Identification of Novel Mutations in the Ryanodine-Receptor Gene (RYR1) in Malignant Hyperthermia: Genotype-Phenotype Correlation

Bernadette M. Manning,[1] Kathleen A. Quane,[1] Helle Ording,[4] Albert Urwyler,[5] Vincenzo Tegazzin,[6] Mary Lehane,[3] John O'Halloran,[2] Edmund Hartung,[7] Linda M. Giblin,[1] Patrick J. Lynch,[1] Pat Vaughan,[1] Kathrin Censier,[5] Diana Bendixen,[4] Giacomo Comi,[9] Luc Heytens,[10] Koen Monsieurs,[10,11] Tore Fagerlund,[12] Werner Wolz,[8] James J. A. Heffron,[1] Clemens R. Muller,[8] and Tommie V. McCarthy[1]

Departments of [1]Biochemistry and [2]Zoology and Animal Ecology, University College Cork, and [3]Department of Anaesthesia, Cork University Hospital, Cork; [4]Department of Anaesthesiology, Herlev Hospital, University of Copenhagen, Copenhagen; [5]Department of Anaesthesia, University Hospital Kantonsspital, Basel; [6]Department of Anaesthesiology and Critical Care Medicine, Traumatologic-orthopaedic Hospital, Padova, Italy; [7]Institute for Anaesthesiology, University of Wurzburg, and [8]Institut fur Humangenetik, Biozentrum, Wurzburg, Germany; [9]Institute of Clinical Neurology, Insituto di Ricovero e Cura a Carattere Scientifico Ospedale Maggiore Policlinico, University of Milan, Milan; [10]Department of Intensive Care, Malignant Hyperthermia Laboratory, and [11]Laboratory of Neurogenetics, Borne-Bunge Foundation, University of Antwerp, Antwerp; and [12]Institute for Medical Genetics, Ulleval University Hospital, Blindem, Norway

Summary

Malignant hyperthermia (MH) is a pharmacogenetic disorder of skeletal muscle that is triggered in genetically predisposed individuals by common anesthetics and muscle relaxants. The ryanodine receptor (RYR1) is mutated in a number of MH pedigrees, some members of which also have central core disease (CCD), an inherited myopathy closely associated with MH. Mutation screening of 6 kb of the RYR1 gene has identified four adjacent novel mutations, C6487T, G6488A, G6502A, and C6617T, which result in the amino acid alterations Arg2163Cys, Arg2163His, Val2168Met, and Thr2206Met, respectively. Collectively, these mutations account for 11% of MH cases and identify the gene segment 6400–6700 as a mutation hot spot. Correlation analysis of the in vitro contracture-test data available for pedigrees bearing these and other RYR1 mutations showed an exceptionally good correlation between caffeine threshold and tension values, whereas no correlation was observed between halothane threshold and tension values. This finding has important ramifications for assignment of the MH-susceptible phenotype, in genotyping studies, and indicates that assessment of recombinant individuals on the basis of caffeine response is justified, whereas assessment on the basis of halothane response may be problematic. Interestingly, the data suggest a link between the caffeine threshold and tension values and the MH/CCD phenotype.

Introduction

Malignant hyperthermia (MH) is a potentially fatal disorder of skeletal muscle. An MH episode can be precipitated in susceptible individuals by exposure to triggering agents, such as inhalational anesthetics or depolarizing muscle relaxants. The clinical features of an MH episode are hyperthermia, accelerated muscle metabolism, contractures, metabolic acidosis, and tachycardia. In the absence of triggering agents, susceptible individuals are normal, although cramps, fatigue, and heat intolerance have been reported (Denborough et al. 1962; Ellis and Heffron 1985; Gronert 1986).

Because of the subclinical nature of the condition, diagnosis in advance of exposure to anesthesia is difficult. However, an in vitro diagnostic test for diagnosis of susceptibility to MH has been developed and is based on the differential contractile response of normal and MH muscle to caffeine (Kalow et al. 1970) and to halothane (Ellis and Harriman 1973). A standardized European in vitro contracture test (IVCT) was established in 1984 (European Malignant Hyperthermia Group 1984). It allows the following diagnoses to be made: MH susceptible (MHS), MH equivocal (MHE), and MH normal (MHN). If a muscle-biopsy strip produces a sustained increase in muscle tension of 0.2 g, at ⩽2% halothane and, independently, at a caffeine concentration of ⩽2 mM, the patient is considered to be MHS. MHN is diagnosed if the 0.2-g threshold is not attained at these concentrations, whereas MHE is diagnosed if the threshold tension is attained with caffeine (MHE[c]) or halothane (MHE[h]) but not with both. The clinical and biochemical relationship between the MHS and MHE phenotypes is unclear.

