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Novel Ryanodine Receptor Mutation That May Cause Malignant Hyperthermia

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Background: ***Malignant hyperthermia*** **(MH) is a hypermetabolic condition caused by a genetic disposition leading to increased Ca^{2+} release from the sarcoplasmic reticulum after exposure to triggering agents. In the authors' ongoing evaluation of patients undergoing MH testing in Austria, they detected a family with a new variant of the ryanodine receptor 1. Guidelines suggest that genetic tests are possible only for individuals from families in which the mutations are known. The aim of this study was to provide functional data that establish a potential link between this new variant and susceptibility to MH, and thus enable application in genetic tests.**

Methods: **Messenger RNA was isolated from skeletal muscle cells grown in culture and used for synthesis of complementary DNA, which served as a template for 23 polymerase chain reactions. The sequences of all reaction products were analyzed. Functional studies in differentiated muscle cells included the Ca^{2+} releasing activity of caffeine and 4-chloro-*m*-cresol. The authors measured the intracellular Ca^{2+} concentration and, in combined patch clamp–Ca^{2+} detection experiments, the voltage dependence of the Ca^{2+} release.**

Results: **In a single family, the authors found a transition from a highly conserved thymine to cysteine at position 11953, leading to the exchange of tryptophan to arginine at position 3985. This variant was absent in 100 MH-nonsusceptible individuals. Functionally, cells carrying this variant were more sensitive to caffeine and 4-chloro-*m*-cresol than wild-type cells and showed a shift in the voltage-dependent Ca^{2+} release to more negative potentials.**

Conclusion: **These data document a role of the new W3985R variant in MH susceptibility.**

MALIGNANT hyperthermia (MH) is a pharmacogenetic disorder characterized by increased Ca^{2+} release in skeletal muscles upon exposure to triggering substances such as volatile anesthetics and the depolarizing neuromuscular blocking drug succinylcholine.[1] Vigorous exercise and stress have also been reported to very rarely cause MH-like episodes.[2] Because of its high sensitivity and specificity,[3] the *in vitro* contracture test (IVCT) of MH susceptibility on a vastus muscle fragment is regarded as the accepted standard. The European protocol of the IVCT differs slightly from the North American protocol; in both cases, muscle bundles are exposed to increasing concentrations of caffeine or halothane. Researchers have long attempted to replace these with a genetic test requiring only a blood sample.[4–6]

Unfortunately, there is no single mutation causing MH susceptibility. Currently, more than 170 variations in the ryanodine receptor 1 (RYR1) gene have been detected that are linked to MH.[7] Although generally MH susceptibility is a result of a defective Ca^{2+} release channel (RYR1) in the sarcoplasmic reticulum, so far one mutation outside the RYR1 gene has also been proven to cause MH. This mutation was found in the skeletal muscle voltage-dependent Ca^{2+} channel[8] which regulates the Ca^{2+} release through the RYR1 channel after depolarization of the sarcolemma. Because linkage analysis seldom provides enough data to classify a variant as causative for MH, additional data about the pathophysiologic effects of a newly described variant are essential.

The huge number of possible mutations causing MH requires the identification of a specific family mutation to perform a genetic test. It is then possible to diagnose susceptibility without performing the IVCT.[9]

Our aim is to provide functional data of a new genetic variant found in one Austrian MH family, to establish a potential link between this receptor variant and MH, and to enable the use of this correlation in future genetic testing.

Materials and Methods

The study was approved by the institutional ethics committee at the Medical University of Vienna (Vienna, Austria). Written informed consent was obtained from all patients scheduled to undergo an IVCT because of suspected MH susceptibility. The IVCT is performed only in cases where there is a family history of MH, or after a suspected MH episode during anesthesia. Individuals with an American Society of Anesthesiologists physical status higher than III and children younger than 16 yr are excluded from the test. The IVCT was performed according to the protocol of the European Malignant Hyperthermia Group, where muscle specimens are exposed to halothane and caffeine. After stretching the muscle to 150% of its initial length and stabilization of the baseline force for at least 15 min, the drugs are added. The concentration of caffeine is increased stepwise to 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 mM; the concentrations of halothane used for the test are 0.11, 0.22, 0.44, and 0.66 mM. The result of the test is a threshold value that is the lowest concentration of a drug that induces a sustained increase in contracture of at least 2 mN. The diagnostic criteria for susceptibility to MH

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(MHS) are a caffeine threshold of 2 mM or less and a halothane threshold of 0.44 mM or less. An individual is diagnosed as MH nonsusceptible (MHN) if the caffeine threshold is 3 mM or more and the halothane threshold is above 0.44 mM. All other test results are MH equivocal to either halothane only or caffeine only.[3] For this study, sequencing of the RYR1 gene was performed only for individuals with a positive (MHS) test result on the IVCT but without a previously identified MH mutation. The family that was found to carry the new genetic variant described here had nine members. Four of them were tested by IVCT (two were diagnosed as MHS and two as MHN), and a DNA sample was available from six family members (fig. 1A). As a control, DNA from blood samples of 100 randomly selected nonrelated individuals who had an MHN result on an IVCT was used to exclude a simple polymorphism.

