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Functional Characterization of a Distinct Ryanodine Receptor Mutation in Human Malignant Hyperthermia-susceptible Muscle*

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Malignant hyperthermia is an inherited autosomal disorder of skeletal muscle in which certain volatile anesthetics and depolarizing muscle relaxants trigger an abnormally high release of Ca^{2+} from the intracellular Ca^{2+} store, the sarcoplasmic reticulum. In about 50% of cases, malignant hyperthermia susceptibility is linked to the gene encoding the skeletal muscle ryanodine receptor/Ca^{2+} release channel (RYR1). To date, eight point mutations have been identified in human RYR1. Although these mutations are thought to lead to an increased caffeine and halothane sensitivity in the contractile response of skeletal muscle, their functional consequences have not been investigated on the molecular level. In the present study, we provide the first functional characterization of a point mutation located in the central part of RYR1, $Gly^{2434} \rightarrow Arg$. Using high affinity [^{3}H]ryanodine binding as the experimental approach, we show that this mutation enhances the sensitivity of RYR1 to activating concentrations of Ca^{2+} and to the exogenous and diagnostically used ligands caffeine and 4-chloro-*m*-cresol. In parallel, the sensitivity to inhibiting concentrations of Ca^{2+} and calmodulin was reduced, transferring the mutant Ca^{2+} release channel into a hyperexcitable state.

Malignant hyperthermia (MH)[1] is a pharmacogenetic skeletal myopathy of humans and swine and is one of the main causes of death due to anesthesia. Predisposed patients are at high risk for undergoing a fulminant MH crisis when exposed to certain volatile anesthetics and depolarizing muscle relaxants commonly used in anesthesia. A point mutation ($Arg^{615} \rightarrow$ Cys) in the skeletal muscle ryanodine receptor (RYR1), which functions as the sarcoplasmic reticulum (SR) Ca^{2+} release channel, has been linked to porcine stress syndrome, an equivalent to human MH (1). Contrary to porcine stress syndrome, human MH is a genetically heterogeneous skeletal muscle disorder. Based on genetic linkage studies, three MH loci are known. The first has been mapped to chromosome 19q12–13.2 encompassing the gene that in homology with the animal model encodes the RYR1 (2, 3), the second has been mapped to chromosome 7q including the gene for the α_2/δ subunit of the skeletal muscle dihydropyridine receptor (4), and the third has been mapped to chromosome 3q13.1 (5). To date, mutations have only been identified in RYR1 that count for approximately 50% of human MH cases (reviewed in Ref. 6). MH mutations seem to cluster in two areas of the RYR1 sequence. Six mutations have been localized in the N-terminal sequence of RYR1 containing a homologous mutation to that identified in porcine MH-susceptible (MHS) muscle. Two further mutations have been found in the central amino acid sequence. *In vitro*, all these mutations induce a hypersensitivity of biopsied muscle to the contracture-triggering agents caffeine and halothane. This enhanced sensitivity is exploited in the diagnostic *in vitro* contracture test (IVCT). According to the European test protocol (7), dissected muscle fiber bundles are exposed to increasing concentrations of caffeine and halothane. Individuals are classified MHS if the sensitivity is increased for both compounds. Ca^{2+} release from human SR vesicles obtained from MHN and MHS muscle samples has been studied in a few approches (8–13). In these experiments, however, MHS muscle samples were collected from genetically nonclassified material. Thus, the observed effects could not be addressed to a single human RYR1 mutation. In the present study, we characterized the functional effects of a human RYR1 mutation that is located in the central part of RYR1, $Gly^{2434} \rightarrow Arg$. (The numbering of amino acids follows the corrected sequence data for the human RYR1 according to Ref. 14.) Our data provide the first definitive evidence that a centrally located mutation is causative for the hypersensitivity of SR Ca^{2+} release in MHS muscle. Part of this work has been submitted in abstract form (15).

