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MUTATION IN BRIEF

HUMAN MUTATION

Functional Characterization of Ryanodine Receptor (RYR1) Sequence Variants Using a Metabolic Assay in Immortalized B-Lymphocytes

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ABSTRACT: Mutations in the *RYR1* gene are linked to malignant hyperthermia (MH), central core disease and multi-minicore disease. We screened by DHPLC the *RYR1* gene in 24 subjects for mutations, and characterized functional alterations caused by some RYR1 variants. Three novel sequence variants and twenty novel polymorphisms were identified. Immortalized lymphoblastoid cell lines from patients with RYR1 variants and from controls were stimulated with 4-chloro-*m*-cresol (4-CmC) and the rate of extracellular acidification was recorded. We demonstrate that the increased acidification rate of lymphoblastoid cells in response to 4-CmC is mainly due to RYR1 activation. Cells expressing RYR1 variants in the N-terminal and in the central region of the protein (p.Arg530His, p.Arg2163Pro, p.Asn2342Ser, p.Glu2371Gly and p.Arg2454His) displayed higher activity compared with controls; this could account for the MH-susceptible phenotype. Cell lines harboring $RYR1^{Cys4664Arg}$ were significantly less activated by 4-CmC. This result indicates that the p.Cys4664Arg variant causes a leaky channel and depletion of intracellular stores. The functional changes detected corroborate the variants analyzed as disease-causing alterations and the acidification rate measurements as a means to monitor Ca^{2+}-induced metabolic changes in cells harboring mutant RYR1 channels. ©2009 Wiley-Liss, Inc.

KEY WORDS: RYR1, malignant hyperthermia, proton release, B-lymphocytes, calcium channel

INTRODUCTION

Mutations in the *RYR1* gene (MIM# 180901), which encodes the skeletal muscle ryanodine receptor (RYR1), the Ca^{2+} release channel of the sarcoplasmic reticulum, are linked to three skeletal muscle disorders, i.e., malignant

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hyperthermia (MH), central core disease (CCD) and multi-minicore disease (MmD). Furthermore, a *de novo* dominant RYR1 missense mutation has been identified in a subject with centronuclear myopathy and clinical features of congenital myopathy and external ophthalmoplegia [Jungbluth et al., 2007]. Malignant hyperthermia (MIM# 145600) is an autosomal dominant hypermetabolic condition that occurs in genetically predisposed subjects during general anesthesia induced by commonly used volatile anesthetics and/or the neuromuscular blocking agent succinylcholine. An MH attack, unless immediately recognized and treated, is often fatal. Malignant hyperthermia susceptibility (MHS) is diagnosed by an *in-vitro* contracture test (IVCT) [European Malignant Hyperpyrexia Group, 1984]. A considerable genetic heterogeneity has been reported for MH. MHS *loci* have been identified on six human chromosomes: 19q13.1 (MHS1; MIM# 180901), 17q11.2-q24 (MHS2; MIM# 154275), 7q21-q22 (MHS3; MIM# 154276), 3q13.1 (MHS4; MIM# 600467), 1q32 (MHS5; MIM# 601887) and 5p (MHS6; MIM# 601888). RYR1 is the main candidate for MHS, since mutations in the *RYR1* gene (MHS1 *locus*) have been identified in more than 50% of affected families [Robinson et al., 2006].

Central core disease (MIM# 117000) is a rare congenital myopathy characterized by muscle hypotrophy and hypotonia in infancy. The histological analysis of muscle samples usually reveals the presence of central core lesions extending the length of type I muscle fibers. The cores are regions characterized by sarcomeric disorganization, absence of mitochondria, and lack of oxidative activity. Multi-minicore disease (MIM# 255320) is a recessive clinically heterogeneous condition; general features include neonatal muscle hypotonia, delayed motor development, generalized muscle weakness, and amyotrophy. Muscle biopsy shows multiple “minicores” of sarcomere disorganization and mitochondria-depletion in most muscle fibers.

Thus far, more than 200 sequence variants have been identified in the *RYR1* gene [Anderson et al., 2008; von der Hagen et al., 2008; Monnier et al., 2008; Sato et al., 2008; Kossugue et al., 2007; Lyfenko et al., 2007; Rossi et al., 2007; Zhou et al., 2007, 2006; Robinson et al., 2006; Wu et al., 2006]. Identification of novel RYR1 variants and their functional characterization help shed light on the molecular bases of the distinct pathophysiological characteristics of each disorder (drug-dependent hyperactivity in MH *versus* muscle weakness and core development in CCD and minicores in MmD [Treves et al., 2008]), and are an aid to the diagnosis of MH. In fact, although the IVCT is the gold standard to establish the risk of MHS, an individual harboring an MH causative mutation can be considered MHS even without an IVCT result (www.emhg.org). Furthermore, genetic analysis is crucial to identify and evaluate the few cases of discordance between genotype, characterized by the presence of a causative mutation, and MHN-typed phenotype [Robinson et al., 2003; Fortunato et al., 1999].

Various methods have been developed to characterize the function of RYR1 variants: analysis of calcium release in human primary myotubes [Wehner et al., 2004, 2002; Girard et al., 2002; Brinkmeier et al., 1999] and in immortalized B-lymphocytes from patients or after expression by transfection in various cell types [Yang et al., 2003; Girard et al., 2001; Censier et al., 1998; Tong et al., 1997], and determination of the channel openings in a ryanodine binding assay [Sambuughin et al., 2001]. Moreover, metabolic tests *in vivo* [Bina et al., 2006; Anetseder et al., 2003, 2002; Textor et al., 2003] and *in vitro* [Klingler et al., 2002] have been developed to distinguish between MHS and MHN muscles. In particular, a metabolic assay *in vitro* based on the measurements of proton release rate has been reported to detect hypermetabolic responses, probably originating from an increased myoplasmic Ca^{2+} concentration, of MHS compared with MHN myotubes [Klingler et al., 2002].

