage studies in North American and southern African pedigrees. The gene for the adult muscle sodium channel (SCN4A) was suggested as a candidate (Levitt et al. 1992; Olckers et al. 1992). However, no molecular evidence for this assumption is presently available. On the other hand, the skeletal muscle dihydropyridine-sensitive L-type voltage-dependent calcium channel (VDCC) has been considered as a candidate for MHS, since this voltage-dependent channel is responsible for the regulation of calcium movement in skeletal muscle (Bean 1984). Furthermore, it has been shown that the skeletal muscle VDCC and the ryanodine receptor form the triad at the level of the junction between the T-tubule and the sarcoplasmic reticulum (Marty et al. 1994; Takekura et al. 1994).

Of the four genes encoding subunits of the skeletal muscle 1,4-dihydropyridine-sensitive VDCC, three—the α1-subunit (CACLN1A3) on chromosome 1q (Gregg et al. 1993*a*), the β-subunit (CACNLB1), and the γ-subunit (CACNLG), the latter two of which are localized on chromosome 17q11.2-q24 (Gregg et al. 1993*b;* Powers et al. 1993), in proximity of the SCN4A locus—have been excluded as candidates in German, British, and Belgium pedigrees (Iles et al. 1993; Sudbrak et al. 1993). However, cosegregation of MHS with the marker D7S849 adjacent to the gene for the α2/δ-subunit of the dihydropyridine-sensitive VDCC (CACNL2A), which map to chromosome 7q21-22 (Powers et al. 1994), was reported in one German pedigree (Iles et al. 1994*a*). To our knowledge, no mutations in this gene have been described to date. More recently, we initiated a European collaborative search for additional MHS loci, using a panel of MHS pedigrees with the MHS trait not linked to the RYR1 gene. A clear linkage between the MHS phenotype and markers defining a 1-cM interval on chromosome 3q13.1 was described (Sudbrak et al. 1995). However, no candidate genes have been identified in this region so far.

We report here an MHS pedigree in which the MHS phenotype is genetically associated with the CACLN1A3 gene, which encodes the α1-subunit of the skeletal muscle VDCC. This represents the first characterization of a mutation responsible for MHS in a gene other than RYR1. Interestingly enough, mutations of the CACNL1A3 that so far have been described have been exclusively associated with hypokalemic periodic paralysis (hypoKPP) (Jurkat-Rott et al. 1994; Ptacek et al. 1994). These results will be discussed in terms of pathological model and allelic diseases.

Patients and Methods

Proband, Pedigree, and IVCT Results

The proband, individual lII-12, 30 years of age, died from a fulminant MH crisis during anesthesia. Patient III-12 developed tachycardia, extra systoles, and hypercapnia 30 min after injection of suxamethonium and administration of isoflurane. After 90 min, hyperthermia (40°C) was observed, and isoflurane was stopped immediately. However, despite administration of dantrolene, the patient died from cardiac arrest. The family was then investigated for MHS, and 18 members of the family were then diagnosed on the basis of IVCT; 10 were classified as MHS, 5 as MHN, and 3 as MHEh.

No distinctive clinical symptoms or histological features evidencing a skeletal muscle disease were identified in this family. The IVCT was performed according to the European standard protocol (European Malignant Hyperthermia Group 1984), and in figure 1 the results are indicated below each tested individual.

DNA Analysis of Microsatellite Markers

Genomic DNA was extracted from whole blood by use of a rapid guanidine method (Jeanpierre 1987). Amplification by PCR was performed according to standard protocols using primers for the microsatellite-repeat markers specified in table 1. For the CACNL1A3.PCR1 amplimer (GDB accession number 196341), PCR was performed in a 25-μl volume containing 0.4 pmol of forward primer 5′ end-labeled with ^{32}P, 200 mM dNTPs, and 0.25 U of *Taq* polymerase (Appligene). Amplification was performed as described previously, except that only 30 cycles were run and that annealing temperature was 62°C. After denaturation with formamide, ^{32}P-5′ end–labeled amplification products were loaded onto a 6% polyacrylamide/8 M urea DNA sequencing gel prior to autoradiography.

Data Analysis

Linkage analysis was performed by use of the 5.2 LINKAGE package (Lathrop and Lalouel 1984). The pedigree files were created with the 1.1 Cyrillic program (Cherwell Scientific Publishing). Parameters were set as defined by the genetics section of the European Malignant Hyperthermia Group and reported elsewhere (Sudbrak et al. 1995): the disease-allele frequency was 1/10,000, the penetrance for the index case was taken as 1.0, and the penetrance of the disease heterozygote was set at .98; the phenocopy rate was .02, and MHE individuals were assigned unknown status (i.e., they do not contribute to the LOD score in this family). In the absence of specific data, allele frequencies for the hypervariable microsatellite markers were set as equal and thus as dependent on the maximum number of alleles reported for each marker.