EXPERIMENTAL PROCEDURES

Materials—*Taq* polymerase was purchased from Pharmacia (Freiburg, Germany), and *Alw*NI was from New England Biolabs (Schwalbach, Germany). A DNA preparation kit was obtained from MWG-Biotech (Ebersberg, Germany). (9,21-^{3}H(N))Ryanodine was purchased from DuPont NEN (Bad Homburg, Germany). Ryanodine was from Calbiochem (Bad Soden, Germany), and protease inhibitors were from Boehringer (Mannheim, Germany). Protein molecular mass standard was purchased from Bio-Rad (München, Germany), and DNA size standard was from MBI Fermentas (St. Leon-Rot, Germany). All other chemicals were of analytical grade. Filter membranes for [^{3}H]ryanodine binding were purchased from Schleicher & Schüll (Dassel, Germany).

Patient Characterization—Skeletal muscle biopsies (*Musculus vastus lateralis*) were taken from a patient who had suffered from a typical MH crisis and from his relatives for the test of susceptibility to MH. DNA was extracted from anticoagulated blood of individuals from this pedigree found to be heterozygous for the RYR1 $Gly^{2434} \rightarrow Arg$ mutation. For control, muscle specimens were obtained from individuals who had undergone muscle biopsy for exclusion of MH susceptibility. Muscle samples were tested according to the protocol of the European Malignant Hyperthermia Group (7). All procedures were in accordance with the Helsinki convention and were approved by the Ethics Commission of the University of Ulm.

Amplification and Digestion of Genomic DNA—Genomic DNA was

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[1] The abbreviations used are: MH, malignant hyperthermia; 4-CmC, 4-chloro-*m*-cresol; CaM, calmodulin; IVCT, *in vitro* contracture test; MHN, malignant hyperthermia negative; MHS, malignant hyperthermia-susceptible; PCR, polymerase chain reaction; RYR1, skeletal muscle ryanodine receptor; SR, sarcoplasmic reticulum; bp, base pair(s); PIPES, 1,4-piperazinediethanesulfonic acid; CCD, central core disease.

isolated from 10 ml of blood from MHN and MHS individuals using a DNA preparation kit (MWG-Biotech). For analysis of mutation G7300A predicting the Gly^{2434} to Arg substitution, flanking primers as designed from the published sequence (14) (Ex45RyR sense 5′-TTCCCTG-CAGCTTTGGTG-3′ and Ex45RyR reverse 5′-GGGTCTCACATG-CATCTC-3′) were used to amplify a 128-bp fragment. PCR was carried out with 50 ng of genomic DNA and 30 pmol primers each in a total volume of 50 μl. The PCR reaction contained 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 2.5 mM $MgCl_2$, 0.01% gelatin, 200 μM of each dNTP, and 1 unit of *Taq* polymerase. PCR amplification conditions were 94 °C for 4 min followed by 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 30 s. The presence or absence of the mutation was detected by polyacrylamide gel electrophoresis of products obtained by digestion of PCR products with *Alw*NI.

Preparation of SR Membranes—MHS muscle samples obtained by biopsy from the *M. vastus lateralis* were collected from five individuals carrying the RYR1 mutation $Gly^{2434} \rightarrow$ Arg. The patients consisted of five males, varying in age from 34 to 69 years. For control, muscle samples were obtained from 28 individuals who were classified MHN according to the European IVCT protocol (7). Biopsied muscle specimens were immediately frozen and stored in liquid nitrogen until use. A microsomal SR fraction was isolated as described previously (16). Isolated membranes were resuspended in 0.3 M sucrose, 10 mM K-PIPES, pH 6.8, rapidly frozen in liquid nitrogen and stored at −70 °C. To prevent proteolysis, the following protease inhibitors were included in various purification steps: 200 μM Pefabloc (4-(2-aminoethyl)benzol-sulfonyl-fluoride), 100 nM aprotinin, 1 μM leupeptin, 1 μM pepstatin A, and 1 mM benzamidine. Protein concentration was determined according to the method described in Ref. 17 using bovine serum albumin as a standard.

[^{3}H]Ryanodine Binding—SR vesicles (at a protein concentration of 400 μg/ml) were incubated with indicated concentrations of [^{3}H]ryanodine in a medium containing 100 mM KCl, 100 μM EGTA, 20 mM Na-PIPES, 200 μM Pefabloc, pH 6.8, for 3 h at 37 °C. Varying concentrations of Ca^{2+}, calmodulin, caffeine, and 4-chloro-*m*-cresol were added to the incubation medium as indicated in the figure legends. Unbound ryanodine was separated from protein-bound ryanodine by filtration of protein aliquots (14 μg) through Schleicher & Schüll GF51 filters presoaked in 1% polyethylenimine. Filters were washed three times with ice-cold buffer solution as described above. Radioactivity remaining with the filters was measured by liquid scintillation counting. Specific binding was calculated as the difference of total and nonspecific binding determined in the presence of a 1000-fold excess of unlabeled ryanodine. Experiments were carried out in duplicate.