The aims of this study were to: 1) screen the *RYR1* gene for mutations in 24 subjects (23 MHS individuals and one with minicores) from southern Italy; 2) characterize the cellular proton release in response to the RYR1 activator 4-chloro-*m*-cresol in EBV-immortalized lymphoblastoid cells; and 3) apply this assay to investigate the metabolic consequences of RYR1variants in EBV-immortalized lymphoblastoid cells from patients carrying *RYR1* gene variants.

MATERIALS AND METHODS

Subjects

Twenty-three unrelated MHS subjects, including 16 probands who experienced an MH event during anesthesia, were enrolled in this study. The MHS status was assessed by the IVCT [Ørding et al., 1997; European Malignant Hyperpyrexia Group, 1984]. One patient was referred with myopathy and minicores. Informed consent was obtained for each patient according to a procedure established by the local Bioethics Institutional Committee.

Mutation screening

Genomic DNA was extracted with the "Nucleon" procedure (Amersham, UK) from peripheral blood samples. PCR primers for the amplification of the 106 exons of the *RYR1* gene and of the intron boundary sequences were designed on the human *RYR1* genomic sequence (AC011469.6, AC067969.1, AC005933.1 accession numbers). Primers and amplification conditions are available on request (salvator@unina.it). Denaturing high performance liquid chromatography (DHPLC) analysis was performed using the WAVE system 3500 (Transgenomic, Omaha, NE). PCR samples from MHS patients were mixed with the wild-type amplicon, denaturated at 95°C for 5 min and then cooled at room temperature to allow heteroduplexes to form. Amplicons with an altered DHPLC eluition profile compared to that obtained from a wild-type amplicon were directly sequenced with dye-terminator chemistry (Applied Biosystems) using an ABI3100 automated sequencer (Applied Biosystems, USA). Nucleotide substitutions were numbered on the cDNA sequence (GenBank NM_000540.2) and on the genomic sequence (AC011469.6, AC067969.1, AC005933.1 accession numbers) using the Mutalyzer program [Wildeman et al., 2008]. For the novel genomic variants, the following criteria were evaluated: segregation of the sequence variant in families performed by restriction analysis, when possible, or by direct DNA sequencing; the absence/presence in 100 normal chromosomes of the same ethnic group by DHPLC analysis; conservation of the amino acid residues replaced across homologous proteins by BLASTP.

Mononuclear cells and EBV-transformed cell lines

Whole blood was collected in EDTA-treated tubes and mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. For infection with Epstein-Barr virus (EBV), mononuclear cells were exposed to supernatants of the B95.8 cell line, according to standard procedures [Neitzel, 1986]. Cells were cultured in Iscove's Modified Dulbecco's Medium (I3390, Sigma-Aldrich) supplemented with 20% fetal bovine serum (CH30160,03, Hyclone) and 1% L-glutamine (G7513, Sigma-Aldrich). All the EBV-immortalized lymphoblastoid cells carrying the *RYR1* gene variants were analyzed to verify the presence of the nucleotide substitution.

Proton release measurements

We measured proton release using the Cytosensor® microphysiometer (Molecular Devices, San Diego) as described [Klingler et al., 2002]. All experiments were carried out with repeated cycles of 9 min applications of 4-chloro-*m*-cresol (4-CmC), with a stepwise increase from 200 µM to 1000 µM, followed by a 9-min washout. For the inhibition experiments with 50 µM cyclopiazonic acid (CPA), 5 µM thapsigargin, or 80 µM dantrolene, the cells were pre-treated for 20 min with thapsigargin or 30 min with the other inhibitors and then stimulated with 4-CmC in the presence of inhibitor. At the end of these experiments the cell were perfused with inhibitor-free medium for 20 min and then stimulated again with 4-CmC to check cell viability.

The following stock solutions were prepared for the experiments with the Cytosensor®: 10 mM 4-CmC (C55402, Sigma-Aldrich); 29.7 mM CPA (239805, Calbiochem) in chloroform; 1.5 mM thapsigargin (586005, Calbiochem) in DMSO; 1 mM dantrolene sodium salt (251680, Calbiochem) in 274 mM mannitol (M4125, Sigma-Aldrich).

Statistical analysis

Results are reported as means and standard error of the mean (SEM) of at least three independent experiments. Significance of the differences between groups was evaluated by the Mann-Whitney test. Differences were considered to be significant when $P<0.05$.

RESULTS

RYR1 gene mutation analysis

DPHLC screening of the 106 exons of the *RYR1* gene in 23 MHS individuals and in one subject with minicores revealed 12 variants (Table 1) and several known and novel polymorphisms (Table 2). Nine sequence variants, c.1589G>A(p.Arg530His), c.1840C>T(p.Arg614Cys), c.6488G>C(p.Arg2163Pro), c.6502G>A(p.Val2168Met), c.6635T>A(p.Val2212Asp), c.7025A>G(p.Asn2342Ser), c.7361G>A(p.Arg2454His), c.11708G>A (p.Arg3903Gln) and c.12700G>C(p.Val4234Leu), were previously associated with MHS status [Robinson et al., 2006], among which three (p.Arg614Cys, p.Val2168Met, p.Arg2454His) were found to be MH-causative

(www.emhg.org), and one, p.Arg530His, was reported [Robinson et al., 2006] as a personal communication without characterization data. Lastly, we found the p.Arg3903Gln sequence variant in a patient who had myopathy associated to altered distribution of oxidative enzymes and minicores in muscle (biopsy analysis, Dr. F. Cornelio, Milan, Italy, personal communication). Three missense changes, c.7112A>G(p.Glu2371Gly), c.7355G>C(p.Arg2452Pro) and c.13990T>C(p.Cys4664Arg) (Table 1), are novel sequence variants; these were absent in more than 100 alleles in MHN subjects and resulted in changes of amino acid residues conserved in all the RYR isoforms of various species. We also identified the variants c.2920G>A(p.Val974Met) and c.9674G>A (p.Arg3225Gln) (Table 2), each detected also in one MHN individual, and c.9457G>A(p.Gly3153Arg) and c.6599C>T(p.Ala2200Val) (Table 2), which did not segregate with the MHS phenotype in the pedigrees we analyzed. Therefore, these sequence variants are probably polymorphisms (Table 2). The variants p.Glu2371Gly and p.Arg2452Pro occurred in two single MHS subjects, whereas p.Arg530His and p.Cys4664Arg segregated with the disease (Fig. 1). Family NA-15, in which p.Cys4664Arg segregated with the MHS phenotypes, also carried the p.Arg3225Gln substitution, and subject II:2 had both sequence variants (Fig. 1). Considering the mutations and putative causative variants identified in this study, each in a single patient, the mutation rate was 50%.