Sera and media for cell culture were obtained from PAA (Linz, Austria). Trypsin-EDTA, glutamax, penicillin, streptomycin, gentamicin, and amphotericin B were obtained from GIBCO (Vienna, Austria); trypsin was obtained from Roche (Vienna, Austria); and fura-2/AM was obtained from Molecular Probes (Invitrogen, Lofer, Austria). Fibronectin and matrigel were purchased from BD Biosciences (Bedford, MA). All other chemicals were obtained from Sigma Aldrich GmbH (Vienna, Austria).

During muscle biopsy for MH diagnosis, surplus muscle bundles (100–350 mg) were obtained for human skeletal muscle cell culture as described previously.[10] In brief, muscle tissue was freed from fat and connective tissue, cut, and digested in collagenase (type IA, 0.5 mg/ml) and trypsin (0.25 mg/ml) in phosphate-buffered salt solution without Ca^{2+} and Mg^{2+}. After centrifugation (200*g*), the cell suspension was resuspended in wash medium (Ham's F12 supplemented with 20% horse serum), filtered through a 70-μm nylon mesh, and finally seeded in growth medium onto 50-ml cell culture flasks for proliferation. Growth and differentiation media were prepared according to Baroffio *et al.*[11] Growth medium contained Ham's F12 supplemented with 15% fetal calf serum, 10 ng/ml epidermal growth factor, 200 ng/ml insulin, 400 ng/ml dexamethasone, 0.5 mg/ml fetuin, 0.5 mg/ml bovine serum albumin, 7 mM glucose, 4 mM glutamax, 200 U/ml penicillin, 200 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. Differentiation medium contained Dulbecco/Vogt modified Eagle minimal essential medium supplemented with 5% horse serum, 4 mM glutamax, 100 ng/ml insulin, and 0.1 μg/ml gentamicin.

Cells were kept at 37°C under 2.5% CO_2 in an incubator, grown close to confluence, and reseeded on 25-mm glass coverslips coated with fibronectin or matrigel. Adherent cells were exposed to differentiation medium to promote fusion of myogenic satellite cells to myotubes in an incubator with 5% CO_2.

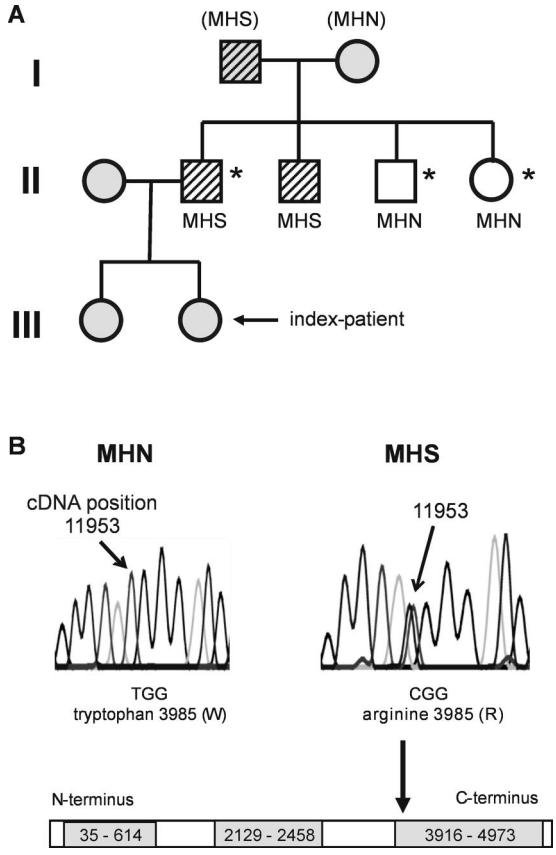

Fig. 1. *In vitro* contracture test (IVCT) and genetic data of the family. (*A*) Pedigree of the family with a new putative heterozygous malignant hyperthermia (MH) mutation in the ryanodine receptor 1 (RYR1) gene. Four individuals of the second generation were tested with the IVCT; two of them were found to be MH susceptible (MHS) and had the W3985R variant (*striped symbols*), whereas the other two were diagnosed as normal (MHN) and did not carry the W3985R variant (*empty symbols*). The diagnosis in parentheses is based not on the IVCT result but on the occurrence of the W3985R variant. Individuals in the third generation were too young for the IVCT, and no blood samples were available. *Gray symbols* = no IVCT was performed. * Q11266E variant. (*B*) A base change in the codon for tryptophan 3985 (TGG → CGG) leads to the insertion of arginine at this position. Below is a linear representation of the primary sequence of the RYR1. Shaded boxes denote the so-called MH regions, domains of the protein that are thought to harbor most causative MH mutations. The new variant is at the beginning of the third MH domain but not related to any other known functional site of the protein. cDNA = complementary DNA.