Molecular-genetic studies of humans and pigs have

Table 1

RYR1 Mutations Identified in MHS and CCD Families

Mutation	Reference	Nucleotide Sequence	Amino Acid Sequence[a]	MH Families with Mutation
Cys35Arg	Lynch et al. (1997)	aagctc t/c gcctggcc	eqlKl C/R LaaEG	1 family
Arg163Cys[b]	Quane et al. (1993)	aaggtc c/t gcgttggg	EGEKV R/C VGDDI	2%
Gly248Arg	Gillard et al. (1992)	tatgag g/a ggggagct	lvyYE g/r Gavct[c]	2%
Gly341Arg	Quane et al. (1994*b*)	aagtac g/a gggagtca	peIKY G/R ESlcf	6%
Ile403Met[b]	Quane et al. (1993)	cgcatgat c/g cacagc	QAARm I/M hsTng	1 family
Tyr522Ser[b]	Quane et al. (1994*a*)	cttctct a/c tgaactc	Ivnll Y/S elLAS	1 family
Arg552Trp	Keating et al. (1997)	ctggat c/t ggctggag	vsKLd r/w leaSS[d]	1 family
Arg614Cys	Gillard et al. (1991)	gctgta c/t gctccaac	NGVAV R/C SnQdl	4%
Arg614Leu	Quane et al. (1997)	gctgta c/a gctccaa	NGVAV R/L SnQdl	2%
Arg2163Cys	This study	cagatc c/t gctcgctg	cLgQI R/C SLLiV	4%
Arg2163His[b]	This study	cagatcc g/a ctcgctg	cLgQI R/H SLLiV	1 family
Val2168Met	This study	ctcatc g/a tgcagatg	RSLLi V/M QMgpQ	7%
Thr2206Met	This study	cacgaga c/t ggtcatg	LgMHE T/M VMeVM	1 family
Gly2435Arg	Keating et al. (1994); Phillips et al. (1994)	ctgctc g/a gacgctgt	LIDLL G/R RCAPE	4%
Arg2436His[b]	Zhang et al. (1993)	ctcggac g/a ctgtgca	IDLLG R/H CAPEm	1 family
Arg2458Cys	Manning et al. (1998)published data	atcctc c/t gctcccctt	iRAiL R/C SLVPl	4%
Arg2458His	Manning et al. (1998)published data	atcctcc g/a ctcccctt	iRAiL R/H SLVPl	4%

[a] Conserved/semiconserved amino acids are indicated by capital letters.
[b] Associated with CCD.
[c] G248 conserved in seven of nine RYR genes.
[d] R552 conserved in eight of nine RYR genes.

established the ryanodine receptor (RYR1) on chromosome 19 as the primary MHS locus (MHS1; MIM 180901) (MacLennan et al. 1990; McCarthy et al. 1990). The RYR1 protein has a subunit size of 560 kD and forms an elaborate tetrameric structure that acts both as a calcium-release channel and a foot structure bridging the gap between the sarcoplasmic reticulum and the t-tubule, in skeletal muscle (reviewed in MacLennan and Phillips 1992). The RYR1 gene is comprised of 106 exons encompassing a total of 160 kb and, as such, is one of the most complex genes characterized, to date (Phillips et al. 1996).

Genetic heterogeneity has been reported in MH. Alternative MHS loci have been proposed to reside on chromosomes 17q11.2-q24 (Levitt et al. 1992) and 7q11.23-q21.1 (Iles et al. 1994), LOD scores in favor of linkage in these cases are marginal. In addition, in a single large pedigree, linkage has been established between the MHS locus and markers on the q13.1 region of chromosome 3 (Sudbrak et al. 1995). More recently, in a second single large pedigree, linkage has been established between the MHS locus and markers on chromosome 1q (Robinson et al. 1997), and putative causative mutations have been identified in a major candidate gene in this region, CACNL1A3, which encodes the α1-

subunit of the dihydropyridine receptor (Monnier et al. 1997).

Mutation screening of the RYR1 gene in MHS individuals has identified 13 mutations, to date. The details of these mutations and their incidence are shown in table 1. Mutations in the RYR1 gene also have been described in central core disease (CCD), a rare inherited myopathy (Shy and Magee in 1956) that is closely associated with MH (Shuaib et al. 1987). Patients with CCD often exhibit hypotonia and proximal muscle weakness in infancy, leading to delayed motor milestones. CCD is a disorder that exhibits substantial variability both clinically and histologically, and the severity of symptoms may vary from normal to severe. Diagnosis of CCD is by histological examination of muscle-biopsy tissue, which shows amorphous central areas (cores) in type 1 fibers, with a relative lack, if not a complete absence, of mitochondria in core regions (Hayashi et al. 1989). Patients with CCD are at risk for MH and, in almost all cases, are diagnosed as MHS by the IVCT.

Autosomal dominant inheritance of the MHS trait has generally been observed in most large European MHS families, although it is unclear whether this will be the case for all MHS families. However, it is not clear what percentage of individuals diagnosed as MHS will actu-

ally develop a clinical episode of MH when exposed to a triggering agent. One report, by Kalow (1987), based on an epidemiological study of MH "at-risk" individuals, estimated that approximately one-third of susceptible individuals develop a clinical episode of MH when exposed to a triggering agent, indicating a penetrance of .33.

The thirteen RYR1 mutations identified, to date, are located in two regions of the RYR1 gene, the amino-terminal and central regions, and indicate that other mutations causing MH and CCD may cluster in these areas. We have analyzed 6 kb covering these regions of the RYR1 gene, and, in this article, we report four novel mutations in the central portion of the gene. IVCT data for RYR1 mutations described elsewhere and for the mutations reported in this article were collected and have allowed us to perform the first major genotype-phenotype correlation for RYR1 mutations and IVCT responses.