Table 1. RYR1 sequence variants identified in the 23 MHS subjects and in the one MmD subject screened

Exon	Nucleotide change	Amino acid change	Role of mutation	References
15	c.1589G>A	p.Arg530His	MHS c.m.*	Robinson et al. 2006
17	c.1840C>T	p.Arg614Cys	MHS c.m.	Robinson et al. 2006
39	c.6488G>C	p.Arg2163Pro	MHS c.m.*	Robinson et al. 2006
39	c.6502G>A	p.Val2168Met	MHS c.m.	Robinson et al. 2006
40	c.6635T>A	p.Val2212Asp	MHS p.c.m.	Galli et al 2006
43	c.7025A>G	p.Asn2342Ser	MHS c.m.*	Robinson et al. 2006
44	**c.7112A>G**	**p.Glu2371Gly**	**MHS c.m.***	**This study**
46	**c.7355G>C**	**p.Arg2452Pro**	**MHS p.c.m.**	**This study**
46	c.7361G>A	p.Arg2454His	MHS c.m.	Robinson et al. 2006
85	c.11708G>A	p.Arg3903Gln	MHS/ MmD p.c.m	Robinson et al. 2006 and this study
91	c.12700G>C	p.Val4234Leu	MHS p.c.m.	Robinson et al. 2006
95	**c.13990T>C**	**p.Cys4664Arg**	**MHS c.m.***	**This study**

c.m.: causative mutation; c.m.*: causative mutation demonstrated in this study; p.c.m: putative causative mutation. Nucleotide numbering reflects cDNA numbering based on cDNA Ref Seq: NM_000540.2, with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. The initiation codon is codon 1.

Table 2. Polymorphisms detected in the MHS population screened

Nucleotide change		Location	Amino acid	Ref SNP ID (NCBI)	References
genomic	cDNA				
g.27135G>T	**c.165+35G>T**	**Intron 2**			**This study**
g.28716G>A	c.270+27G>A	Intron 3		rs3745843	
g.30855C>T	c.573C>T	Exon 7	p.Asp191	rs892054	
g.30876A>G	c.594A>G	Exon 7	p.Leu198	rs12985668	
g.30952_30953insC	**c.631+39_40insC**	**Intron 7**			**This study**
g.35004T>C	c.1077T>C	Exon 11	p.Ala359	rs10406027	
g.41447T>C	c.1441-24T>C	Intron 13		rs7254832	
g.41778G>A	c.1668G>A	Exon 15	p.Ser556	rs2288888	
g.41811G>C	c.1672+29G>C	Intron 15		rs2288889	
g.44559C>G	c.2167+30C>G	Intron 18		rs2071086	
g.45499C>T	c.2286C>T	Exon 19	p.Pro762	rs3745847	
g.49592A>G	c.2678-67A>G	Intron 20		rs2304148	
g.52376G>A	**c.2920G>A**	**Exon 24**	**p.Val974Met**		**This study**
g.52399G>A	c.2943G>A	Exon 24	p.Thr981	rs2228069	
g.52435C>T	c.2979C>T	Exon 24	p.Asn993	rs2228070	
g.55276C>T	c.3456C>T	Exon 26	p.Ile1152	rs11083462	
g.61548T>C	c.4161-6T>C	Intron 28		rs55845760	
g.64095C>T	**c.4443C>T**	**Exon 30**	**p.Asn1481**		**This study**
g.72251C>T	c.5360C>T	Exon 34	p.Pro1787Leu	rs34934920	
g.75487A>G	c.5622A>G	Exon 35	p.Glu1874		Robinson et al. 2006
g.75714C>G	c.5814+35C>G	Intron 35		rs16972654	
g.76880A>G	c.6039A>G	Exon 37	p.Lys2013	rs2228068	
g.78776G>T	c.6178G>T	Exon 38	p.Gly2060Cys	rs35364374	
g.80697C>T	**c.6384C>T**	**Exon 39**	**p.Tyr2128**		**This study**
g. 80871C>T	**c.6548+10C>T**	**Intron 39**			**This study**
g.82501C>T	c.6599C>T	Exon 40	p.Ala2200Val		Robinson et al. 2006
g.85932C>T	c.7089C>T	Exon 44	p.Cys2363	rs2228071	