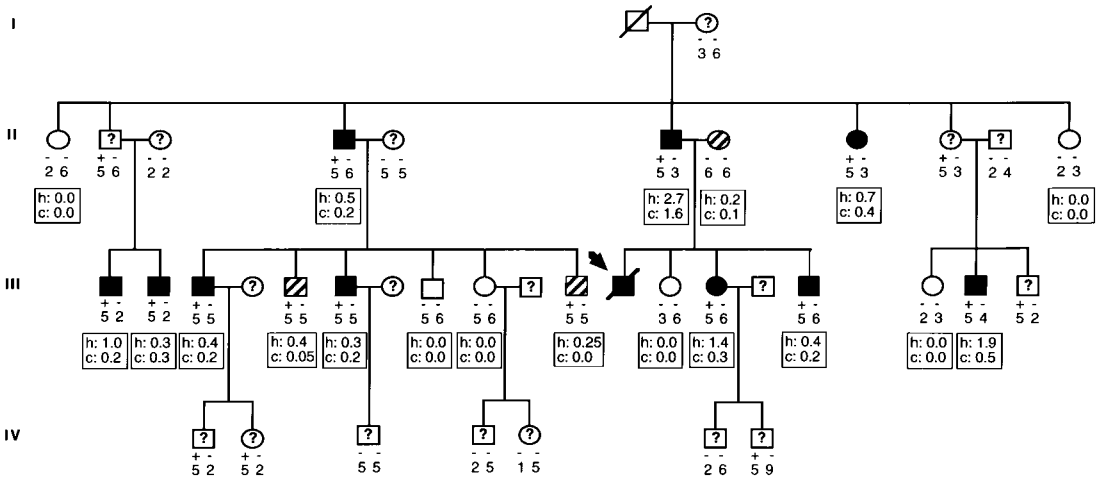

Figure 1 Segregation, in the VE2GR pedigree, of the Arg1086His mutation with the MHS trait. Blackened symbols denote patients tested with the IVCT and phenotyped as MHS; unblackened symbols denote those typed as MHN; hatched symbols denote those typed as MHEh; and unblackened symbols with a question mark denote untested family members (i.e., disease status is unknown). The index patient (III-12), marked by an arrow, died from a fulminant MH crisis. The presence or absence of the Arg1086His mutation is indicated by a plus sign (+) or a minus sign (−), together with the result of genotyping for polymorphic marker CACNL1A3.PCR1. Results of IVCT are reported in boxes below each tested individual. Values indicated (expressed in g) correspond to muscle-fiber contractures in the presence of either 2% halothane (h) or 2 mM caffeine (c).

Analysis of Genomic DNA and cDNA

Blood samples and muscle biopsy were obtained with the appropriate informed consent of the patients, in agreement with French ethical rules. Total RNA was extracted from a 100-mg vastus lateralis muscle-biopsy sample of MHS patient III-14, as described elsewhere (Chomczynski and Sacchi 1987). Random hexamer–primed cDNA was synthesized from 1 μg of total RNA in a final volume of 50 μl, by use of a first-strand cDNA synthesis kit (Stratagene). Reverse transcription–PCR was performed in a final volume of 50 μl, by use of 1.5 μl of the reaction as template, 1 μM primers, and 0.5 U of *Taq* polymerase (Appligene). Amplification parameters were as follows: initial denaturation at 94°C for 2 min; 35 cycles of 94°C for 30s, 58°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. Twelve overlapping fragments were amplified—(153-795), (675-1235), (1188-1680), (1558-2176), (2054-2605), (2558-3230), (3037-3692), (3448-4019), (3925-4480), 4368–4925), (4752-5320), and (5257-6021). Base assignment was done according to the cDNA described by Hogan (Hogan et al. 1994) (Genbank accession number 33798). Alternatively, exons were amplified from genomic DNA by use of exon-specific intronic primers (Hogan et al. 1996) (Genbank accession numbers U30666–U30707).

Table 1

Two-Point LOD-Score Values for Linkage between MHS and Markers from Potential Chromosomal Candidate Regions

Chromosome and Microsatellite Marker	LOD Score at Recombination Fraction 0	Reference
1q:		
CACLN1A3.PCR1	+4.38	Gregg et al. (1993*a*)
3q:		
D3S1281	−7.97	Subdrak et al. (1995)
D3S1616	−7.96	
7q:		
D7S660	−1.6	Iles et al. (1994*a*)
D7S849	−3.01	
D7S254	−4.92	
17q:		
D17S518	−5.36	Levitt et al. (1992)
D17S250	−5.44	
NM23	−5.46	
19q:		
D19S191	−.95	
D19S220	−6.27	

Direct Sequencing of PCR Products

PCR-amplified fragments obtained from cDNA or genomic DNA were purified by use of the Qiaex DNA-purification system (Diagen). In order to eliminate any sequence error resulting from polymerase activity, three independently amplified products of each fragment were sequenced by use of an ABI 373 apparatus and the PCR primers as sequencing primers.

Mutation Analysis

For extensive screening of the Arg1086His mutation, analysis of the G→A transition was alternatively per-

formed on genomic DNA by restriction digestion using *Hha*I. A 226-bp-long fragment encompassing the mutation was amplified by use of the 25i5 forward primer, 5′-CTTGGTGCTGACCTGTCCTGTT, and the 25i3 reverse primer, 5′-GATCAGACATTTTTCTCCTGGGG. Digestion of the normal allele generates two fragments, 190 and 36 bp long, whereas the mutated allele is not cut. Digested fragments were analyzed on a 6% acrylamide gel.

Results

MHS Phenotype in the VE2GR Family: Association with the CACLN1A3 Gene on Chromosome 1q31

Combined pairwise LOD scores with informative markers adjacent to previously identified MHS loci on chromosomes 19q, 17q, 7q, and 3q are listed in table 1. Negative LOD-score values obtained with the different markers indicate exclusion of MHS from these loci in the VE2GR family. Since (i) a well-documented MH crisis had been observed in the index case, (ii) exclusion of previously described MHS loci was established, and (iii) the pedigree was large enough to allow for an individual LOD score >3.0 with positive linkage, the VE2GR pedigree was included in a systematic human-genome linkage study using a panel of MHS families. This was part of a collaborative European project aimed at the identification of additional MHS loci. Preliminary data obtained from this general search pointed to the 1q31 region as a potential region for an MHS locus in this family. The CACNL1A3 gene encoding the α1-subunit of the skeletal muscle VDCC has been mapped to chromosome 1q31-32 (Gregg et al. 1993*a*) and internal polymorphic markers for this gene (CACNL1A3.PCR1 and CACNL1A3.PCR2) have been identified (Gregg et al. 1993*a;* Iles et al. 1994*b*). We tested these markers in the VE2GR family. Only the CACNL1A3.PCR1 marker worked in our study and proved to be fully informative in the family. A clear segregation of allele 5 was obtained with the MHS phenotype (fig. 1). Two-point LOD-score calculation between this marker and the MHS trait in this family generated a LOD-score value of 4.38 at 0 cM, compared with the 4.46 maximum LOD-score value obtained in simulation studies using MSIM, a LINKAGE-based package.