Polyacrylamide Gel Electrophoresis—Protein samples were denatured in Laemmli buffer at 95 °C for 3 min and separated in 3.5–15% gradient SDS/polyacrylamide minigels. Gels were stained with Coomassie Brilliant Blue. DNA fragments were separated in linear 10% polyacrylamide gels. Gels were stained with ethidium bromide.

Miscellaneous Methods—Free concentrations of Ca^{2+} were calculated using the computer program and binding constants described in Ref. 18. Dose-response curves were fitted using nonlinear curve-fitting routines based on the Marquardt-Levenberg algorithm. The data represent the means ± S.D. of two different MHN and MHS SR preparations.

RESULTS

Human skeletal muscle specimens were obtained from patients who underwent muscle biopsy for the test of susceptibility to MH. For this purpose, the caffeine and halothane sensitivity of dissected fiber bundles of biopsied muscle were tested according to the European IVCT protocol. MHS samples were collected from individuals of one pedigree heterozygous for the RYR1 $Gly^{2434} \rightarrow$ Arg mutation. To investigate the presence of the mutation in this pedigree, the 128-bp region spanning the G7300A mutation was amplified and subjected to restriction enzyme analysis. The base exchange results in the creation of an *Alw*NI restriction site (19). Digestion of PCR-amplified DNA fragments resulted in MHS individuals in two additional bands of 100 and 28 bp (Fig. 1). Fig. 1 shows that the mutation segregated with MH. All patients who were classified MHS in IVCT carried the point mutation in RYR1. To investigate the functional consequences of this mutation at the molecular level, a microsomal SR fraction was isolated from MHS muscle and as control from MHN samples and utilized for high affinity

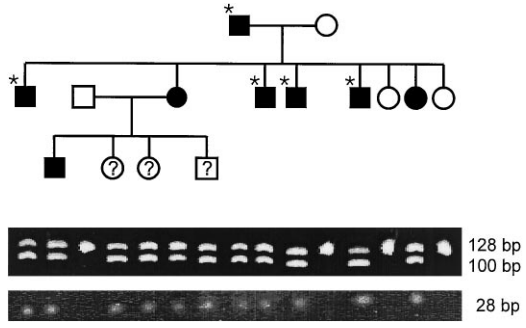

FIG. 1. **Pedigree of an MH family heterozygous for the RYR1 $Gly^{2434} \rightarrow$ Arg mutation with corresponding polyacrylamide gel.** A 10% polyacrylamide gel was loaded with fragments of PCR-amplified genomic DNA following digestion with *Alw*NI. The gel was stained with ethidium bromide. The upper and lower part was cut out for demonstration. PCR products of MHN individuals show a nondigested PCR product of 128 bp. In MHS patients' DNA, cleavage of the normal 128-bp PCR fragment generates two additional bands of 100 and 28 bp. The results of the IVCT test are indicated by *filled* (MHS) and *open* (MHN) symbols. A *question mark* denotes untested individuals. Skeletal muscle biopsies of individuals labeled with an *asterisk* were taken for SR preparation. *Circles*, females; *squares*, males.

[^{3}H]ryanodine binding.

Fig. 2 shows a Coomassie-stained SDS/polyacrylamide gel that was loaded with aliquots of SR vesicles of MHN and MHS muscle samples. The overall gel pattern was not different for MHN and MHS vesicles. RYR1s were separated as single high molecular mass bands of an estimated size of about 450 kDa with no detectable degradation products.

The [^{3}H]ryanodine affinity of isolated SR vesicles from MHN and MHS muscle was determined in the presence of an activating Ca^{2+} concentration of 10 μM. Scatchard analysis revealed a single class of high affinity binding sites for both tissues (Fig. 3). The affinity of MHN vesicles (K_d = 47.0 ± 3.7 nM, n = 4) was approximately 1.5-fold lower compared with MHS vesicles (K_d = 31.7 ± 3.9 nM, n = 2). No significant differences between MHN and MHS vesicles were found for the maximal activation of [^{3}H]ryanodine binding (for B_{max}(MHN), 1.37 ± 0.12 pmol/mg protein (n = 4) *versus* 1.44 ± 0.05 pmol/mg protein (n = 2) for B_{max}(MHS)).