Nucleotide change		Location	Amino acid	Ref SNP ID (NCBI)	References
genomic	cDNA				
g.85941C>T	c.7098C>T	Exon 44	p.Pro2366	rs2229147	
g.86189C>T	c.7260C>T	Exon 45	p.His2420	rs12973632	
g.86794G>T	**c.7324-48G>T**	**Intron 45**			**This study**
g.87112G>T	c.7500G>T	Exon 47	p.Ala2500	rs2228072	
g.87139G>A	c.7527G>A	Exon 47	p.Val2509	rs2071088	
g.87236C>G	c.7614+10C>G	Intron 47		rs2960323	
g.89143C>T	c.7863C>T	Exon 49	p.His2621	rs2229142	
g.89152C>T	c.7872C>T	Exon 49	p.Arg2624	rs1469698	
g.89234A>G	c.7926+28A>G	Intron 49		rs1469699	
g.90506G>A	c.7977G>A	Exon 50	p.Thr2659	rs2229144	
g.90953_90955delCCT	c.8068-30_32delCCT	Intron 50		rs10532729	
g.91034T>C	c.8118T>C	Exon 51	p.Ile2706	rs2960340	
g.91106T>C	c.8190T>C	Exon 51	p.Asp2730	rs2915951	
g.91571G>A	c.8337G>A	Exon 53	p.Glu2779	rs2915952	
g.91610G>A	**c.8376G>A**	**Exon 53**	**p.Arg2792**		**This study**
g.91662A>G	c.8400+28A>G	Intron 53		rs2915953	
g.92216C>T	c.8541+34C>T	Intron 54		rs2960342	
g.92219T>G	c.8541+37T>G	Intron 54		rs2960343	
g.92221G>C	**c.8541+39G>C**	**Intron 54**			**This study**
g.92236A>G	c.8541+52A>G	Intron 54		rs2915954	
g.92586T>C	c.8589T>C	Exon 55	p.Ser2863	rs2229146	
g.92620G>A	**c.8616+7G>A**	**Intron 55**			**This study**
g.92635G>C	**c.8616+22G>C**	**Intron 55**			**This study**
g.92672C>T	c.8617-35C>T	Intron 55		rs2960344	
g.92673T>C	**c.8617-34T>C**	**Intron 55**			**This study**
g.92810T>G	c.8692+28T>G	Intron 56		rs2960345	
g.92960_92961insTCA	c.8693-105_104insTCA	Intron 56		rs35973146	
g.92996A>T	**c.8693-69A>T**	**Intron 56**			**This study**
g.93055G>C	c.8693-10G>C	Intron 56		rs2915958	

Nucleotide change		Location	Amino acid	Ref SNP ID (NCBI)	References
genomic	cDNA				
g.93205T>A	c.8816+16T>A	Intron 57		rs2915959	
g.93231A>C	c.8816+42A>C	Intron 57		rs2960346	
g.94097_94098insC	c.8932+34_35insC	Intron 58		rs5828009	
g.97960G>A	**c.9172+114G>A**	**Intron 61**			**This study**
g.98287C>T	c.9173-21C>T	Intron 61		rs2960338	
g.98321A>G	c.9186A>G	Exon 62	p.Pro3062	rs2071089	
g.98704G>A	**c. 9457G>A**	**Exon 63**	**p.Gly3153Arg**		**This study**
g.102442G>A	**c.9674G>A**	**Exon 65**	**p.Arg3225Gln**		**This study**
g.105619C>T	c.10188C>T	Exon 67	p.Asp3396	rs2229145	
g.105655C>T	c.10218C>T	Exon 67	p.Tyr3406		Robinson et al. 2006
g.105697G>A	**c.10259+7G>A**	**Intron 67**			**This study**
g.109668G>T	**c.10440+122G>T**	**Intron 69**			**This study**
g.8585C>T	c.10687-7C>T	Intron 72		rs2960354	
g.9458A>G	c.10938-86A>G	Intron 74		rs6508806	
g.15671C>G	c.11266C>G	Exon 79	p.Gln3756Glu	rs4802584	
g.17914C>T	**c.11608+201C>T**	**Intron 83**			**This study**
g.24356T>A	c.11754T>A	Exon 85	p.Thr3918	rs45613041	
g.11427C>T	c.13317C>T	Exon 91	p.Ala4439		Robinson et al. 2006
g.16392C>G	c.13671C>G	Exon 94	p.Ser4557	rs35959206	
g.32386G>T	**c.15021+37G>T**	**Intron 105**			**This study**

Nucleotide location was determined using the Mutalyzer program (Wildelman et al., 2008) from the genomic sequences AC011469.6 (from intron 2 to intron 69), AC067969.1 (from intron 72 to exon 85) and AC0059333.1 (from exon 91 to intron 105) and from cDNA sequence Ref Seq: NM_000540.2 (nucleotides were numbered on cDNA with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. The initiation codon is codon 1).

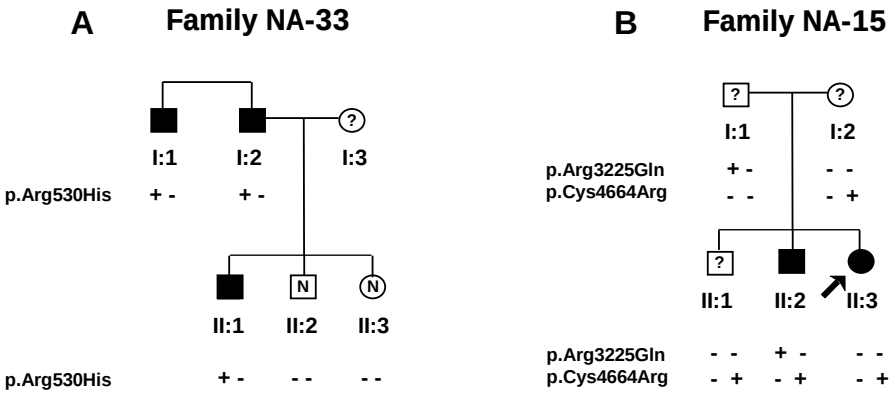

Figure 1. Pedigrees of families NA-33 (A) and NA-15 (B) showing the segregation of RYR1 sequence variants, which are listed on the left of each panel. Solid symbols: MHS individuals identified by the IVCT; N: individuals MHN typed by the IVCT; ?: untested members of the pedigree; +/–: presence or absence of the sequence variants. The arrow indicates the index case who experienced an MH episode.