Arg1086His Mutation in the CACLN1A3 Gene: Association with the MHS Phenotype

We isolated the cDNA of the CACNL1A3 gene from a skeletal muscle biopsy of MHS individual III-14. Alternatively, sequencing of genomic DNA was performed on amplified exonic DNA. The CACNL1A3 cDNA was amplified by PCR, in overlapping products of ~600 bp. Direct sequencing of the PCR products obtained from the CACNL1A3 cDNA yielded 10 sequence changes (table 2). All the sequence modifications were confirmed by analysis of the genomic DNA. Sequence changes leading to modification of amino acids at positions 265, 574, 627, 628, 918, 919, 1180, 1671, 1815, and 1840 are present as homozygous changes. Except for modification of amino acids at positions 1815 and 1840, all substitutions that we report lead to changes in amino acid composition that are strictly conserved in genes encoding the dihydropyridine-sensitive VDCC from rabbit, rat, carp, *Drosophila,* and human skeletal muscle (Tanabe et al. 1987; Grabner et al. 1991; Zheng et al. 1995; Hogan et al. 1994). Therefore, it is likely that these amino acid corrections correspond to the actual sequence and do not represent infrequent polymorphisms. On the other hand, two polymorphisms present at the heterozygous level were also found. The first corresponds to a A1598T substitution and results in a His→Leu substitution at amino acid residue 458 (table 3). This polymorphism corresponds to a sequence variation between exonic and cDNA sequences that has been described elsewhere (Gregg et al. 1993*a*). The second is a C→T substitution at position 4840 and results in an Arg→Cys change at amino acid 1539. Since it has been reported that Arg→Cys mutations in the RYR1 gene are associated with MHS (Gillard et al. 1991; Quane et al. 1994*b*), we critically checked the distribution of this polymorphism in the VE2GR family. This C4840T substitution was found in both MHN and MHS individuals, and its segregation clearly failed to follow the MHS phenotype. Therefore, this sequence modification is likely to represent a simple polymorphism.

The final DNA alteration detected was the substitution of an A for G3333 in the cDNA, resulting in an Arg1086His mutation in the α1-subunit of the skeletal muscle VDCC (fig. 2). The mutation is localized at the very beginning of exon 25 and abolishes a *Hha*I restriction site. Accordingly, we screened for the presence or the absence of this site, by constructing PCR primers in the flanking introns, whose boundaries are located, respectively, 2 bp upstream of the mutation and 157 bp downstream of the mutation. PCR amplification yielded a fragment of 226 bp, which, in the MHS family that we studied, was fully cleaved in normal individuals and was 50% cleaved in heterozygous individuals (fig. 3). This mutation replaces an amino acid that is strictly conserved in brain and endocrine human VDCC isoforms and in all species in which the CACNL1A3 cDNA has been sequenced so far (fig. 4).

We found the Arg1086His mutation as a heterozygous trait in 10 individuals of the family (II-4, II-6, II-8, III-1, III-2, III-3, III-6, III-14, III-16, and III-18; fig. 1) who were diagnosed for MHS by use of IVCT. We also found the mutation in two obligate carriers (II-2 and II-9) and in two MHEh individuals (III-5 and III-

Table 2

Sequence Corrections in CACNL1A3 cDNA, and Predicted Amino Acid Sequence

	Amino Acid Position											
Sequence	265	505	574	627	628	918	919	1180	1671	1814	1815	1840
Original:[a]												
Amino acid	Cys	Cys	Arg	Ser	Ser	Ala	Arg	Asn	Ala	Leu	Gly	Asp
cDNA	TGC	TGC	CGC	TCG	AGC	GCT	AGG	AAC	GCG	CTC	GGA	GAC
Corrected:[b]												
cDNA	TG<u>G</u>	TG<u>T</u>	<u>G</u>GC	T<u>A</u>C	<u>G</u>GC	G<u>T</u>G	<u>CA</u>G	<u>G</u>AC	<u>C</u>CG	CT<u>G</u>	G<u>C</u>A	GA<u>G</u>
Amino acid	<u>Trp</u>	<u>Cys</u>	<u>Ala</u>	<u>Tyr</u>	<u>Gly</u>	<u>Val</u>	<u>Gln</u>	<u>Asp</u>	<u>Gly</u>	<u>Leu</u>	<u>Ala</u>	<u>Glu</u>
Amino acid:												
Rabbit[c]	Trp	Cys	Ala	Tyr	Gly	Val	Gln	Asp	Gly	Leu	Val	Gln
Rat[c]	Trp	Cys	Ala	Tyr	Gly	Val	Gln	Asp	Gly	Leu	Val	Gln
Carp[d]	Trp	Cys	Ala	Tyr	Gly	Val	Gln	Asp	Val	Leu	Val	Ser
Drosophila[e]	Trp	Cys	Ala	Tyr	Gly	Val	Gln	Asp	Gly	Leu	Arg	Ser

[a] Hogan et al. (1994)
[b] Changes from original sequence are underlined.
[c] Tanabe et al. (1987).
[d] Grabner et al. (1991).
[e] Zheng et al. (1995).