Patients and Methods

Patients

The pedigrees of the families we investigated are shown in figure 1. At age 12 years, the proband in pedigree D1 exhibited symptoms consistent with the onset of an MH crisis, while undergoing opthalmic surgery for the first time. Ten minutes after initiation of anesthesia with halothane and succinylcholine, body temperature rose to 37.4°C, accompanied by myoglobinurea, masseter spasm, and ventricular arrythmia. The patient's maximal heart rate was measured at 178 beats per min (bpm), his maximal potassium level was 4.8 mEq/liter, and his maximal creatine kinase (CK) level was 6,0520 U/liter. Dantrolene therapy was not undertaken to reverse the course of the crisis, and the patient survived without sequela. The proband in pedigree D2 had an MH crisis at age 27 years, while undergoing surgery (orthopedic) for the first time, with halothane and succinylcholine. The maximal temperature recorded during the MH crisis was 39.2°C. The anesthesia duration prior to development of the MH crisis was 90 min. Other characteristics of this crisis included masseter spasm, inappropriate tachypnea, and sinus tachycardia. The maximal heart rate was measured at 150 bpm, and the maximal CK level was >2,500 U/liter. Dantrolene therapy was not undertaken.

The S6 pedigree is of Swiss descent. A detailed description of the proband in this pedigree has been published previously by Urwyler et al. (1994).

The proband in the Irish pedigree, Ir4, experienced an MH crisis while undergoing orthopedic surgery. After initiation of anesthesia with isoflurane and succinylcholine, masseter spasm developed. Clinical symptoms included myoglobinurea and cola-colored urine during the perioperative period. The maximal CK level was measured at 40,700 U/liter. Rapid reversal of the crisis was achieved without dantrolene intervention, and the proband survived without sequela.

The proband in the Italian pedigree, It2, had undergone eight previous surgical procedures, under general anesthesia, before presenting with a fulminant MH crisis. On six of these previous occasions, an MH-triggering anesthetic had been used. Histological examination of the muscle revealed a predominance of type I fibers, with central cores present in many fibers, indicating the presence of asymptomatic CCD. A complete clinical picture of this case is documented in the study by Tegazzin et al. (1994).

All IVCTs were performed according to the standardized protocol. In this protocol, muscle strips are standardized to a 15–25-mm length and a 100-mg weight. The appropriate informed consent was obtained from all subjects.

RYR1 Mutation Analysis

Genomic DNA was isolated from whole blood lysate with a phenol/chloroform extraction followed by isopropanal precipitation. Total RNA was isolated from frozen MHS muscle-biopsy samples, by acid guanidinium-phenol-chloroform extraction (Chomczynski and Sacchi 1987). First-strand cDNA was synthesized by use of the Promega reverse-transcription system and random primers. PCR amplification of overlapping RYR1 fragments of 250–400 bp in length was performed by use of primer sets designed from the published human RYR1 sequence (Zorzato et al. 1990).

The primer sets chosen covered the gene sections -10 to 3088 and 5114 to 8052. The PCR reactions for SSCP analysis were performed as outlined elsewhere (Quane et al. 1993). Nondenaturing gel electrophoresis for SSCP detection was undertaken by use of the following three conditions: 6% polyacrylamide:bisacrylamide (99:1) with 5% glycerol, at room temperature and at 4°C, and without glycerol, at 4°C (Orita et al. 1989).

The four mutations described were detected in the RYR1 fragment 6379–6766. This fragment was amplified by use of the primers 6379F (5'-CAG TAC GAC GGG CTG GGT GAG-3') and 6766R (5'-GCC ACT GTT CTC CAG CAG GTA-3'). Direct sequencing was performed as described by Quane et al. (1993), by use of primer 6379F.