Genetic Analysis

RNA was isolated from differentiating muscle cells on day 4, after changing from growth medium to differentiation medium, with the Peqgold total RNA kit from Peqlab (Erlangen, Germany). RNA was then reverse transcribed into complementary DNA (cDNA) using the SuperScript First-Strand cDNA Synthesis System (Invitrogen) according to the protocols of the manufacturers. cDNAs were used as templates for amplification of parts of the RYR1 sequence with the polymerase chain reaction technique. Oligonucleotide primers according to McCarthy[12] were used to amplify 23 overlapping sequences of the RYR1 and the 25i5 and 25i3 primers for

detection of the R1086H substitution in the α_1 subunit of the L-type Ca^{2+} channel.[8] The length of the polymerase chain reaction products was 600–1,000 bp.

Polymerase chain reaction products were then purified with the Spin PCRapid purification kit (Invitek, Berlin, Germany) and sequenced by a commercial sequencing laboratory (DI Martin Ibl, Vienna, Austria). Both strands of all polymerase chain reaction products were sequenced.

Determination of Ca^{2+} Concentration

Cells were incubated with the Ca^{2+}-sensitive fluorescence dye fura-2/AM. The loading buffer was Tyrode solution (137 mM NaCl, 5.6 mM glucose, 5.4 mM KCl, 2.2 mM $NaHCO_3$, 1.1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 10 mM HEPES–Na, 1.8 mM $CaCl_2$, pH 7.4) supplemented with 2.5–5 μM fura-2/AM and 0.025% F-127 Pluronic. After incubation in loading buffer for 45–60 min at 37°C, unloaded dye was washed out, and coverslips were placed into a perfusion chamber of a Nikon fluorescence microscope at 400× magnification (Nikon, Vienna, Austria). Only cells that reacted to depolarization solution HK (Tyrode solution with 60 mM KCl and 80 mM NaCl, no Ca^{2+} added) with an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were used for experiments, assuming a skeletal muscle–specific excitation–contraction coupling. Fluorescence intensity was monitored at an emission wavelength of 510 nm by altering excitation wavelengths between 340 and 380 nm using a monochromator (VisiTech, Sunderland, United Kingdom).

For measurement of $[Ca^{2+}]_i$, images were recorded with a sample interval of 0.2–2 s. Stored images were analyzed using the QC2000 software package from VisiTech. For determination of $[Ca^{2+}]_i$, regions of interest were defined covering the entire visible area of a cell. The light intensity values from these specified areas were integrated and exported into the Sigma Plot program (SPSS Inc., Erkrath, Germany). Background subtraction, ratioing, and calculation of $[Ca^{2+}]_i$ were performed off-line. Resting $[Ca^{2+}]_i$ was defined by the average of the first 10 data points before application of any substances. The value of a $[Ca^{2+}]_i$ transient was determined by the peak value reached within the time of substance application.

Calibration of fura-2 fluorescence signals to calculate $[Ca^{2+}]_i$ values was performed according to Grynkiewicz *et al.*[13] To obtain the parameters for the equation, we used the pentapotassium salt of fura-2 (5 μM) in a solution mimicking the intracellular milieu.[14]

Dose–response curves were assessed by application of increasing concentrations of substances to a cell and measurement of the $[Ca^{2+}]_i$. After washout of the substance and return of $[Ca^{2+}]_i$ to resting levels, the next higher concentration was applied. Least square fitting of dose–response curves to the Hill equation was performed with the Sigma Plot program.

Electrophysiology

Voltage-dependent Ca^{2+} release was determined in fura-2/AM–loaded cells clamped to a holding potential of −80 mV. From this potential, depolarization steps from −50 mV up to +30 mV were applied in 20-mV increments. The depolarization lasted for 2 s and was followed by a 13-s repolarization interval. The voltage-induced Ca^{2+} release was normalized to the maximum Ca^{2+} release obtained during a test pulse series and averaged for the respective diagnostic group. For patch clamping the cells, an Axopatch 1D connected to an Axon Digidata 1200 and a personal computer with pClamp6 (Axon Inc., Foster City, CA) were used. The patch electrodes (PG10150-4; WPI, Sarasota, FL) had a resistance from 0.8 to 2 MΩ and were filled with intracellular solution (130 mM KCl, 1 mM$MgCl_2$, 10 mM HEPES–K^+, 4 mM ATP–K_2, pH 7.2, and 10 mg/ml Chelex 100). As a bathing solution, Tyrode solution was used.