11) who presented a strong response to halothane only. These two MHEh patients, whose status was based on IVCT, were MHS patients on the basis of haplotyping with the CACLN1A3.PCR1 marker and the Arg1086His mutation as polymorphisms. The mutation was absent from six members of the family diagnosed as MHN after IVCT. The LOD score for linkage between the mutation in CACNL1A3 and MHS was 4.38, at a recombination fraction of 0. We extended the analysis to additional members of the family and found the mutation in three children (IV-1, IV-2, and IV-7) who had not yet been subjected to the diagnostic test. The mutation was not found in 100 independent MHN chromosomes belonging to nonrelated IVCT-negative patients from pedigrees

Table 3

Polymorphisms in CACNL1A3 Coding Sequence

	Amino Acid Position	
Sequence	458	1539
Original:[a]		
Amino acid	His	Arg
cDNA	CAC	CGC
Found:		
cDNA	CAC/CTC	CGC/TGC
Amino acid	His/Leu	Arg/Cys
Amino acid:		
Rabbit[b]	Leu	Arg
Rat[b]	Leu	Arg
Carp[c]	Glu	Arg
Drosophila[d]	Asp	Arg

[a] Hogan et al. (1994).
[b] Tanabe et al. (1987).
[c] Grabner et al. (1987).
[d] Zheng et al. (1995).

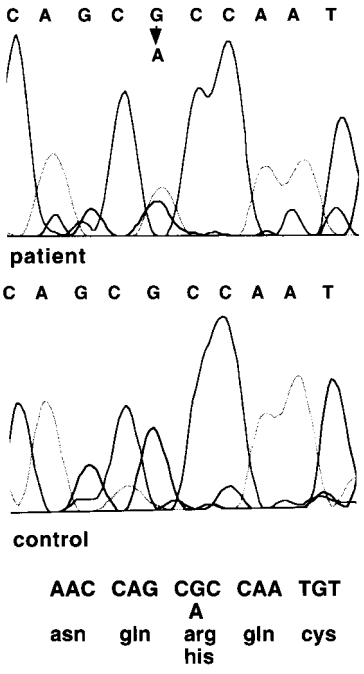

Figure 2 DNA sequencing of the cDNA of the CACNL1A3 gene. The ABI sequencing profile shows a heterozygous G→A substitution at position 3333 in MHS patients that is not observed in MHN patients. The DNA sequence of the coding strand with the amino acid translation is shown below the graph.

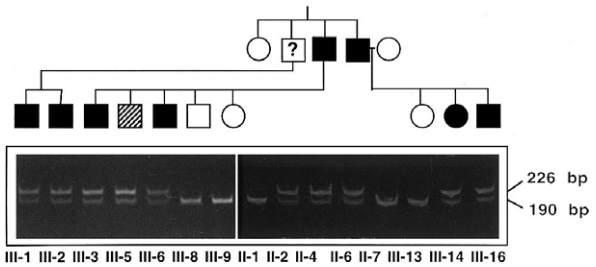

Figure 3 Screening of the G→A mutation at position 3333, by analysis of the presence or the absence of an *Hha*I restriction-endonuclease site in the 226-bp PCR fragment from genomic DNA of individuals from a grouping within the pedigree described in figure 1. Cleavage of the 226-bp fragments generated 190- and 36-bp fragments. Because of the gel composition used and the ethidium bromide visualisation, the 36-bp fragment did not give a neat signal on gels; therefore, only ethidium bromide fluorescent signals corresponding to the uncut 226-bp fragment and to the 190-bp fragment generated after *Hha*I digestion are shown in the figure. All MHS patients and the MHE patient are heterozygous for the G3333A mutation; and all MHN patients are homozygous for the normal sequence. Symbols and numbering are as defined in figure 1.

at risk for MHS. The mutation was also absent in a population of 50 unrelated MHS individuals belonging to MHS families mostly linked to the RYR1 locus.

Discussion

This report represents the first genetic evidence of a mutation affecting a voltage-dependent calcium channel of the skeletal muscle and associated with MHS. Results obtained in a French MHS pedigree with the MHS trait defined according to the European IVCT protocol provide direct evidence that MHS is associated with an Arg1086His mutation in the product of the CACNL1A3 gene on chromosome 1q31. The Arg1086His mutation in CACNL1A3 appears to be a private mutation, restricted so far to a single French pedigree among >50 French and Italian MHS families that we have tested. This situation is not unusual, since it was also the case for some RYR1 mutations (Zhang et al. 1993; Quane et al. 1994*b*) and for the reports linking MHS with other loci (Iles et al. 1994*a;* Sudbrak et al. 1995). Together with the clear segregation of the mutation with the MHS trait and the fact that the mutation affects a well-conserved amino acid, the absence of the mutation in 100 unrelated MHN chromosomes contributes to exclude this mutation as being a simple polymorphism. MHS is characterized by both allelic and genetic heterogeneities. As a matter of fact, >10 different mutations of the RYR1 gene have been reported, illustrating a clear-cut allelic heterogeneity in RYR1. On the other hand, at least four different loci have been associated with MHS. However, none of them has offered direct proof—that is, a two-point LOD score >+3.0 or a molecular defect—that a gene other than RYR1 is involved in MHS. Therefore, our data represent the first ironclad report of locus heterogeneity for MHS.