Proton release measurements in EBV-immortalized normal lymphoblastoid cells

The rate of extracellular acidification in chemically stimulated cultured myocytes was reported to be significantly higher in MHS myotubes than in MHN myotubes [Klingler et al., 2002]. Previous studies have also demonstrated that human B-lymphocytes express RYR1 [Sei et al., 1999] and could be used as a model to test the effect of sequence variants on RYR1 function [Sei et al., 2002, 1999; Girard et al., 2001]. We therefore applied the proton release assay in EBV-immortalized lymphoblastoid cells from patients carrying RYR1 variants. First, we characterized proton release in response to 4-CmC. The latter compound specifically activates the RYR1 channel and can be used to discriminate between MHN and MHS phenotypes [Herrmann-Frank et al., 1996]. Lymphoblastoid cells from normal subjects responded to 4-CmC by increasing the proton secretion rate (Fig. 2). The cellular response to the triggering agent was independent of extracellular calcium since it was not affected by the addition of 15 mM EGTA to the medium (data not shown).

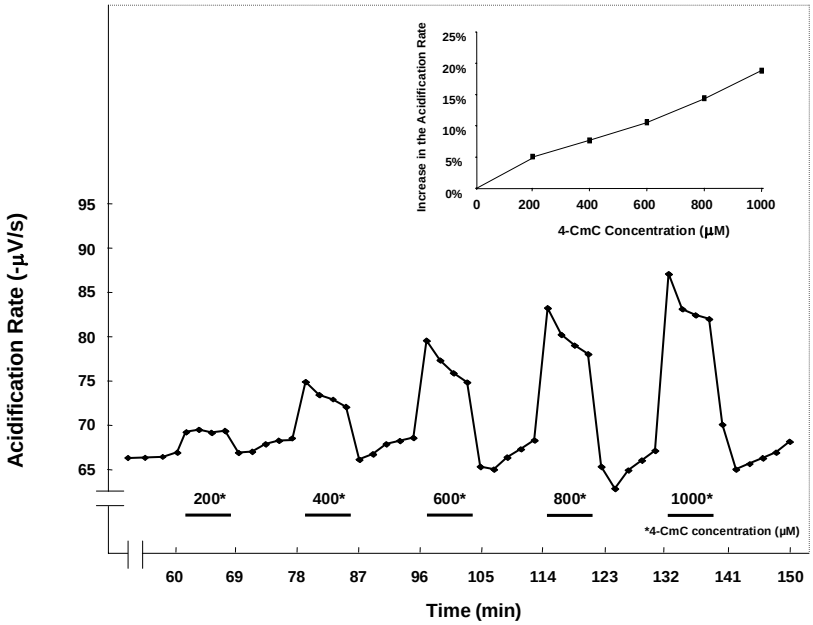

Figure 2. Acidification rate recorded in lymphoblastoid cells from an MHN control. The bars indicate perfusion of the chamber with the medium containing the indicated concentrations of 4-CmC. The increase in the acidification rate expressed as percentage of predrug value plotted *versus* 4-CmC concentration is shown in the inset.

To evaluate whether the metabolic response to 4-CmC was due to Ca^{2+} release from the endoplasmic reticulum, the cells were treated with CPA or with thapsigargin, which are SERCA pump blockers that deplete Ca^{2+} stores by blocking Ca^{2+} uptake and allowing passive leakage of Ca^{2+} [Duke et al., 1998; Thastrup et al., 1990]. Lymphoblastoid cells from an MHN subject were stimulated with 600 μM 4-CmC after preincubation with 50 μM CPA or 5 μM thapsigargin. The proton release rate increased in the first 2 min after the addition of CPA or thapsigargin to the perfusion medium (not shown) as a result of the block of the Ca^{2+} flux from the cytosol to the endoplasmic reticulum, and stabilized to basal values in about 15 min. Stimulation with 600 μM 4-CmC in the presence of CPA or thapsigargin reduced the response rate by about 50% (Fig. 3) as a result of Ca^{2+} store depletion due to the continuous SERCA pump inhibition [Duke et al., 1998]. These data confirm that endoplasmic reticulum Ca^{2+} is essential for the full cellular response to 4-CmC in the microphysiometer system.

Dantrolene is an RYR1 antagonist that blocks calcium release from the sarco/endoplasmic reticulum stores and is the only specific agent available for the treatment of an MH attack. Thus, we used dantrolene to evaluate the effect of RYR1 channel opening on the acidification response to 4-CmC. Dantrolene treatment reduced the response to 600 μM 4-CmC by about 70% (Fig. 3), which indicates that RYR1 plays a major role in 4-CmC-induced acidification.