Parameters for the voltage-dependent Ca^{2+} release were assessed using a Boltzmann equation, where the Ca^{2+} concentration reached during a step depolarization was normalized to the maximum $[Ca^{2+}]_i$ observed during a series of depolarizations.

Whole cell patch experiments for determination of the kinetics of L-type Ca^{2+} currents were performed using a HEKA EPC-9 patch clamp amplifier with the Pulse software package (HEKA Elektronik, Lambrecht, Germany). Patch electrodes were filled with a solution containing 145 mM CsOH, 135 mM aspartic acid, 11 mM EGTA, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES–Na, 2 mM K_2ATP, and 5 mM Na_2 creatine phosphate, pH 7.2; series resistance compensation was at least 50%. To measure currents through Ca^{2+} channels, Ba^{2+} was used as a charge carrier; the extracellular solution contained 115 mM CsCl, 15 mM $BaCl_2$, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES–Na, and 2.5 mM 4-aminopyridine, pH 7.4. L-type currents were determined by digital subtraction of current traces obtained in the presence of 10 μM of the specific L-type Ca^{2+} channel blocker nifedipine. Least square fitting of the current–voltage relation was performed according to a Boltzmann equation combined with a linear function describing the total voltage-dependent conductance:

$$I(x) = G_{max} \cdot (x - E_{rev}) \cdot (1/(1 + e^{-(x - E_{0.5})/k}))$$

where I(x) is the current at a certain membrane potential, x is the membrane potential, G_{max} is the maximum total Ca^{2+} conductance, E_{rev} is the reversal potential, $E_{0.5}$ is the half activation voltage, and k is the slope factor.

Statistical Analysis

Data regarding cells from two individuals with the new receptor variant were pooled and compared with data

regarding cells from a total of 11 individuals without the variant. Because of a high variability in the Ca^{2+}-releasing properties between single cells—which exceeds the variability between cells of different individuals—results of single-cell experiments were treated as independent for statistical analysis. For statistical analysis, we used Statgraphics plus 4.0 (Statistical Graphics Corp., Rockville, MD). Tests for normal distribution included the chi-square goodness-of-fit test, the test for Shapiro–Wilk W, and the z score for skewness and kurtosis. Means of normally distributed data were compared using the Student *t* test. For other data, the Mann–Whitney test was used to compare the medians. Significant difference was assumed for $P < 0.05$. Data are given as medians with 25th and 75th percentiles or as mean ± SE.

Results

IVCT and Genetic Testing

Four members of the family were tested with the IVCT after a 6-yr-old girl showed signs of a possible MH crisis during an adenotomy. The anesthesia was performed with less than 3% isoflurane and 70% nitrous oxide and oxygen in combination with 40 mg succinylcholine intravenously. The girl developed a tachycardia (252 beats/min), masseter spasm, and mydriasis without pupillary reflexes. No hyperthermia or generalized skeletal muscle rigidity was observed. The creatine phosphokinase values increased to 11,000 U/l. After immediate termination of anesthesia, the girl recovered without further symptoms. Because of her age, no IVCT was performed. The father of the girl and her uncle tested positive for MH on the IVCT (fig. 1A). Both showed slightly increased creatine phosphokinase values at rest (127 and 209 U/l, respectively) but were otherwise without obvious neuromuscular symptoms, and histologic tests were not performed. The threshold concentration of the MHS individuals for halothane was 0.11 mM (0.5% vol/vol). The thresholds for caffeine were reached at 1.0 and 1.5 mM, respectively. Two other members of this family were tested and diagnosed as MHN on the IVCT. The genetic analysis of the RYR1 of the two MHS members of the family yielded a nucleotide exchange in exon 87 at position 11953 from thymine to cytosine, which causes a change of the amino acid tryptophan at position 3985 to arginine (fig. 1B).

The new W3985R variant was found only in the two MHS individuals and not in any of the MHN individuals of that family or in 100 further individuals diagnosed MHN from other families. In the RYR1 gene of three family members, a further base change was found at position 11266 from cytosine to guanine (exon 79), which results in the change of amino acid 3756 from a glutamine to glutamate. This change in the protein sequence has already been described in an equivocal individual but was not regarded as important for the pathophysiology of MH.[15] In accord, we found this variant not only in one of the MHS individuals, but also in both nonaffected MHN family members, indicating that Q3756G is not a mutation involved in the pathophysiology of MH.