Three individuals (IV-1, IV-2, and IV-7) too young to have an IVCT carry the mutation. Identification of the CACNL1A3 gene mutation allows a direct genetic diagnosis for those patients in the VE2GR family who are at risk for MHS. Among the three individuals who were diagnosed as MHE by IVCT, two (III-5 and III-11) have the mutation and can be considered MHS from a genetic point of view (i.e., on the basis of mutation and haplotyping). The third MHE patient (II-7) does not have the mutation and is unrelated to the first generation of this MHS family. Despite the fact that the gene frequency for MHS is estimated at only 1/10,000, one cannot formally exclude the presence of another MHS-associated mutation in this individual, which might explain the partial response to halothane. Conversely, in some of our study's MHS families in which the disease has been clearly associated with a mutation in the RYR1 gene, we have observed that individuals can test as MHEh or MHEc even in the absence of the causative mutation (data not shown).

IVCT results obtained in this family in which MHS is associated with the Arg1086His mutation in the α1-subunit of the skeletal muscle VDCC did not differ significantly from those obtained in families in which MHS is associated with an RYR1 mutation. Interestingly, skeletal muscle VDCC mutations might cause hypoKPP (MIM 170400; McKusick 1990), and three different mutations of the CACNL1A3 gene have been associated with the disease (Jurkat-Rott et al. 1994; Ptacek et al. 1994). HypoKPP belongs to a particular group of skeletal muscle diseases that are due to an abnormal function of ion channels: the "muscle ion-channel diseases." This disorder is transmitted as an autosomal dominant trait and is characterized by episodic weakness associated with decreased serum potassium concentration. Thus, defects in CACLN1A3 can lead to various symptoms and to various disease nomenclatures. On the basis of these studies, at least some forms of MHS and hypoKPP

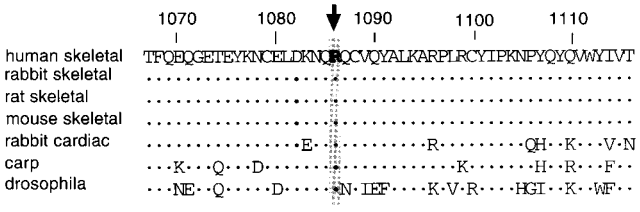

Figure 4 Amino acid sequence of the α1-subunits and related isoforms in the region flanking the residue Arg1086. This region corresponds to the cytoplasmic domain of the dihydropyridine receptor, localized between the S6 transmembranous sequence of domain III and the S1 transmembrane segment of domain IV (Hogan et al. 1994). Residues identical to the human sequence are indicated by dots.

appear to be allelic disorders. A similar situation exists with hypoKPP and paramyotonia congenita, both of which result from mutations in the adult sodium-channel gene SCN4A (Ptacek et al. 1993), and with MHS and CCD. In the latter situation, both pathologies are caused by mutations in the RYR1 gene (Quane et al. 1993; Zhang et al. 1993), with some mutations being associated with both phenotypes in some patients whereas other mutations are associated specifically with MHS. However, in contrast to the situation with MHS and CCD, a study investigating the relation of MHS with hypoKPP indicated that the latter is not associated with MHS (Lehmann-Horn and Iaizzo 1990). In agreement with that report, no clinical symptoms or biological signs that might be associated with hypoKPP have been observed in the VE2GR family. However, in the literature a case has been reported in which a patient with hypoKPP experienced an MH episode. This patient was the only case of hypoKPP in his family and was classified as MHEh after IVCT was performed on a muscle biopsy (Lambert et al. 1994).

The hypoKPP mutations that so far have been described occur in the S4 segments of domains II (Arg528His) and IV (Arg1239His and Arg1239Gly) (fig. 5). The S4 segments of these voltage-gated ion channels are thought to function as voltage sensors (Catterall 1988). Since the EC coupling and calcium-conducting properties of the dihydropyridine-sensitive VDCC are voltage sensitive, it was postulated that these mutations, which involve the loss of an arginine, a positively charged amino acid, in the S4 segments may alter one or both of these functions. On the other hand, on the basis of studies of chimeric dihydropyridine receptors, it has been shown that the putative cytoplasmic region between domains II and III of the α1-subunit of the skeletal muscle VDCC is an important determinant of skeletal-type excitation-contraction (EC) coupling (Tanabe et al. 1990), whereas the cytoplasmic region between domains I and II might be involved in interactions with the β-subunit of the skeletal muscle VDCC (Pragnell et al. 1994). The α1-subunit domain I-II linker contributes also to a PKC-dependent up-regulation of the channel activity, through a direct interaction with a G-protein βγ complex (De Waard et al. 1997; Zamponi et al. 1997). The α1-subunit of the skeletal muscle VDCC is an in vivo substrate for many protein kinases, and several phosphorylation sites have been characterized within the COOH terminal region of the protein (De Jongh et al. 1989). It is interesting, however, to note that two isoforms of the α1-subunit have been described