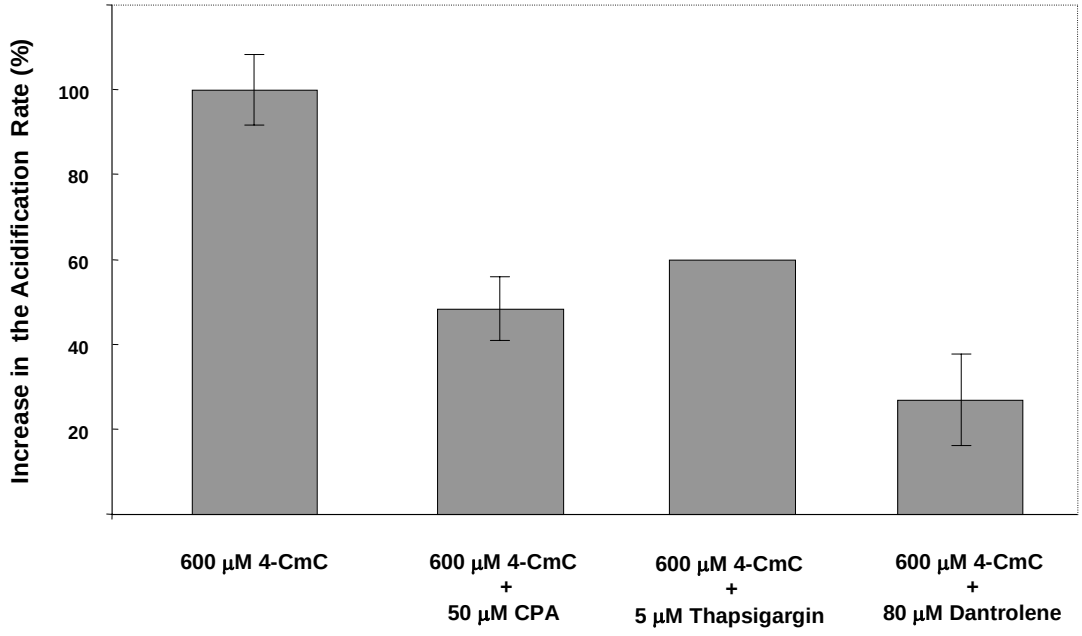

Figure 3. Effect of cyclopiazonic acid (CPA), thapsigargin and dantrolene on the acidification rate in response to 4-CmC in lymphoblastoid cells from an MHN control. Increases in the acidification rate in response to 600 μM 4-CmC after pretreatment with 50 μM CPA or 5 μM thapsigargin or 80 μM dantrolene are expressed as percentage of the response in the absence of inhibitors. Error bars show the SEM except for the third column where only one experiment could be performed.

Proton release measurements in EBV-immortalized lymphoblastoid cells carrying RYR1 channel variants

B-lymphocytes from patients with $RYR1^{Arg530His}$ (subject II:1 of family NA-33, Fig. 1), $RYR1^{Arg2163Pro}$ (proband of family NA-3 [Fortunato et al., 2000]), $RYR1^{Asn2342Ser}$, $RYR1^{Glu2371Gly}$, $RYR1^{Arg2454His}$ and $RYR1^{Cys4664Arg}$ variants were available and were immortalized with EBV. Regarding the $RYR1^{Cys4664Arg}$ variant, three cell lines, MHS5, MHS6 and MHS7, were prepared from subjects II:1, II:2 and II:3, respectively, of family NA-15 (Fig.1).

The cell lines were used for the functional characterization using proton release measurements in response to 4-CmC. Two cell lines were used as controls, named MHN1 from an unrelated MHN subject and MHN10 from subject II:2 of family NA-33 (Fig. 1). Metabolic responses to 4-CmC were significantly higher in the cell line with the $RYR1^{Arg530His}$ mutant channel (Fig. 4) and in the cell lines harboring RYR1 channels with the other sequence variants located at the central region of the protein than in wild-type RYR1 cell lines (Fig. 5). On the contrary, the metabolic responses to 4-CmC were lower in the three cell lines carrying $RYR1^{Cys4664Arg}$ than in controls (Fig. 5).

However, the response to 800 μM 4-CmC of the MHS6 cell line with two substitutions, p.Arg3225Gln and p.Cys4664Arg, each transmitted from one parent (Fig. 1), did not significantly differ from controls. The basal metabolism was unaffected, since the acidification rate of all the three cell lines expressing $RYR1^{Cys4664Arg}$ recorded in the absence of 4-CmC was indistinguishable from that of the MHN control cells (data not shown). Mutations in the C-terminal membrane-spanning domain have been reported to show a smaller thapsigargin-induced increase in cytosolic [Ca^{2+}], in the absence of external Ca^{2+}, than controls [Treves et al., 2008; Lynch et al., 1999]. This result has been taken to indicate Ca^{2+} store depletion due to "leaky" mutant RYR1 channels, since the signal obtained with the SERCA blocker reflects the size of the rapidly releasable intracellular Ca^{2+} stores [Tilgen et al., 2001]. To determine if the$RYR1^{Cys4664Arg}$ variant reacts in a similar way, we analyzed the acidification response of the MHS5 cell line to thapsigargin. The early increase (in the first 2 min) after treatment with the SERCA pump blocker was about 50% lower (data not shown) in this cell line than in the control. This result is consistent with partial Ca^{2+} store depletion in the cell line expressing $RYR1^{Cys4664Arg}$, as confirmed by Ca^{2+} release measurements (unpublished results).

We next used dantrolene to evaluate the contribution of RYR1 activation to the increase of the acidification rate of cell lines harboring RYR1 variants. The response to 600 μM 4-CmC was inhibited in each variant (Fig. 6). Moreover, the extent of inhibition of the hyperactivated mutant RYR1 channels tended to correlate inversely with the extent of 4-CmC activation in the absence of dantrolene (Fig. 6, inset).