Ca^{2+} Release Experiments

For this study, differentiated skeletal muscle cells from two MHS individuals and two individuals of the same

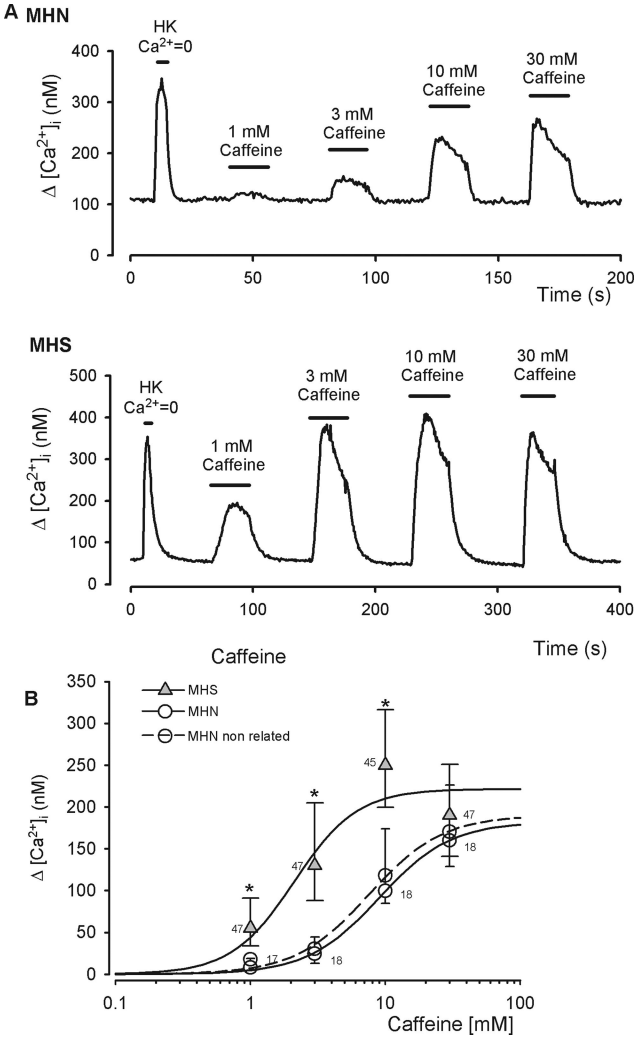

Fig. 2. Sensitivity against caffeine. (*A*) Time course of the intracellular Ca^{2+} concentration in differentiated human skeletal muscle cells. HK Ca^{2+} = 0 is a Ca^{2+}-free solution with elevated potassium, producing a depolarization of the cell. The observed increase in Ca^{2+} is due to a release and not an influx of Ca^{2+}, which is typical for the skeletal muscle type excitation contraction coupling. Increasing concentrations of caffeine resulted in increasing Ca^{2+} transients (MHN = cell from a normal individual; MHS = cell from an individual diagnosed as susceptible to malignant hyperthermia). (*B*) The concentration–response curves for caffeine showed a left shift for cells with the mutated RYR1. *Lines* are least square fits through the symbols according to the Hill equation. The *dashed line* shows the sensitivity of muscle cells derived from nonrelated MHN individuals. *Figures* next to the symbols give the number of experiments. Data are given as median with 25th and 75th percentiles. * Significant difference between MHS and MHN cells (Mann–Whitney test, $P < 0.05$).

family without the genetic variant were used as well as cells from nine nonrelated MHN individuals. All MHN cells were tested for absence of the W3985R mutation.

The resting Ca^{2+} levels in differentiated primary skeletal muscle cells were not different between cells from individuals diagnosed as MHS or MHN on the IVCT. The mean resting Ca^{2+} concentration in MHN cells was determined to be 82 ± 4 nM (SEM, n = 121 cells of two related and 6 nonrelated individuals), whereas MHS cells showed a resting Ca^{2+} concentration of 81 ± 4 nM (SEM, n = 74 cells of two related individuals with the W3985R variant).

To specifically activate a Ca^{2+} release through the RYR1, caffeine and 4-chloro-*m*-cresol (4-CmC) were used, and the sensitivity of muscle cells against these substances was determined. Changes in the $[Ca^{2+}]_i$ due to application of 1–30 mM caffeine were monitored (fig. 2A), and the maximum effects on the $[Ca^{2+}]_i$ were used to calculate a concentration–response curve (fig. 2B) according to the Hill equation. The EC_{50} value of MHS cells was 1.4 mM caffeine (1.1 mM, 2.0 mM, n = 30 cells from two individuals with the W3985R variant), whereas MHN cells of the same family reacted only at 6.9 mM caffeine (5.5 mM, 10.2 mM, n = 18 cells from two related individuals) with a half-maximum increase in $[Ca^{2+}]_i$ (table 1). The maximum $[Ca^{2+}]_i$ reached during application of caffeine did not differ significantly between the two diagnostic groups. Cells from nonrelated MHN individuals (n = 8 cells from three individuals) reacted to caffeine in a similar way as those derived from the two MHN family members (fig. 2B). For 4-CmC, the difference in sensitivity against the Ca^{2+}-releasing potency of the drug was less pronounced (fig. 3). At a concentration of 68.1 μM 4-CmC (61.1 μM, 83.6 μM, n = 31 cells from two individuals with the W3985R variant), MHS cells showed half-maximum Ca^{2+} release; MHN cells, however, reacted only at 108 μM 4-CmC (88 μM, 150 μM, n = 23 cells from two related individuals) with a comparable release of Ca^{2+}. Again, no change in the amount of released Ca^{2+} was detected when comparing MHN and MHS cells.