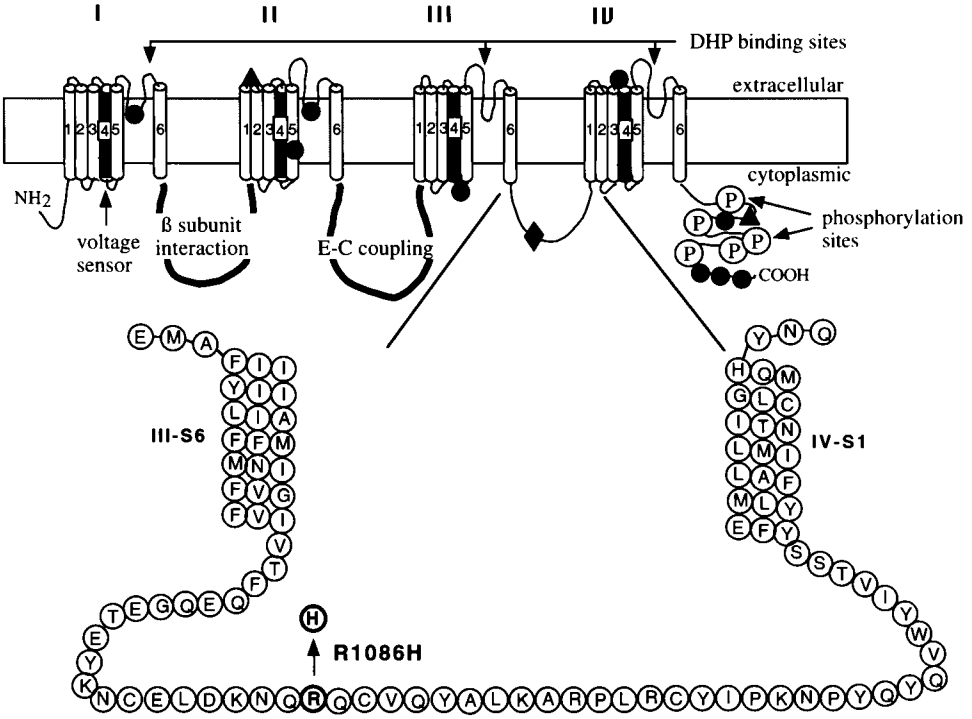

Figure 5 Schematic representation of the α1-subunit of the skeletal muscle dihydropyridine receptor. Sequences involved in both EC coupling and interaction with the β-subunit of the dihydropyridine receptor are shown in boldface. S4 transmembrane segments of the different domains that operate as voltage sensors are blackened, and dihydropyridine binding sites and phosphorylation sites present in the C-terminal region are indicated. Positions of the sequence modifications are represented: black dots denote homozygous corrections; black triangles denote heterozygous polymorphisms; and black diamonds denote Arg1086His mutation.

in adult skeletal muscle. The minor, full-length form contains 1,873 amino acids, whereas the major form, truncated in the COOH terminal region, is 1,685 amino acids long (De Jongh et al. 1991). Three dihydropyridine binding sites have been described between the S5 and S6 transmembrane segments of domains I, III, and IV (Kalasz et al. 1993).

Except on exposure to halogenated anesthetics and succinyl choline, most MHS individuals do not usually present symptoms related to the muscle ion-channel diseases. This suggests that a mutation associated with the MHS trait does not dramatically affect the essential functions of the α1-subunit. In effect, as shown in the model presented in figure 5, the Arg1086His mutation, which is localized in the domain III-IV linker, belongs neither to one of the membranous domains involved in the voltage-gated channel, nor to the EC coupling domain, nor to potential phosphorylation sites. Interestingly, the mutation is localized in a region present in both adult isoforms but, along with the EC domain, absent in the newborn isoform (Malouf et al. 1992). On the other hand, the α-subunit of the sodium channel and the α1-subunit of the L-type calcium channel have a close transmembrane organization. The domain III-IV linker has been implicated in sodium-channel inactivation, and several mutations in this region have been associated with paramyotonia congenita and myotonia fluctuans (Barchi 1995). Hence, this region might be a mutation hot spot in both skeletal muscle sodium- and calcium-channel genes, even if the functional role of this region is less documented in the skeletal muscle dihydropyridine-sensitive VDCC.

On the basis of coimmunoprecipitation experiments and freeze-fracture studies, it has been reported that the skeletal muscle VDCC interact with the ryanodine receptor to form the triad (Marty et al. 1994; Takekura et al. 1994). A direct, reciprocal, energetic interaction between RYR-1 and the skeletal muscle VDCC recently has been demonstrated elsewhere (Nakai et al. 1996). When the proposed model for the structure of the α1-subunit is taken into account, a region spanning amino acids V1065–S1118 and located between domains III and IV represents a putative cytoplasmic region that may interact directly or indirectly with the RYR-1 receptor. Therefore, one can hypothetize that the Arg1086His mutation might affect the interaction between the α1-subunit of the skeletal muscle VDCC and the RYR-1 receptor present in the sarcoplasmic reticulum. This modification of interaction between receptors could mimic the modifications generated by mutations in the RYR-1 protein that have been described in MHS families. This situation, in which the same MHS phenotype could be associated with defects in one of the proteins forming the triad at the T-tubule/sarcoplasmic-reticulum junction, reflects that encountered with muscular dystrophies and the dystophin-associated proteins in the skeletal muscle (Sunada and Campbell 1995). In the latter case, almost identical clinical phenotypes are associated with defects of genes encoding functionally associated proteins. Expression studies of the mutated subunit and biochemical investigations will provide more insight into the interactions between the skeletal muscle VDCC and the RYR receptor in the regulation of calcium movements in skeletal muscle fibers.

Acknowledgments

We thank all family members for their participation in this study, J. Lerale for technical assistance, Dr. C. Mueller for helpful discussions and MSIM simulation studies, and Dr. A. Fuchs for critical reading of the manuscript. This work was supported in part by grants from Association Française contre les Myopathies, DRRC-CHU Grenoble, and Fondation Daniel Ducoin.