The binding of [^{3}H]ryanodine is greatly influenced by ligands of RYR1 that activate or inhibit SR Ca^{2+} release (20–22). Because the amount of MHS muscle was very limited (<2.2 g), further analysis was restricted to the investigation of modulators for which an abnormal sensitivity has been observed in MHS ($Arg^{615} \rightarrow$ Cys) porcine muscle (reviewed in Ref. 23).

Fig. 4 shows the dependence of high affinity [^{3}H]ryanodine binding on cytoplasmic Ca^{2+} concentration. Ca^{2+} activated [^{3}H]ryanodine binding more potently in MHS vesicles, but the maximum binding was reached at 10 μM Ca^{2+} for both vesicle types. Whereas the threshold of activation for MHN vesicles was around 1 μM Ca^{2+} (pCa = 6), binding to MHS vesicles was distinctly activated by this Ca^{2+} concentration. The largest differences for activating Ca^{2+} concentrations were found between a pCa of 5.0 and 5.5 and for inhibiting concentrations between a pCa of 4.3 and 3.5. Higher concentrations inhibited binding to both vesicle types to almost the same extent. Binding to MHN vesicles was half-maximally activated at 3.6 μM, whereas the EC_{50} for binding to MHS vesicles was 3-fold lower (1.2 μM). In parallel, MHS vesicles were approximately 2-fold less sensitive for inhibiting Ca^{2+} concentrations (for MHN, IC_{50} = 135 μM (n = 6), and for MHS, IC_{50} = 282 μM (n = 3)).

Calmodulin (CaM) inhibits SR Ca^{2+} release when the release channel (RYR1) is previously activated by Ca^{2+} (24–30). For the experiments described here, [^{3}H]ryanodine binding was initially activated by 10 μM Ca^{2+}. CaM inhibited Ca^{2+}-acti-

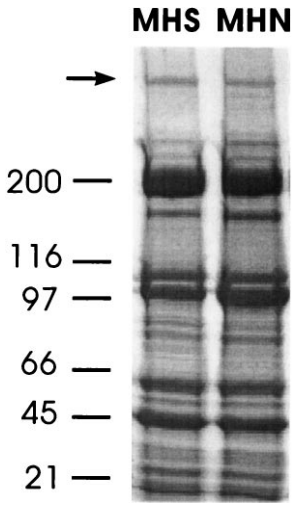

FIG. 2. **Electrophoretic analysis of SR vesicles from MHN and MHS skeletal muscle.** Aliquots (60 μg) of MHN and MHS SR vesicles were separated on a 3.5–15% SDS/polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue. Molecular mass standards (migration indicated on the *left-hand side*) were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and trypsin inhibitor (21 kDa). The *arrow* indicates the position of RYR1.