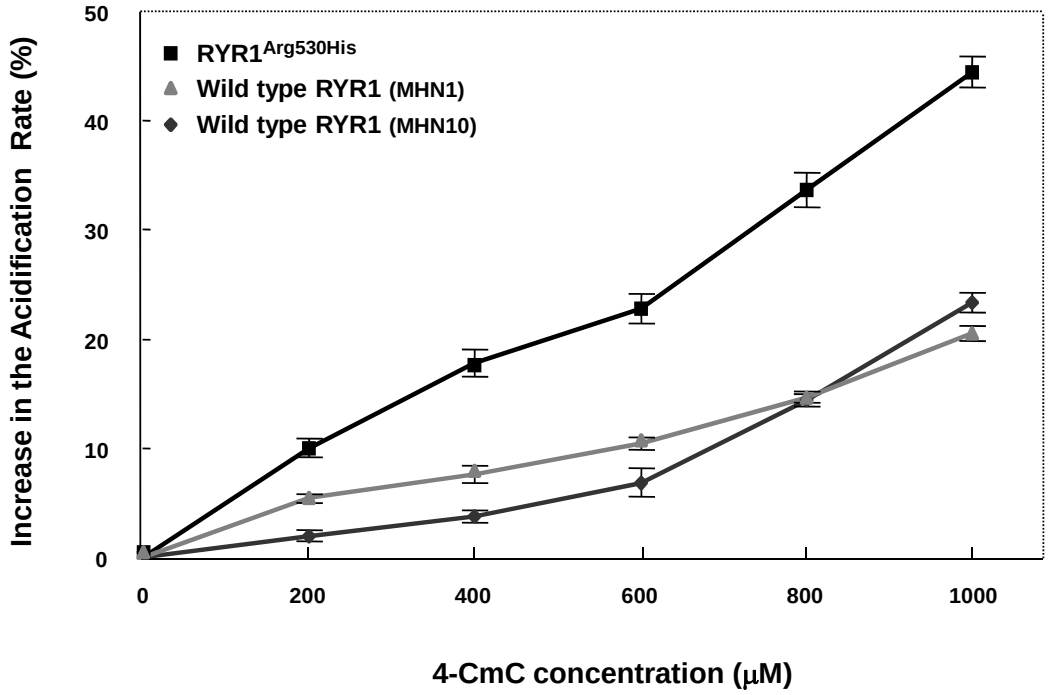

Figure 4. Acidification rate of lymphoblastoid cells from two MHN controls and from a patient with the $RYR1^{Arg530His}$ channel stimulated with 4-CmC. The increase in the acidification rate is reported as percentage of the predrug value. Squares: cells with $RYR1^{Arg530His}$; triangles: control cells MHN1; diamonds: control cells MHN10 (see text under Results). Error bars show the propagation of the SEM; at all the measured concentrations the responses were significantly different ($P<0.05$) from the controls.

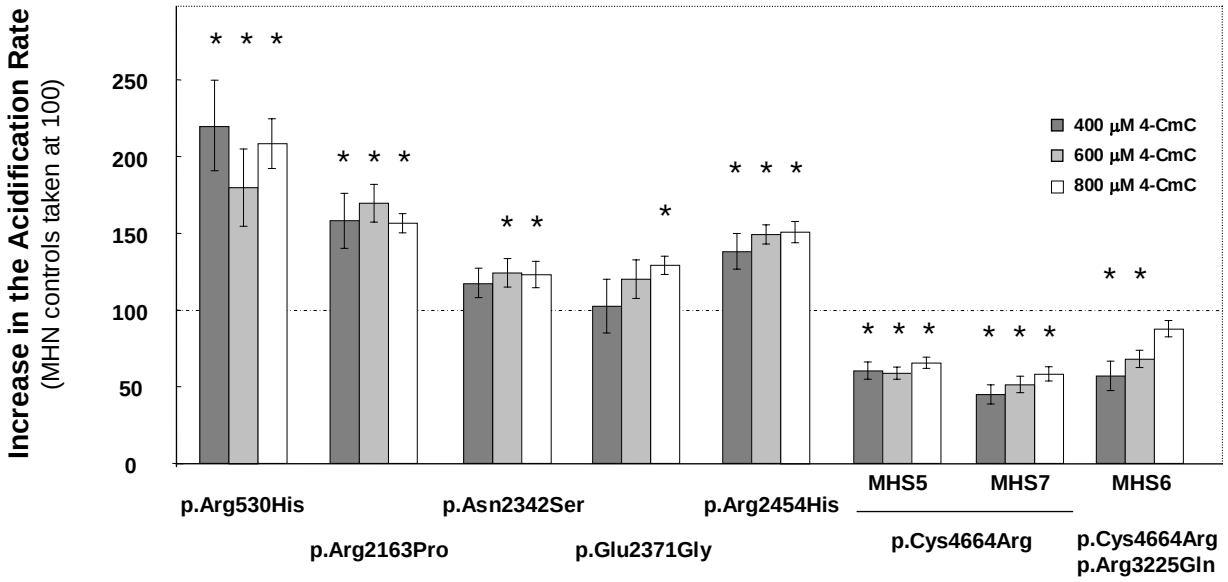

Figure 5. Increase in the acidification rate of RYR1 variants. The increase in the acidification rate value of lymphoblastoid cells from normal subjects is taken as 100. Cell lines carrying RYR1 variants p.Arg530His, p.Arg2163Pro, p.Asn2342Ser, p.Glu2371Gly, p.Arg2454His, p.Cys4664Arg and p.Cys4664Arg/p.Arg3225Gln were stimulated with 400 µM, 600 µM or 800 µM 4-CmC as indicated. Each block of three bars corresponds to a cell culture preparation derived from a RYR1 variant patient as indicated. MHS5, MHS7 and MHS6 correspond to three different cell lines (see text). Error bars show the propagation of the SEM; asterisks indicate responses significantly different ($P<0.05$) from the controls.

DISCUSSION

RYR1 gene screening showed that no RYR1 sequence variant is prevalent in MHS subjects and families from southern Italy. To examine the function of RYR1 channel variants in EBV-immortalized lymphoblastoid cells, we recorded extracellular acidification, as a measure of the energy metabolism of activated cells, with a sensitive pH-metric biosensor. RYR1 is expressed in human B lymphocytes where it functions as a Ca^{2+}-release channel during the B-cell receptor–stimulated Ca^{2+} signaling process [Sei et al., 1999], and Ca^{2+} homeostasis is altered in B cells of MHS individuals [Sei et al., 2002; Girard et al., 2001]. We demonstrate that the increase in the acidification rate of immortalized lymphoblastoid cells in response to 4-CmC is mainly due to the activation of the RYR1 calcium channel; therefore this assay can be used to analyze the activity of RYR1 in this cellular system.