References

Bean BP (1984) Nifedipine block of cardiac calcium channels: high affinity binding to the inactivated state. Proc Natl Acad Sci USA 81:6388–6392

Catterall WA (1988) Structure and function of voltage-sensitive ion channels. Science 242:50–61

Chomczynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

De Jongh KS, Merrick DK, Catterall WA (1989) Subunits of purified calcium channels: a 212-kDa form of α1 and partial amino acid sequence of a phosphorylation site of an independent β subunit. Proc Natl Acad Sci USA 86:8585–8589

De Jongh KS, Warner C, Colvin AA, Catterall WA (1991) Characterization of the two forms of the α1-subunit of skeletal muscle L-type calcium channels. Proc Natl Acad Sci USA 88:10778–10782

Deufel T, Golla A, Iles D, Meindl A, Meitinger T, Schindelhauer D, DeVries A, et al (1992) Evidence for genetic heterogeneity of malignant hyperthermia susceptibility. Am J Hum Genet 50:1151–1161

De Waard M, Liu H, Walker D, Scott VES, Gurnett CA, Campbell KP (1997) Direct binding of G-protein bg complex to voltage-dependent calcium channels. Nature 385: 446–450

European Malignant Hyperpyrexia Group (1984) A protocol for the investigation of malignant hyperpyrexia (MH) susceptibility. Br J Anaesth 56:1267–1269

Gillard EF, Otsu K, Fujii J, Khanna VK, DeLeon S, Derdemezi J, Britt BA, et al (1991) A substitution of cysteine for arginine 614 in the ryanodine receptor is potentially causative of human malignant hyperthermia. Genomics 11:751–755

Grabner M, Friedrich K, Knaus HG, Striessnig J, Scheffauer F, Staudinger R, Koch WJ, et al (1991) Calcium channels from Cyprinus carpio skeletal muscle. Proc Natl Acad Sci USA 88:727–731

Gregg RG, Couch F, Hogan K, Powers PA (1993*a*) Assignment

of the human gene for the α1-subunit of the skeletal muscle DHP-sensitive Ca2+ channel (CACNL1A3) to chromosome 1q31-q32. Genomics 15:107–112

Gregg R, Powers P, Hogan K (1993*b*) Assignment of the human gene for the β subunit of the voltage dependent calcium channel (CACNLB1) to chromosome 17 using somatic cell hybrids and linkage mapping. Genomics 15:186–187

Hogan K, Gregg RG, Powers PA (1996) The structure of the gene encoding the human skeletal muscle α1-subunit of the dihydropyridine-sensitive L-type calcium channel (CACNL1A3). Genomics 31:392–394

Hogan K, Powers PA, Gregg RC (1994) Cloning of the human muscle α1-subunit of the dihydropyridine-sensitive L-type calcium channel (CACNL1A3). Genomics 24:608–609

Iles DE, Lehmann-Horn F, Scherer SW, Tsui LC, Weghuis DO, Suijkerbuijk RF, Heytens L, et al (1994*a*) Localization of the gene encoding the α2/δ subunits of the L-type voltage-dependent calcium channel to chromosome 7q and analysis of the segregation of flanking markers in malignant hyperthermia susceptible families. Hum Mol Genet 3:969–975

Iles DE, Segers B, Heytens L, Sengers RCA, Wieringa B (1992) High-resolution physical mapping of four microsatellite repeat markers near the RYR1 locus on chromosome 19q13.1 and apparent exclusion of the MHS locus from this region in two malignant hyperthermia susceptible families. Genomics 14:749–754

Iles DE, Segers B, Olde Weghuis D, Suijkerbuijk R, Mikala G, Schwartz A, Wieringa B (1994*b*) Refined localization of the α1-subunit of the skeletal muscle L-type voltage dependent calcium channel (CACNL1A3) to human chromosome 1q32 by in situ hybridization. Genomics 19:561–563

Iles DE, Segers B, Sengers RCA, Monsieur K, Heytens L, Halsall PJ, Hopkins PM, et al (1993) Genetic mapping of the β1- and γ-subunits of the human skeletal muscle L-type voltage-dependent calcium channel on chromosome 17q and exclusion as candidate genes for malignant hyperthermia susceptibility. Hum Mol Genet 2:863–868

Jeanpierre M (1987) A rapid method for the purification of DNA from blood. Nucleic Acids Res 15:9611

Jurkat-Rott K, Lehmann-Horn F, Elbaz A, Heine R, Gregg RG, Hogan K, Powers PA, et al (1994) A calcium channel mutation causing hypokalemic periodic paralysis. Hum Mol Genet 3:914–917

Kalasz H, Watanabe T, Yabana H, Itagaki K, Naito K, Nakayama H, Schwartz A, et al (1993) Identification of 1,4-dihydropyridine binding domains within the primary structure of the α1-subunit of the skeletal muscle L-type calcium channel. FEBS Lett 331:177–181

Kalow W (1987) Inheritance of malignant hyperthermia—a review of published data. In: Britt BA (ed) Malignant hyperthermia. Martinus-Nijhoff, Amsterdam and Boston, pp 155–180

Lambert C, Blanloeil Y, Krivosic-Horber R, Berard L, Reyford H, Pinaud M (1994) Malignant hyperthermia in a patient with hypokalemic periodic paralysis. Anesth Analg 79: 1012–1014

Larach M (1989) Standardization of the caffeine halothane muscle contracture test. Anesth Analg 69:511–515

Lathrop GM, Lalouel JM (1984) Easy calculations of lod scores and genetic risks on small computers. Am J Hum Genet 36:460–465

Lehmann-Horn F, Iaizzo PA (1990) Are myotonias and periodic paralyses associated with susceptibility to malignant hyperthermia? Br J Anaesth 65:692–697

Levitt RC, Olckers A, Meyers S, Fletcher JE, Rosenberg H, Isaacs H, Meyers DA (1992) Evidence for the localization of a malignant hyperthermia susceptibility locus (MHS2) to human chromosome 17q. Genomics 14:562–566

MacLennan DH, Duff C, Zorzato F, Fujii J, Phillips M, Korneluk RG, Frodis W, et al (1990) Ryanodine receptor gene is a candidate for predisposition to malignant hyperthermia. Nature 343:559–561