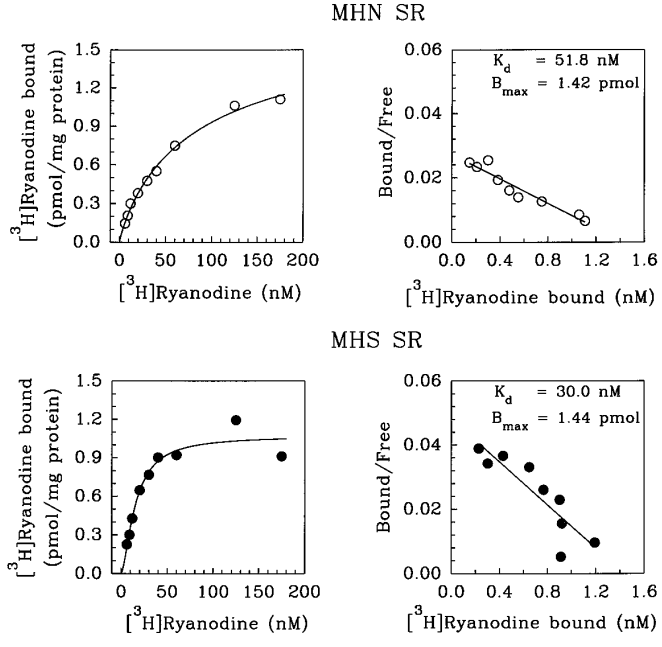

FIG. 3. **Specific binding of [^{3}H]ryanodine to MHN and MHS skeletal muscle SR vesicles.** Binding was carried out in 0.1 M KCl, 10 μM Ca^{2+}, pH 6.8, and the indicated concentrations of [^{3}H]ryanodine as described under "Experimental Procedures." *Right*, corresponding Scatchard plots. The symbols represent the means of one representative experiment carried out in duplicate.

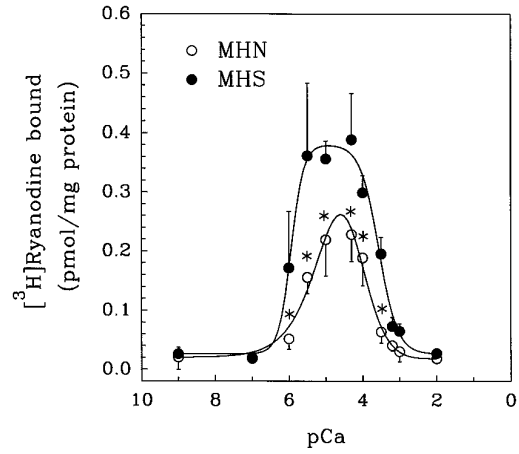

FIG. 4. **Dependence of high affinity [^{3}H]ryanodine binding on cytosolic Ca^{2+}.** Ca^{2+} dependence of [^{3}H]ryanodine binding in the presence of 12 nM [^{3}H]ryanodine and indicated concentrations of free Ca^{2+}. Data were fitted acoording to the following equation: $B = B_{max} \times \{[Ca]^{n1}/(k_1 + [Ca]^{n1}) - [Ca]^{n2}/(k_2 + [Ca]^{n2})\}$, where B corresponds to bound [^{3}H]ryanodine and B_{max} corresponds to maximally bound [^{3}H]ryanodine, [Ca] is free Ca^{2+} concentration, k_1 and k_2 are the binding constants for the Ca^{2+} activating and inhibitory sites, and n_1 and n_2 are the corresponding Hill coefficients. Resulting half-maximal activating and inhibiting concentrations were: MHN, EC_{50} = 3.55 μM, IC_{50} = 135 μM (n = 6); MHS, EC_{50} = 1.20 μM, IC_{50} = 282 μM (n = 3), where n represents the number of experiments carried out in duplicate. Data points labeled with *asterisks* are significantly different on the $p < 0.05$ level (Student's t test).

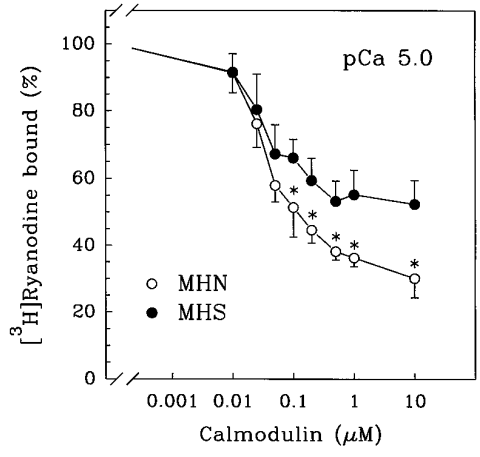

FIG. 5. **Inhibition of [^{3}H]ryanodine binding by CaM.** [^{3}H]Ryanodine binding was performed in the presence of 12 nM [^{3}H]ryanodine at a pCa of 5. The data were normalized to the amount of [^{3}H]ryanodine bound in the absence of CaM. Data points represent the means ± S.D. of five experiments of MHN SR vesicles and four experiments for MHS vesicles. An *asterisk* indicates significant differences between corresponding data points at $p < 0.05$ (Student's t test).

vated binding in a concentration-dependent manner (Fig. 5). Contrary to the porcine $Arg^{615} \rightarrow Cys$ mutation (30), the human $Gly^{2434} \rightarrow Arg$ mutation resulted in a loss of sensitivity for CaM. In MHN vesicles, CaM inhibited binding to about 30% of control, whereas binding to MHS vesicles was only reduced to 50%. Significant differences were found in the presence of CaM concentrations greater than 0.1 μM.