The metabolic responses of mutant RYR1 channels analyzed in this study were of two types, i.e. increase or decrease of proton release rate compared with the wild-type channel. The response seems to depend on the location of the mutation. The metabolic response was higher in RYR1 channels with mutations located in the N-terminal and the central regions of the protein, and these higher metabolic responses can account for the MHS phenotype. In our tests we included cells expressing $RYR1^{Arg2454His}$, whose functional behavior has been already characterized by another method [Monnier et al., 2005]. The higher acidification rate in response to 4-CmC *versus* the wild-type channel detected in our experiments is in line with the higher sensitivity to caffeine detected by Ca^{2+}-release measurements [Monnier et al., 2005]. This result indicates that the increase in proton release triggered by 4-CmC results from Ca^{2+} release and is an indirect indicator of altered Ca^{2+} metabolism. On the contrary, 4-CmC-induced activation was significantly lower in the cell lines harboring $RYR1^{Cys4664Arg}$, and our results indicate that p.Cys4664Arg leads to a leaky channel with depletion of intracellular stores. The p.Cys4664Arg mutation is located at the C-terminal region of the protein in the M6 transmembrane segment [Zorzato et al., 1990]. Other mutations at the C-terminus of RYR1 and associated with the CCD phenotype have been reported to lead to channels that are non- or hypo-reactive to chemical stimulation [Brini et al., 2005; Ducreux et al., 2004; Tilgen et al., 2001; Lynch et al., 1999]. In contrast, the p.Thr4826Ile mutation, associated to the MHS phenotype, was reported to have a higher 4-CmC and caffeine sensitivity compared to the wild-type RYR1 in transfected human myotubes [Yang et al. 2003]. The p.Cys4664Arg mutation has been identified in an MH family (Fig. 1) and no signs of myopathy compatible with CCD were found in the medical history of the patients.

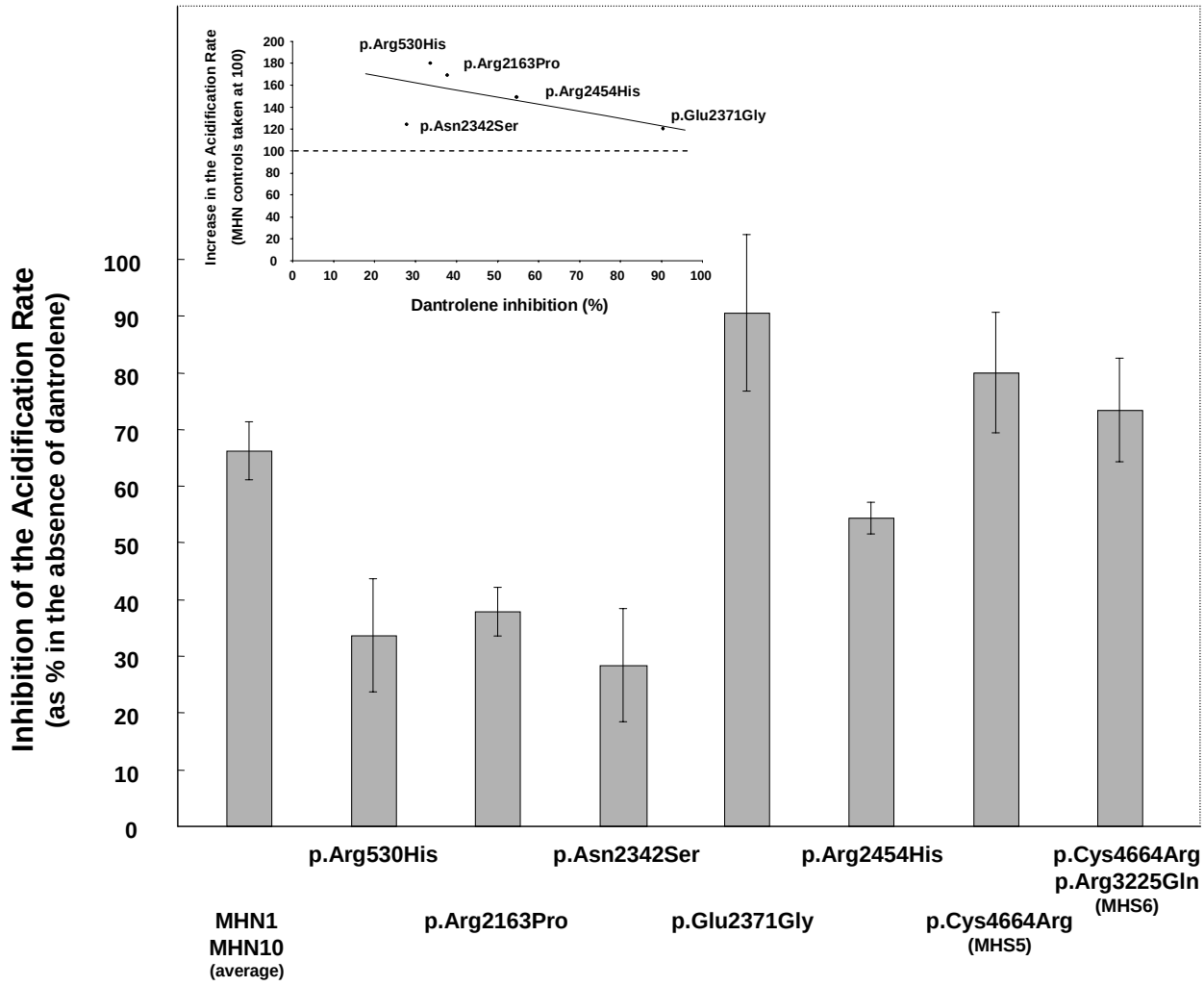

Figure 6. Dantrolene inhibition of the acidification rate of RYR1 variants stimulated with 600 µM 4-CmC in the presence of 80 µM dantrolene. The inhibition is expressed as percentage of the acidification rate in the absence of inhibitor. Error bars show the propagation of the SEM. The inverse correlation between the % of the increase in the acidification rate at 600 µM 4-CmC and the % of inhibition by dantrolene (80 µM) for the various RYR1 variants is shown in the inset.

In conclusion, we identified three novel sequence variants in the *RYR1* gene. Using the drug-induced proton release rate of lymphoblastoid cells harboring some of the sequence variants identified, we demonstrate that these genetic alterations are – as expected for impairments of the Ca^{2+} release channel – associated with changes in metabolic function. They, therefore, fulfill the requirements of the "guidelines for the molecular genetic detection of susceptibility to malignant hyperthermia" [Urwyler et al., 2001] for predictive genetic testing.

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