A 64-bp product of exon 39, encompassing the mutations C6486T, G6487A, and G6501A, was amplified from genomic DNA by use of primers 6464F (5'-TGC TCG AGT GCC TCG GCC AGAT-3') and 6506R (5'-TTC TCC TCC TGG GGG CCC ATCT-3'). The mutation C6617T is located in exon 40 of the RYR1 gene,
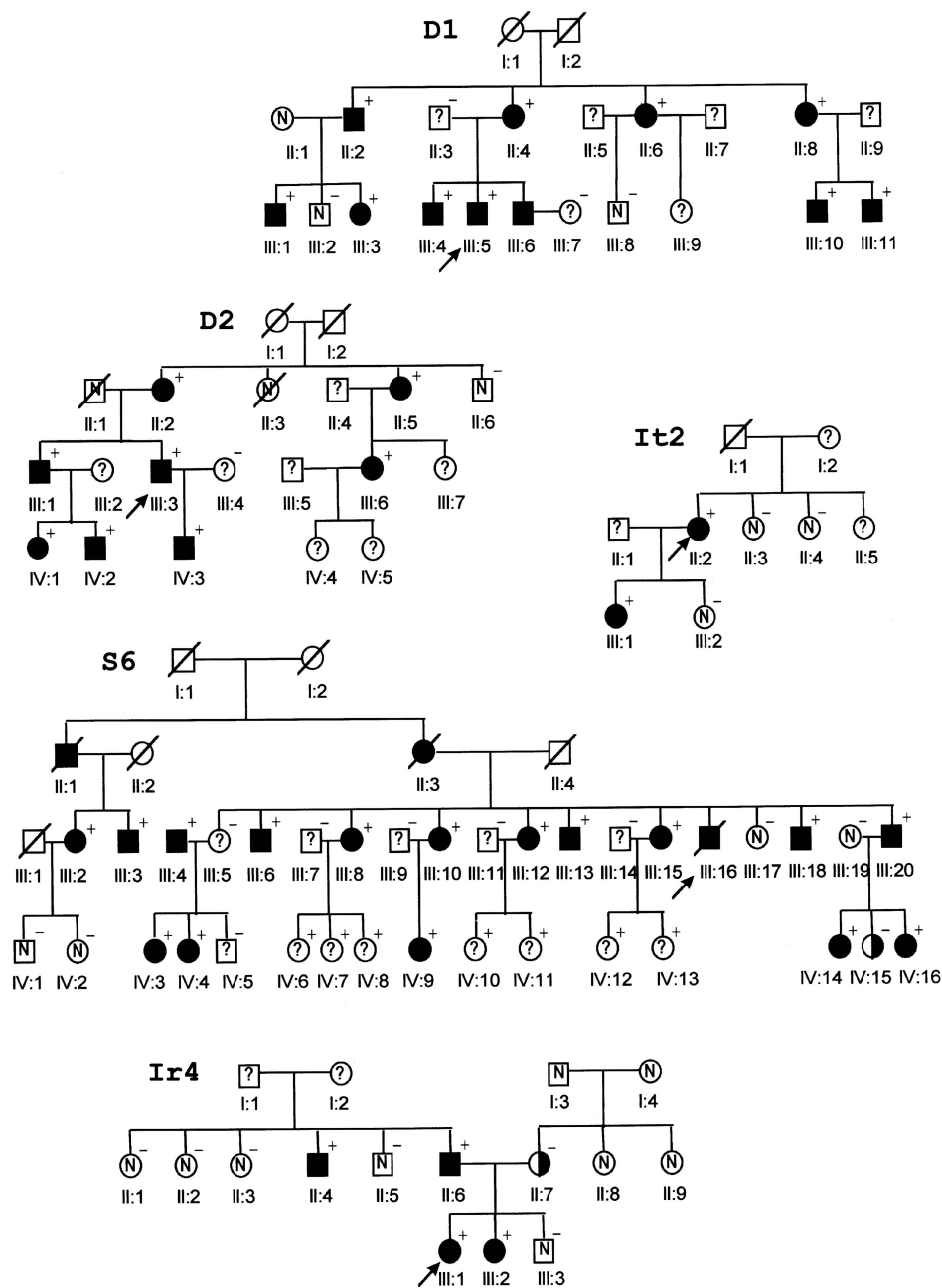
Figure 1   Segregation of the RYR1 mutations Arg2163Cys (in pedigrees D1 and D2), Arg2163His (in pedigree It2), Val2168Met (in pedigree S6), and Thr2206Met (in pedigree Ir4). Blackened symbols indicate individuals who are MHS; unblackened symbols indicate individuals who are MHN; half-blackened symbols indicate individuals who are MHE(h); and a question mark (?) indicates individuals for whom disease status is unknown. Segregation of the respective mutations also is indicated: a plus sign (+) indicates that an individual is heterozygous for the mutant allele, and a minus sign (−) indicates that an individual is homozygous for the normal allele.

and a 74-bp product encompassing this mutation was amplified by use of primers 6588F (5′-CCT GAT GAG GGC GCT GGG CAT-3′) and 6641R (5′-TGG ACT CGC CGC CCC CGA GGA-3′). Standard PCR conditions were used for amplification (denaturation at 94°C

for 1 min, annealing at 64°C for 1 min [both primer sets], and extension at 72°C for 1 min).

Because of the close proximity of the three adjacent mutations in exon 39 and the unavailability of a restriction site for the C6617T mutation in exon 40, SSCP

analysis was the method of choice for detection of these mutations. The optimal electrophoretic conditions for detection of these mutations were 10% polyacrylamide:bisacrylamide (99:1) with 5% glycerol, at 4°C and 50 W.

Statistical Analysis

IVCT data were collected for all published RYR1 mutations, with the exception of Arg2435His and Gly248Arg, which are rare in the European MHS population. The IVCT results were recorded as four separate entities—namely, the concentrations of caffeine and halothane at which a threshold tension of 0.2 g is reached and the tensions generated by 2% (0.44 mM) halothane and 2 mM caffeine. All data analysis was undertaken by use of the Minitab statistical package (Ryan et al. 1985). Comparison of mean values of caffeine threshold concentration versus halothane threshold concentration and of the tensions generated at 2 mM caffeine versus 2% halothane, for the different mutations, was undertaken by use of a two-sample *t*-test. Variation in the IVCT response was determined by use of one-way analysis of variants. Pairwise comparison of IVCT data for each mutation was performed by use of a Tukey pairwise-comparison test ($P > .05$). Direct comparison of the IVCT response for individuals carrying mutations Arg614Cys and Arg614Leu was completed by use of the student's two-sample *t*-test.

Results

RYR1 Mutation Analysis

Screening for novel mutations in the regions −10 to 3088 and 5114 to 8052 was undertaken by use of the SSCP technique (Orita et al. 1989). The previously published RYR1 mutations (table 1) were not detected in these samples. The majority of aberrant SSCP patterns detected in these regions were due to commonly occurring polymorphisms (Gillard et al. 1992), since several MHS individuals were homozygous for either allele, and/or the SSCP pattern was present in normal controls.

Four unique SSCP patterns were detected in MHS individuals from families D1, D2, It2, S6, and Ir4, in the fragment 6379–6766. Direct sequencing of this amplified fragment identified the presence of four mutations: C6487T in the individuals from families D1 and D2, G6488A in the individual from family It2, G6502A in the individual from family S6, and C6617T in the individual from family Ir4. These mutations result in the amino acid alterations Arg2163Cys, Arg2163His, Val2168Met, and Thr2206Met, respectively. The C6617T mutation also was detected independently in pedigree Ir4, by use of a novel mutation–scanning

method (authors' unpublished data). Full details showing the DNA and amino acid sequences flanking these mutations and those reported elsewhere are shown in table 1.