The physiologic stimulus of Ca^{2+} release in skeletal muscle cells is the depolarization of the membrane. The activation of the dihydropyridine receptor and the relay of this signal *via* physical interaction are thought to open the RYR1, thus leading to Ca^{2+} release.[16] In combined patch clamp–Ca^{2+}-detection experiments (fig. 4A), the maximum Ca^{2+} release in MHN cells was observed at the highest depolarization potential of +30 mV, and the potential where the half-maximum $[Ca^{2+}]_i$ occurred ($E_{0.5Ca}$) was determined to be −27 ± 3.6 mV (SEM, n = 6 cells from three individuals). In MHS cells, the maximum $[Ca^{2+}]_i$ was reached at a depolarization potential of +10 mV, and the $E_{0.5Ca}$ was −41 ± 4.0 mV (SEM, n = 6 cells from two individuals with the W3985R variant; $P < 0.05$; fig. 4B). To exclude the possibility that this shift is the result of altered Ca^{2+} channel kinetics, we determined the current–voltage relation of Ba^{2+} currents through L-type Ca^{2+} channels in myotubes from MHS and MHN individuals. The current–voltage curves of MHS and MHN Ba^{2+} currents were identical (fig. 4C).

In addition, we did not detect in MHS cells the Arg His mutation at residue 1086 of the α_1 subunit of the L-type Ca^{2+} channel—which is known to cause MH[8]—further corroborating the causative role of the new RYR1 variant for MH.

Discussion

The RYR1 plays a central role in releasing Ca^{2+} from the sarcoplasmic reticulum. MH, central core disease (CCD), and minicore disease have all been linked to mutated RYR1 channels. We do not fully understand the mechanisms of mutations in impairing channel functions to the point of pathologic manifestations. So far, mutations leading to MH and/or CCD have been found to cluster in three distinct regions of the channel protein (MH/CCD regions 1–3), with region 1 (AA35-614) and region 2 (AA2129-2458) located in the myoplasmic domain of the channel, and region 3 (AA3916-4973) being part of the putative transmembrane and luminal domain of the protein.[17] Although much effort has been made to elucidate the three-dimensional structure of the Ca^{2+} release channel,[18] its relation to the primary sequence is

Table 1. Parameters for Ca^{2+} Release

	Maximum Ca^{2+} Increase	Hill Coefficient	EC_{50}	n
MHN caffeine	191 (135, 255) nM	1.9 (1.5, 2.6)	6.9 (5.5, 10.2) mM	18
Mann–Whitney test	U = 406, P = 0.193	U = 412, P = 0.159	U = 7, $P < 0.001$	
MHS caffeine	231 (186, 282) nM	2.5 (1.7, 4.1)	1.4 (1.1, 2.0) mM	30
MHN 4-CmC	249 (193, 300) nM	3.5 (1.9, 5.2)	108 (88, 150) μM	23
Mann–Whitney test	U = 411, P = 0.349	U = 467, P = 0.109	U = 603, $P < 0.001$	
MHS 4-CmC	273 (217, 319) nM	2.4 (1.7, 3.8)	68.1 (61.1, 83.6) μM	31

Parameters for Ca^{2+} release by caffeine and 4-chloro-*m*-cresol (4-CmC). Data are median with 25th and 75th percentiles in parentheses, and n is the number of experiments used for calculation of parameter values according to a Hill equation. The malignant hyperthermia susceptible (MHS) group contains experiments from two individuals with the W3985R variant, whereas in the malignant hyperthermia nonsusceptible (MHN) group cells of two individuals of the same family without the variant were used. Results of the Mann–Whitney test are given between the compared values.