MacLennan DH, Phillips M (1992) Malignant hyperthermia. Science 256:789–794

Malouf NN, McMahon DK, Hainsworth CN, Kay BK (1992) A two-motif isoform of the major calcium channel subunit in skeletal muscle. Neuron 8:899–906

Marty I, Robert M, Villaz M, DeJongh KS, Lai Y, Catteral WA, Ronjat M (1994) Biochemical evidence for a complex involving dihydropyridine receptor and ryanodine receptor in triad junctions of the skeletal muscle. Proc Natl Acad Sci USA 91:2270–2274

McCarthy TV, Healy JMS, Heffron JJA, Lehane M, Deufel T, Lehmann-Horn F, Farrall M, et al (1990) Localization of the malignant hyperthermia locus to human chromosome 19q12-13.2. Nature 343:562–564

McKusick VA (ed) (1990) Mendelian inheritance in man. Johns Hopkins University Press, Baltimore and London

Nakai J, Dirksen RT, Nguyen HT, Pessah IN, Beam KG, Allen PD (1996) Enhanced dihydropyridine receptor channel activity in the presence of ryanodine receptor. Nature 380: 72–74

Olckers A, Meyers DA, Meyers S, Taylor EW, Fletcher JE, Rosenberg H, Isaacs H, et al (1992) Adult muscle sodium channel α-subunit is a candidate gene for malignant hyperthermia susceptibility. Genomics 14:829–831

Powers P, Liu S, Hogan K, Gregg R (1993) Molecular characterization of the gene encoding the γ subunit of the human skeletal muscle DHP-sensitive Ca2+ channel (CACNLG), cDNA sequence, gene structure and chromosomal location. J Biol Chem 268:9275–9279

Powers P, Scherer SW, Tsui L, Gregg RG, Hogan K (1994) Localization of the gene encoding the α2/δ subunit (CACNL2) of the human skeletal muscle voltage dependent calcium channel to chromosome 7q21-22 by somatic cell hybrid analysis. Genomics 19:192–193

Pragnell M, de Waard M, Mori Y, Tanabe T, Snutch TP, Campbell KP (1994) Calcium channel β-subunit binds to a conserved motif in the I-II cytoplasmic linker of the α1-subunit. Nature 368:67–70

Ptacek LJ, Gouw L, Kwiecinski H, McManis PG, Mendell J, George AL, Barchi RL, et al (1993) Sodium channel mutations in paramyotonia congenita and hyperkalemic periodic paralysis. Ann Neurol 33:300–307

Ptacek LJ, Tawil R, Griggs RC, Engel AG, Layzer RB, Kwiecinski H, McManis PG, et al (1994) Dihydropyridine receptor mutations cause hypokalemic periodic paralysis. Cell 77: 863–868

Quane KA, Healy JMS, Keating KE, Manning BM, Couch FJ,

Palmucci LM, Doriguzzi C, et al (1993) Mutations in the ryanodine receptor gene in central core disease and malignant hyperthermia. Nat Genet 5:51–55

Quane KA, Keating KE, Healy JMS, Manning BM, Krivosic-Horber R, Krivosic I, Monnier N, et al (1994*a*) Mutation screening of the RYR1 gene in malignant hyperthermia: detection of a novel tyr to ser mutation in a pedigree with associated central cores. Genomics 23:236–239

Quane KA, Keating KE, Manning BM, Healy JMS, Monsieurs K, Heffron JJA, Lehane M, et al (1994*b*) Detection of a novel common mutation in the ryanodine receptor gene in malignant hyperthermia: implication for diagnosis and heterogeneity studies. Hum Mol Genet 3:471–476

Sudbrak R, Golla A, Hogan K, Powers PA, Gregg RG, Du Chesne I, Lehmann-Horn, et al (1993) Exclusion of malignant hyperthermia susceptibility (MHS) from a putative MHS2 Locus on chromosome 17q and of the α1-, β1-, and γ-subunits of the dihydropyridine receptor calcium channel as candidates for the molecular defect. Hum Mol Genet 2: 857–862

Sudbrak R, Procaccio V, Klausnitzer M, Curran JL, Monsieurs K, Van Broeckhoven C, Ellis R, et al (1995) Mapping of a further malignant hyperthermia susceptibility locus to chromosome 3q13.1. Am J Hum Genet 56:684–691

Sunada Y, Campbell K (1995) Dystrophin-glycoprotein complex: molecular organization and critical roles in skeletal muscle. Curr Opin Neurol 8:379–384

Takekura H, Bennett L, Tanabe T, Beam KG, Franzini-Armstrong C (1994) Restoration of junctional tetrads in dysgenic myotubes by dihydropyridine receptor DNA. Biophys J 67: 793–803

Tanabe T, Beam KG, Adams BA, Niidome T, Numa S (1990) Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling. Nature 346:567–569

Tanabe T, Takeshima H, Mikami A, Flockerzi V, Takahashi H, Kangawa K, Kojima M, et al (1987) Primary structure of the receptor for calcium channel blockers from skeletal muscle. Nature 328:313–318

Zamponi GW, Bourinet E, Nelson D, Nargeot J, Snutch TP (1997) Crosstalk between G proteins and protein kinase C mediated by the calcium channel α1-subunit. Nature 385: 442–446

Zhang Y, Chen HS, Khanna VK, De Leon S, Phillips M, Schappert K, Britt BA, et al (1993) A mutation in the human ryanodine receptor gene associated with central core disease. Nat Genet 5:46–51

Zheng W, Feng G, Ren D, Eberl DF, Hannan F, Dubald M, Hall LM (1995) Cloning and characterization of a calcium channel α1-subunit from *Drosophila melanogaster* with similarity to the rat brain type D isoform. J Neurosci 15: 1132–1143