In order to analyze these mutations in genomic DNA, it was necessary to design two primer sets for amplification of the mutations, since C6487T, G6488A, and G6502A are located in exon 39 whereas the fourth mutation, C6617T, occurs in exon 40. When these primer sets were designed, the intron/exon boundaries of the human RYR1 gene were not available, and primers were selected on the basis of trial and error. Primer pairs 6464F and 6506R were used successfully to amplify a 64-bp region encompassing the three mutations in exon 39, and primer pairs 6588F and 6641R were used to amplify the 74-bp region encompassing the C6617T mutation in exon 40.

SSCP analysis was employed and allowed differential detection of each mutation. By use of SSCP analysis, these candidate mutations were shown to be absent in 200 normal chromosomes analyzed (data not shown). Of 70 available MHS cDNA samples, the Arg2163His mutation was detected in one Belgian MHS individual (10B), in addition to the index case. The Val2168Met mutation was identified in an additional three Swiss samples and in one German sample, indicating that this mutation may be one of the most common MHS mutations in the European population or that there may be a founder effect in the Swiss/German population. The Thr2206Met mutation was detected in a German MHS individual. These additional, apparently unrelated, individuals bearing the Val2168Met mutation and the Thr2206Met mutation were the only members of their families tested for susceptibility to MH, by use of the IVCT. Thus, it was not possible to perform haplotype analysis to establish whether the relatively common Val2168Met mutation arose through a founder effect.

Segregation Analysis of the Novel Mutations

Segregation analysis of the mutations Arg2163Cys, Arg2163His, Val2168Met, and Thr2206Met was performed in pedigrees D1 and D2, It2, S6, and Ir4, respectively (fig. 1). The candidate mutations segregated with the MHS phenotype, in all cases. Individual IV:15 of pedigree S6 was diagnosed as MHE(h) (fig. 1). The Val2168Met mutation was absent in this individual.

The IVCT data of the individuals from the five pedigrees in figure 1 are shown in table 2. The IVCT results are presented under four headings—namely, the concentrations of caffeine and halothane required to generate a threshold tension of 0.2 g in each muscle strip and the tension generated in each muscle strip at 2 mM caffeine and at 2% (0.44 mM) halothane.

Table 2

IVCT Data for MHS and MHE Members of MH Pedigrees Bearing the Novel RYR1 Mutations Arg2163Cys (Pedigrees D1 and D2), Arg2163His (Pedigree It2), Val2168Met (Pedigree S6), and Thr2206Met (Pedigree Ir4)

| | TENSION | | THRESHOLD CONCENTRATION | | | |
| | At 2 mM Caffeine (g) | At 2% [.44 mM] Halothane (g) | Caffeine (mM) | Halothane[a] (%) | IVCT STATUS | RYR1 MUTATION[d] |
FAMILY AND INDIVIDUAL						
D1:						
II:2	1.2	1.05	.5	.5	MHS	+
II:4	1.4	1.3	.5	1.0	MHS	+
II:6	1.1	3.9	1.5	.5	MHS	+
II:8	1.05	2.7	1.0	.5	MHS	+
III:1	.5	1.2	1.0	.5	MHS	+
III:3	.9	1.4	1.5	2.0	MHS	+
III:4	1.5	2.9	.5	.5	MHS	+
III:5	.9	2.5	1.0	.5	MHS	+
III:6	.9	1.0	1.0	.5	MHS	+
III:10	1.2	1.85	1.0	.5	MHS	+
III:11	1.9	2.6	1.0	.5	MHS	+
D2:						
II:2[b]	2.3	1.3	<4	.5	MHS	+
II:5	.9	1.1	1.0	1.0	MHS	+
III:1[b]	3.6	2.6	<4	.5	MHS	+
III:3[b]	3.8	1.1	<4	1.0	MHS	+
III:6	1.3	2.2	.5	.5	MHS	+
IV:1	1.4	2.7	1.0	.5	MHS	+
IV:2	.2	1.05	2.0	.5	MHS	+
IV:3	.5	.65	1.5	.5	MHS	+
It2:						
II:2	1.88	6.94	1.5	.5	MHS	+
III:1	1.48	.74	.5	1.0	MHS	+
S6:						
III:2	.5	2.3	2.0	.5	MHS	+
III:3	.2	1.0	2.0	1.0	MHS	+
III:4	.5	2.8	1.5	.5	MHS	+
III:6	.9	.6	2.0	.5	MHS	+
III:8	1.6	2.0	2.0	.5	MHS	+
III:10	.2	1.8	2.0	.5	MHS	+
III:12	.8	2.0	1.0	.5	MHS	+
III:13	.9	2.8	1.5	.5	MHS	+
III:15	.2	1.1	2.0	.5	MHS	+
III:16[c]	...	...	...	...	...	...
III:18	1.2	1.0	1.0	.5	MHS	+
III:20	.6	.7	1.0	.5	MHS	+
IV:3	.8	.5	.5	.5	MHS	+
IV:4	.4	1.8	1.5	1.0	MHS	+
IV:9	2.0	4.5	1.5	.5	MHS	+
IV:14	.2	1.3	2.0	.5	MHS	+
IV:15	.1	.6	4.0	.5	MHE(h)	−
IV:16	.5	.8	2.0	1.0	MHS	+
Ir4:						
II:4	.35	1.4	1.5	1.0	MHS	+
II:6	2.0	3.34	1.0	1.0	MHS	+
II:7	.1	.3	4.0	2.0	MHE(h)	−
III:1	1.4	3.25	1.0	1.0	MHS	+
III:2	.35	1.67	1.5	.5	MHS	+

[a] The data 0.5%, 1.0%, and 2.0% are equivalent to 0.11 mM, 0.22 mM, and 0.44 mM halothane, respectively.