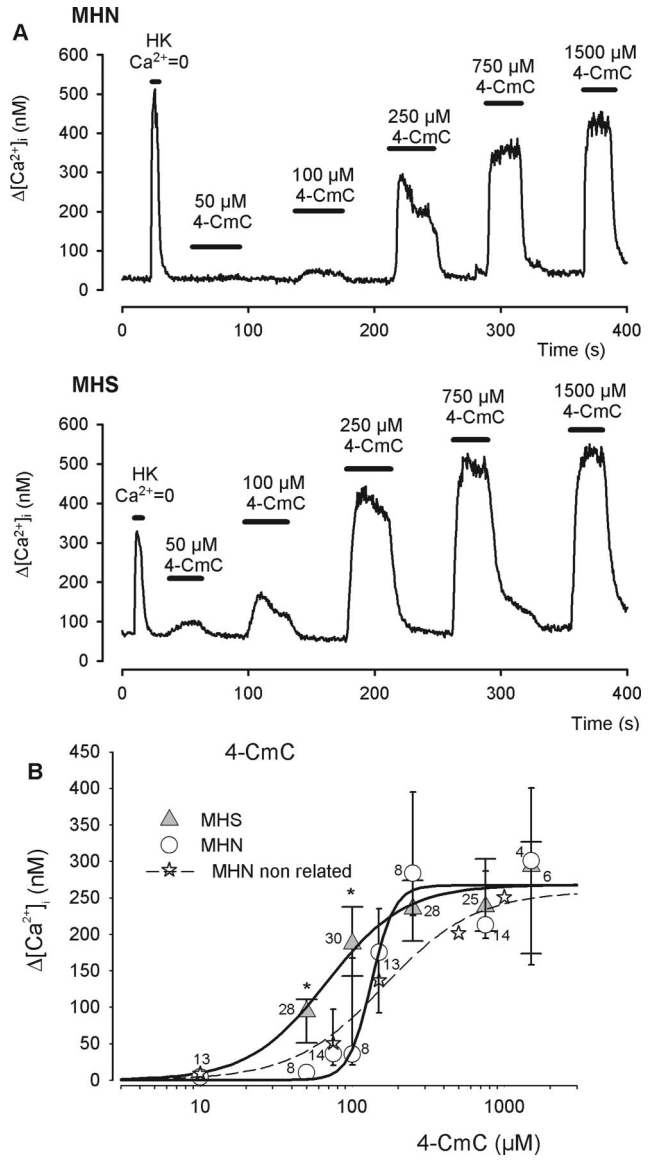

Fig. 3. Sensitivity against 4-chloro-*m*-cresol (4-CmC). (*A*) Effect of 4-CmC on the intracellular Ca^{2+} concentration in differentiated muscle cells (MHN = cell from a normal individual; MHS = cell from an individual diagnosed as susceptible to malignant hyperthermia). (*B*) Concentration–response curve of 4-CmC. MHS cells show a higher sensitivity than MHN cells, especially at low concentrations of 4-CmC. The *dashed line* shows the sensitivity of muscle cells derived from nonrelated MHN individuals. Fit to symbols as in figure 2B. Data are given as median with 25th and 75th percentiles. * Significant difference between MHS and MHN cells (Mann–Whitney test, $P < 0.05$).

still a matter of research. Mutations in highly conserved regions within the gene are of high interest because they are of functional importance and thus shed light on the structure–function relations of such domains.

The site of the new proposed mutation is at the beginning of the classic MH domain 3 and highly conserved, not only between different species but also between the three known isoforms of the RYR. The importance of this domain for MH is also highlighted by findings from Robinson *et al.*,[7] who found variants at AA3986 and 3990 in two MH families. Although the linkage between MH and those variants has yet to be established, sequence changes in this region of the RYR seem to render the channel unstable. The exchange of AA3985 from the aromatic tryptophan to the basic arginine makes this site more hydrophilic and could disrupt a possible interdomain interaction. Such interactions have been proposed to explain the stabilizing effect of dantrolene on the channel,[19] as well as the destabilizing effect of MH mutations found in the DP4 region (corresponding to Leu 2442-Pro 2477) of the RYR1.[20]

A similar destabilizing effect by the new proposed mutation is indicated by the fact that the sensitivity of Ca^{2+} release against 4-CmC and caffeine is enhanced (figs. 2 and 3). These two compounds are known to directly activate the RYR, and it is therefore plausible that the change in the primary sequence of the RYR leads to this increased sensitivity.

The physiologic stimulus for activating the RYR1 is the depolarization of the cell. Depolarization is sensed by the voltage-gated L-type Ca^{2+} channel in the plasma membrane and relayed to the Ca^{2+} release channel. MHS cells with the W3985R variant in the RYR1 showed a shift in the voltage-dependent Ca^{2+} release to more negative potentials (fig. 4), indicating either destabilization of the RYR and opening due to minute changes in L-type Ca^{2+} channel conformation, or an increased sensitivity of the channel against activating concentrations of Ca^{2+} as already shown for MHS muscle.[21,22]

Another possibility for the observed early release of Ca^{2+} could be a shift in the voltage-dependent gating of the L-type Ca^{2+} channel toward more negative potentials. This was ruled out by voltage clamp experiments that revealed no differences of L-type channel kinetics in MHS cells and MHN cells.

To our knowledge, the two individuals carrying the new RYR1 variant do not have muscle fasciculations or other muscle-related abnormalities. This might occur when there is a shift of Ca^{2+} release to more negative potentials, resulting in spontaneous Ca^{2+} release events. However, the firing threshold for action potentials is around −60 mV, so even if there is more release of Ca^{2+} already at −50 mV due to the variant (fig. 4B), this release would take place only after an action potential has been triggered. So the difference between the thresholds for eliciting an action potential and releasing Ca^{2+} still seems to be big enough to enable regular muscular function.