In the following experiments, the effects of the two exogenous RYR1 activators, caffeine and 4-chloro-*m*-cresol, which are used for the diagnosis of MH, were investigated. Experiments were carried out in the presence of a free Ca^{2+} concentration of 0.1 μM, which is below the threshold of activation for both vesicle types.

Similar to Ca^{2+}, caffeine stimulated [^{3}H]ryanodine binding to a higher level to MHS than to MHN vesicles (Fig. 6, *left*). Major differences between MHN and MHS vesicles were observed for concentrations starting at 5 mM. The stimulatory effect, however, was weak compared with caffeine-activated [^{3}H]ryanodine binding to porcine SR vesicles (Table I). The calculated EC_{50} values were about 24.9 mM for MHN and 9.5 mM for MHS vesicles.

It has recently been shown that 4-chloro-*m*-cresol (4-CmC) is a potent and specific activator of RYR1 that can be used as a diagnostic tool to distinguish between MHN and MHS muscle (16, 31–33). Comparable with caffeine, 4-CmC stimulated

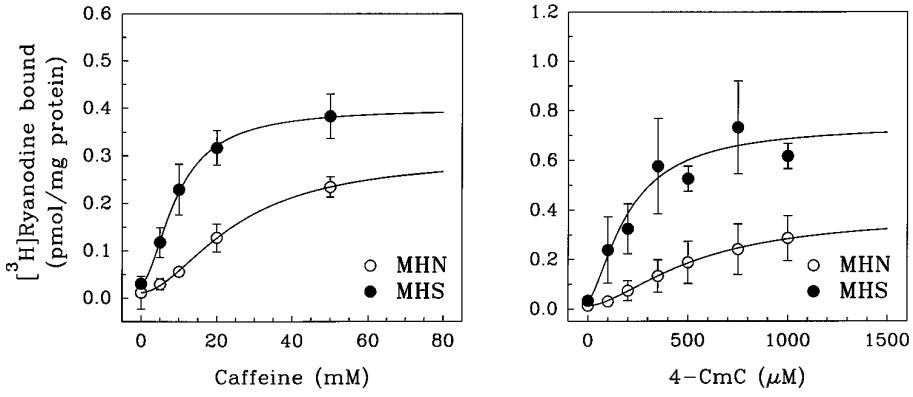

FIG. 6. **Activation of [^{3}H]ryanodine binding by caffeine and 4-CmC.** The activating effect of caffeine (*left*) and 4-CmC (*right*) was investigated in the presence of 0.1 μM Ca^{2+} and 12 nM [^{3}H]ryanodine. Data were fitted according to the Hill equation: EC_{50}/n_{app} (caffeine): MHN, 24.9 mM/1.8 (n = 3), and MHS, 9.5 mM/1.8 (n = 3); EC_{50}/n_{app} (4-CmC): MHN, 535 μM/1.7 (n = 8), and MHS, 190 μM/1.6 (n = 2). The data are derived from n experiments performed in duplicate. If no *error bars* (S.D.) are shown, they are encompassed within the symbol.

TABLE I

Comparison of the functional effects of the porcine $Arg^{615} \rightarrow Cys$ with the human $Gly^{2434} \rightarrow Arg$ mutation

[^{3}H]Ryanodine binding was performed in low salt (0.1 M KCl), pH 6.8, at 12 nM [^{3}H]ryanodine as described under "Experimental Procedures." Data were taken from experiments that were carried out under exactly the same conditions for the $Arg^{615} \rightarrow Cys$ and $Gly^{2434} \rightarrow Arg$ mutation.