[b] Tested prior to introduction of standardized protocol in 1984.

[c] MH death.

[d] +, − indicate presence or absence of a RYR1 mutation, respectively.

Table 3

Comparison of IVCT Mean Values of Caffeine Threshold Concentration versus Those of Halothane Threshold Concentration, for Different RYR1 Mutations

	THRESHOLD CONCENTRATION (SEM)			
	Caffeine	Halothane[b]		*P*
MUTATION (*n*)	(mM)	(%)	*t*	VALUE[a]
Cys35Arg (10)	1.80 (.11)	1.35 (.22) [.3]	1.80	.095
Arg163Cys (4)	.750 (.14)	.625 (.25) [.14]	.65	NS
Gly341Arg (17)	1.08 (.15)	.85 (.14) [.19]	1.14	NS
Arg552Trp (14)	1.07 (.16)	.96 (.09) [.21]	.58	NS
Arg614Cys (21)	1.42 (.11)	1.00 (.12) [.22]	2.69	.01
Arg614Leu (8, 7)[c]	.81 (.12)	.50 (0) [.11]	2.33	.05
Arg2163Cys (16)	1.03 (.11)	.65 (.08) [.14]	2.72	.01
Val2168Met (19)	1.58 (.10)	.61 (.05) [.13]	8.56	<.001
Thr2206Met (4)	1.25 (.14)	.87 (.13) [.19]	1.96	.11
Gly2435Arg (13)	1.11 (.14)	.84 (.15) [.18]	1.29	NS
Arg2458Cys (6)	1.83 (.11)	.66 (.08) [.14]	8.68	<.001

[a] NS = not significant.
[b] The halothane concentration in millimorgans is given in brackets.
[c] Caffeine and halothane data were obtained for eight and seven samples, respectively.

Statistical Analysis of IVCT Data for Known and Novel RYR1 Mutations

For the statistical analysis, two mutations (Arg2163His and Arg2458His) were excluded because the sample size (two) was too small. Also, IVCT data greater than the threshold values for MHE individuals bearing known RYR1 mutations were included, although this actually amounted to the inclusion of IVCT data for only three MHE(h) individuals from a pedigree with the Arg2458Cys mutation (Manning et al. 1998). Homozygous MHS individuals were excluded in the analysis. For analysis of mutations reported elsewhere, IVCT data for genotyped individuals from European MH centers were used.

Comparison of the mean values of caffeine threshold concentration versus those of halothane threshold concentration (i.e., the concentrations of caffeine and halothane required to attain a threshold tension of 0.2 g in the IVCT) for each RYR1 mutation, by use of a two-sample *t*-test, is presented in table 3. The analysis shows a significant difference between the thresholds for mutations Arg614Cys, Arg614Leu, Arg2163Cys, Val2168Met, and Arg2458Cys. The differences observed for Cys35Arg and Thr2206Met approached statistical significance (*P* = .095 and *P* = .11, respectively) and may be biologically significant. In all cases, for the mutations mentioned, the halothane threshold was lower than the caffeine threshold.

Comparison of the mean values of contracture tension generated at 2 mM caffeine versus at 2% (0.44 mM) halothane, by use of the same test, is presented in table 4. The analysis shows a significant difference between

the tensions for mutations Cys35Arg, Arg614Cys, Val2168Met, and Arg2458Cys. The differences observed for Arg614Leu and Thr2206Met approached statistical significance (*P* = .06 and *P* = .08, respectively) and may be biologically significant. For all cases for which a significant difference was observed, the tensions recorded at 2% (0.44 mM) halothane were higher than the tensions recorded at 2 mM caffeine.