Moreover, the resting Ca^{2+} concentration of MHS cells was not increased, and the net Ca^{2+} release was not diminished, indicating that the sarcoplasmic reticulum Ca^{2+} content is not affected by the variant. Some experimental evidence indicates elevated resting $[Ca^{2+}]_i$ for

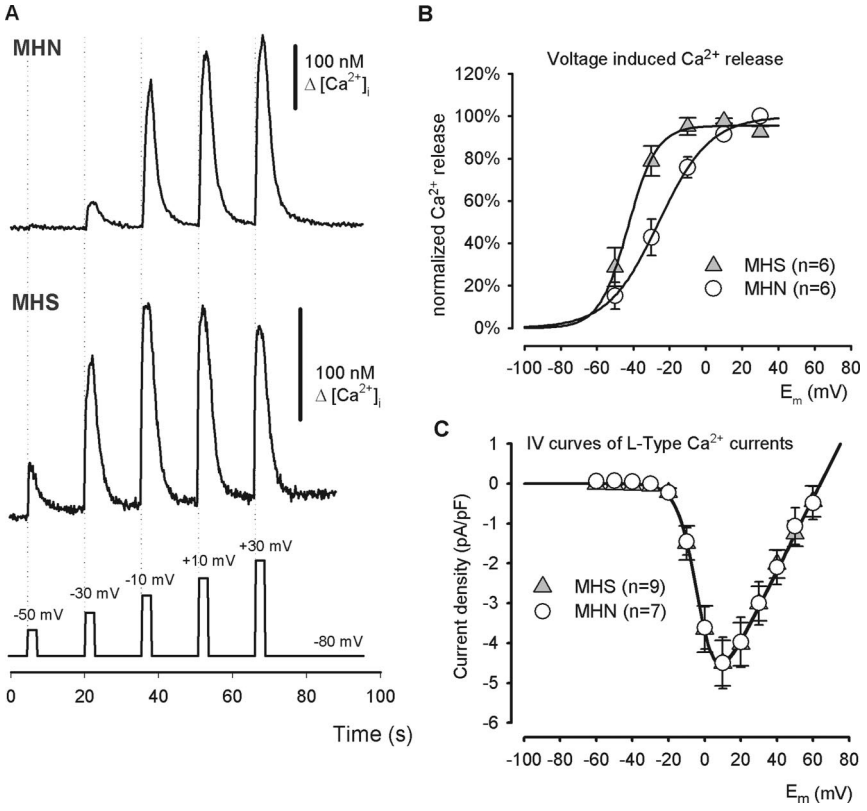

Fig. 4. Voltage-dependent Ca^{2+} release. (*A*) Myotubes were patch clamped at a holding potential of −80 mV and depolarized to increasing potentials for 2 s. Changes in intracellular Ca^{2+} concentrations were monitored fluorometrically. Activation of the voltage dependent Ca^{2+} channels results in an intracellular release of Ca^{2+}. (MHN = cell from a normal individual; MHS = cell from an individual diagnosed as susceptible to malignant hyperthermia). (*B*) The voltage-dependent Ca^{2+} release is shifted to more negative potentials in MHS cells. (*C*) This shift is not due to a change in the properties of voltage-activated L-type Ca^{2+} channel. The current–voltage relation shows the same activation kinetics in MHS and MHN cells.

MHS cells,[23–25] whereas other reports did not find alterations. Conflicting reports may arise from different methods used for the determination of $[Ca^{2+}]_i$ but also from the diversity of functional impairments due to different channel mutations. According to a model proposed by Dirksen and Avila,[26] MH in the absence of CCD arises from RyR1 mutations that result in overactive release channels that do not cause a net change in steady state sarcoplasmic reticulum Ca^{2+} content (so-called compensated leak channels), but predispose the muscle to increased Ca^{2+} release upon exposure to triggering substances. Mutations that enhance the RyR1 activity or sensitivity to an extent sufficient to result in a partial depletion of the sarcoplasmic reticulum Ca^{2+} store would decrease the maximum Ca^{2+} release and lead to elevated resting $[Ca^{2+}]_i$ (decompensated leak). Such leaky channels have been postulated to cause CCD. Therefore, this new variant would belong to the MH-only mutations with no leak or a compensated leak, and indeed, individuals carrying this variant do not have CCD.

The classification of this genetic variant as a mutation that causes MH requires the identification of a family not related to the family presented here, in which MH susceptibility is associated with this RyR1 variant. This will happen only if other centers that examine the RyR1 also examine exon 87. More work of this type is needed to improve the sensitivity of genetic testing for MH susceptibility.

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