Statistical analysis of IVCT responses for the Arg614Cys and the Arg614Leu mutations, by use of a two-sample *t*-test, showed that Arg614Leu had a significantly lower caffeine threshold (*t* = 3.66, *P* = .002) and halothane threshold (*t* = 4.13, *P* = .0005) than Arg614Cys. Comparison of the tension values at 2 mM caffeine and at 2% (0.44 mM) halothane showed that differences approached statistical significance (*t* = 2.01, *P* = .07 and *t* = 1.96, *P* = .08, respectively) and indicated that the Arg614Leu mutation is likely to yield higher tensions than the Arg614Cys mutation.
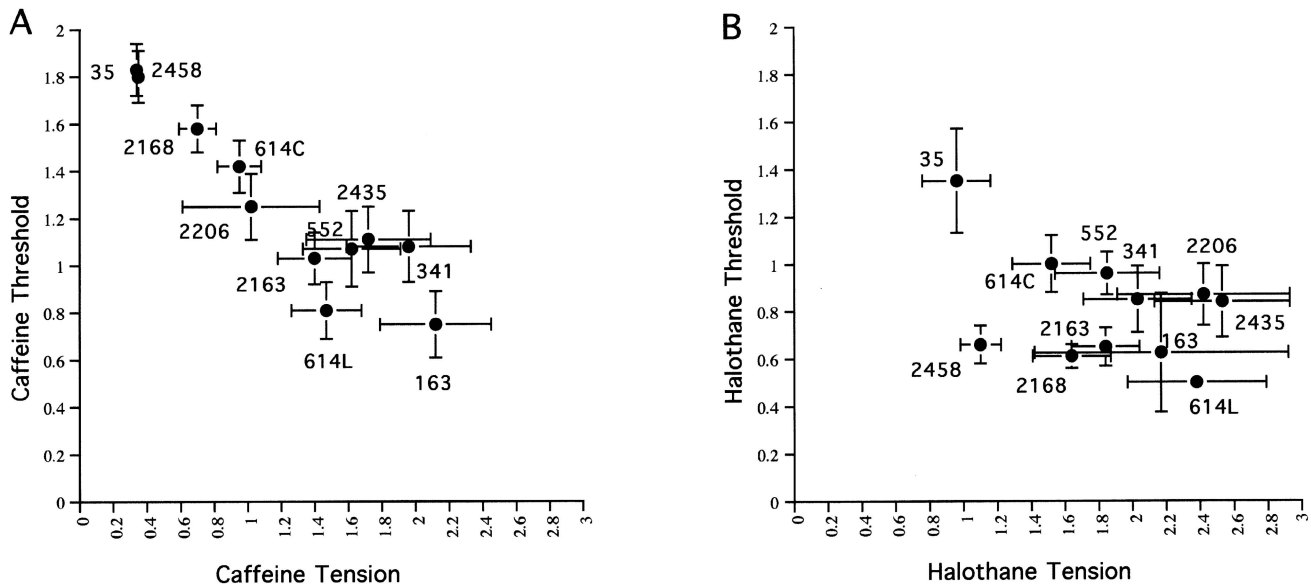
The caffeine threshold, halothane threshold, tension generated at 2 mM caffeine, and tension generated at 2% (0.44 mM) halothane were compared between the different mutations, by use of the ANOVA test and Tukey pairwise comparison (*P* > .05). The results of this analysis show that there is significant variation in the four IVCT measurements for all of these mutations. Because of the variation, it was not possible to critically assess statistical differences between the IVCT responses associated with mutations in the N-terminal region versus those in the central region of the RYR1 gene. Nonetheless, direct examination of the data suggests that mutations in the central portion of the gene may be associated with a more sensitive halothane phenotype.

Table 4

Comparison of IVCT Mean Values of Tension at 2 mM Caffeine versus those of Tension at 2 mM Halothane, for Different RYR1 Mutations

	TENSION (SEM) (g)			
	At 2 mM	At 2% [.44 mM]		*P*
MUTATION (*n*)	Caffeine	Halothane	*t*	VALUE[a]
Cys35Arg (10)	.35 (.05)	.96 (.20)	2.93	.01
Arg163Cys (4)	2.12 (.33)	2.17 (.75)	.06	NS
Gly341Arg (17)	1.96 (.37)	2.03 (.32)	.14	NS
Arg552Trp (14)	1.62 (.29)	1.85 (.31)	.56	NS
Arg614Cys (21)	.95 (.13)	1.52 (.23)	2.16	.04
Arg614Leu (8, 7)[b]	1.47 (.21)	2.38 (.41)	2.12	.06
Arg2163Cys (16)	1.40 (.22)	1.84 (.20)	1.50	NS
Val2168Met (19)	.70 (.11)	1.64 (.23)	3.73	<.001
Thr2206Met (4)	1.02 (.41)	2.42 (.51)	2.12	.08
Gly2435Arg (13)	1.72 (.37)	2.53 (.40)	1.50	NS
Arg2458Cys (6)	.34 (.06)	1.10 (.12)	5.69	<.001

[a] NS = not significant.
[b] Caffeine and halothane data were obtained for eight and seven samples, respectively.

Figure 2   Correlation analysis of IVCT threshold and tension values for different RYR1 mutations in MHS individuals. *A*, Caffeine threshold (in millimorgans) and tension (in grams) values ($r = .91$, df 9, $P > .001$) *B*, Halothane threshold (as percentages) and tension (in grams) values ($r = .32$, df 9, no significant correlation). Mutations are denoted by their location in the RYR1 gene. Arg614Cys and Arg614Leu are denoted by 614C and 614L, respectively.

Statistical analysis of a larger data set will be necessary to clarify this point.

Correlation analysis was performed for IVCT threshold and tension values for caffeine and halothane, for each mutation. A graphical representation of the data from the correlation analysis is shown in figure 2*A* and *B*. For caffeine, there was a statistically significant correlation between threshold and tension values for each mutation ($r = .91$, df 9, $P > .001$). For halothane, no significant correlation between threshold and tension values was observed ($r = .32$, df 9). Correlation analysis also was performed for the IVCT threshold values of both agents, but no significant correlation was observed ($r = .35$). However, a correlation was observed for the IVCT tension values of both agents ($r = .72$, $P > .05$).

Discussion

In the work reported here, we have identified four novel mutations in the RYR1 fragment 6379–6766 in MHS individuals. The combined incidence of these four novel mutations among MHS individuals may be as high as 11%. These results (a) identify exon 39 of the RYR1 gene as a high priority exon, for mutation screening, and (b) confirm that MHS mutations cluster in the central region of the RYR1 gene, as well as in the amino-terminal region.

These mutations satisfy the genetic criteria for causative mutations, and comparison of the ryanodine-receptor isoforms sequenced, to date, shows that the amino acids Arg2163 and Val2168, as well as several amino acids in their immediate vicinity, are conserved across RYR1, RYR2, and RYR3 isoform sequences available from GenBank (accession numbers D17389, D21070, D21071, D45899, J05200, M91451, U50465, U97329, X15750, X95267, X98330, and Y07749) (table 1).

The Arg2163His mutation seems to confer susceptibility to MH and predisposition to CCD in an individual, since cores were present in type I fibers in the proband (II:2) in family It2. In addition, no other mutation was detected in the RYR1 regions −10 to 3088 and 5114 to 8052 in this individual, indicating that, at least in the MH/CCD regions of the RYR1 gene, a second mutation in addition to the Arg2163His mutation is not likely to be present in the proband (II:2). Individual III:1, the daughter of the proband (II:2), is asymptomatic for CCD, even though she also has the segregating mutation. Similar observations for other RYR1 mutations in MHS/CCD pedigrees have been documented elsewhere (Quane et al. 1993, 1994*a*; Zhang et al 1993). It is unclear why cores develop in the muscle of some MHS individuals. If CCD and susceptibility to MH are manifestations of a common mechanistic defect, then presumably the ge-

netic background of each individual may influence the level of manifestation of the phenotype.

To examine the correlation between genotype and phenotype, we undertook a full statistical analysis of IVCT data generated for the RYR1 MHS mutations reported here and for the mutations reported elsewhere. We observed that the halothane threshold for 5 of 11 RYR1 mutations was significantly lower than the caffeine threshold (table 3). Similarly, the tensions generated at 2% (0.44 mM) halothane for 4 of 11 RYR1 mutations was significantly higher than the tensions generated at 2 mM caffeine (table 4). By contrast, we did not observe any case in which the caffeine threshold was significantly lower than the halothane threshold or in which the tension generated at 2 mM caffeine was significantly higher than the tension generated at 2% (0.44 mM) halothane. This suggests that response to halothane may be a more sensitive indicator of susceptibility to MH than response to caffeine or, alternatively, that muscle generally may be more sensitive to halothane-induced contracture than to caffeine-induced contracture. Interestingly, at the Cork MH center, the number of MHE(h) diagnoses is much greater than the number of MHE(c) diagnoses (51 vs. 4). These results suggest that the probability of an MHE(h) individual carrying a segregating mutation will be different than the probability of an MHE(c) individual carrying such a mutation. In agreement with this notion, examination of MHS families studied, to date, in which an RYR1 mutation is segregating with the MHS phenotype shows that the segregating mutation is present in 2 (33%) of 6 MHE(c) individuals and in 4 (22%) of 18 MHE(h) individuals.

Correlation of the IVCT threshold values with the tension values showed a highly significant correlation for caffeine but not for halothane. In addition, no significant correlation was observed between the IVCT threshold values of both agents, whereas a significant correlation was observed between the IVCT tension values of both agents. Indeed, considering that the IVCT is a relatively crude bioassay and that the data points were collected from laboratories throughout Europe, such a good correlation reinforces the quality of the standardized IVCT. The different responses observed for halothane and caffeine suggest a different mechanism of action for these agents. Several explanations could be proposed to explain the lack of a significant correlation between halothane threshold and tension values. Variability in halothane measurements is a realistic explanation, since halothane measurements can be problematic. Halothane is a volatile agent and does not dissolve in auqeous solutions as well as caffeine does. Thus, it is more difficult to control steady-state concentrations of halothane. In addition, the IVCT is performed with four concentrations of caffeine (0.5 mM, 1.0 mM, 1.5 mM,

and 2.0 mM) and three concentrations halothane (0.5% [0.11 mM], 1% [0.22 mM], and 2% [0.44 mM]). Thus, the degree of accuracy achieved for analysis of halothane is less than that achieved for the caffeine data. Alternatively, these data may suggest a different mechanism of action for halothane at lower concentrations than at higher concentrations. Importantly, these data are of major significance for the assessment of potential false-positive diagnoses of susceptibility to MH, in genetic studies, since they indicate the difficulty of differentiating potential false-positive diagnoses on the basis of a halothane response. However, the analysis suggests that examination of the caffeine IVCT response is likely to be a more reliable approach to this difficult problem in MH genetics.

The data presented in figure 2A suggest that mutations veering toward the left of the graph might produce a milder MH phenotype than mutations veering toward the right. Because of the wide variety of surgical and anesthetic procedures and the fact that anesthesia is aborted as rapidly as possible after the appearance of any MH-like symptoms, differences in the severity of clinical episodes of MH are difficult to score, assess, and compare. However, it is interesting to note that, in the graph in figure 2A, the Arg163Cys mutation is in the most rightward position and is the only mutation associated with symptomatic CCD (Quane et al. 1993). Thus, the RYR1 mutations producing the most sensitive caffeine phenotypes with the highest tension values may possibly predispose the individual to MH and CCD. Analysis of the IVCT data of other RYR1 mutations associated with CCD will be necessary, to test this hypothesis. A small percentage of individuals with the Arg614Cys mutation are now known to test as MHN in the European IVCT (Deufel et al. 1995), indicating that this mutation may be relatively mild. This mutation is in the third most leftward position on the graph. Furthermore, the Cys35Arg mutation is in one of the most leftward positions on the graph. Two individuals homozygous for this mutation have been reported (Lynch et al. 1997). Similarly, one individual homozygous for the Arg614Cys mutation has been reported (Deufel et al. 1995). In each of these cases, the homozygous individual has been clinically normal, indicating that these mutations may be relatively mild.

The central region of the RYR1 protein is predicted to be cytoplasmic and has been ascribed many important functions, including those associated with a phosphorylation site at Serine2844 (Suko et al. 1993) and with a calmodulin binding site (Chen et al. 1993; O'Driscoll et al. 1996). Although it is relatively clear that RYR1 mutations that are causative of the MHS phenotype lead to hypersensitive gating of the channel, by agonists, the exact mechanism by which these mutations and the other

reported RYR1 mutations cause such hypersensitive gating is unclear.

Acknowledgments

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