$Arg^{615} \rightarrow Cys$ (porcine RYR1)[a]	$Gly^{2434} \rightarrow Arg$ (human RYR1)
High affinity [^{3}H]ryanodine binding	
MHN, K_d = 43.3 nM	MHN, K_d = 47.0 nM
MHS, K_d = 11.1 nM	MHS, K_d = 31.7 nM
Ca^{2+} dependence	
MHN, EC_{50} = 2.60 μM	MHN, EC_{50} = 3.55 μM
MHS, EC_{50} = 0.96 μM	MHS, EC_{50} = 1.20 μM
MHN, IC_{50} = 270 μM	MHN, IC_{50} = 135 μM
MHS, IC_{50} = 560 μM	MHS, IC_{50} = 282 μM
Caffeine dependence	
MHN, EC_{50} = 10.7 mM	MHN, EC_{50} = 24.9 mM
MHS, EC_{50} = 3.7 mM	MHS, EC_{50} = 9.5 mM
4-Chloro-*m*-cresol dependence	
MHN, EC_{50} = 395 μM	MHN, EC_{50} = 535 μM
MHS, EC_{50} = 193 μM	MHS, EC_{50} = 190 μM

[a] Data were taken from Ref. 16.

[^{3}H]ryanodine binding to MHS SR with higher affinity than to MHN SR (Fig. 6, *right*). Contrary to caffeine, the absolute level of activation was about 2-fold higher, and the resulting EC_{50} values were approximately 20-fold lower. MHS vesicles were about 3-fold more sensitive (EC_{50} = 190 μM (n = 3)) compared with MHN (EC_{50} = 535 μM (n = 8)). The largest differences in activation were observed at 4-CmC concentrations between 200 and 500 μM.

DISCUSSION

The sarcoplasmic reticulum (SR) is the major element in skeletal muscle that regulates the release and uptake of myoplasmic Ca^{2+}. SR Ca^{2+} release is mediated by the high molecular weight ligand-gated Ca^{2+} release channel (RYR1), which is biochemically characterized by its high affinity for the plant alkaloid ryanodine (recent reviews in Refs. 34 and 35). The protein complex comprises four identical subunits each consisting of about 5000 amino acids as deduced by cloning and sequencing of the cDNA (36, 37). Hydropathy plots suggested 4 (rabbit RYR1) to 10 (human RYR1) hydrophobic segments, the ion pore forming segments in the C-terminal part, comprising about 10–20% of the receptor molecule. The remainder of the protein has been assigned to the cytoplasmic side of the SR membrane. Human RYR1 mutations have been linked to two skeletal muscle diseases, malignant hyperthermia and central core disease (CCD) (6, 38–40). Both disorders have been associated with an abnormally high release of Ca^{2+} from SR, which is probably due to an altered function of the mutant Ca^{2+} release channel. Six of these mutations ($Arg^{163} \rightarrow Cys$ (MH, CCD), $Gly^{248} \rightarrow Arg$ (MH), $Gly^{341} \rightarrow Arg$ (MH), $Ile^{403} \rightarrow Met$ (MH, CCD), $Tyr^{522} \rightarrow Ser$ (MH, CCD), and $Arg^{614} \rightarrow Cys$ (MH)) have been identified in the N-terminal part of the receptor and two ($Gly^{2434} \rightarrow Arg$ (MH) and $Arg^{2435} \rightarrow His$ (MH, CCD)) in the central part. Although the functional effects of a RYR1 mutation in the N-terminal part of the receptor ($Arg^{614} \rightarrow Cys$) have been studied in detail in the corresponding animal model in porcine skeletal muscle (23), the functional consequences of genetically defined human mutations and subsequently of a mutation located in the central part of the amino acid sequence of RYR1 has not yet been investigated.

An A for G7300 transition in the RYR1 gene leads to the replacement of a conserved Gly by an Arg at position 2434 in the amino acid sequence. This mutation has been identified in four Caucasian (19) and four Canadian pedigrees (41). Comparing the presence or the absence of the mutation in the pedigree investigated in the present study with the results of the *in vitro* contracture test (Fig. 1) revealed that the $Gly^{2434} \rightarrow Arg$ mutation precisely segregates with the MHS phenotype.

Because the amount of MHS muscle sample was very limited (<2.2 g), we used high affinity [^{3}H]ryanodine binding to isolated SR vesicles to study the effect of this mutation on SR Ca^{2+} release. Binding of [^{3}H]ryanodine reflects the functional state of the SR Ca^{2+} release channel because ligands that have been shown to activate or inhibit the Ca^{2+} release channel modulate [^{3}H]ryanodine binding in a similar way (20–22). Using this highly reproducible functional approach, we were able to investigate the effects of some major endogenous and pharmacological ligands of RYR1.

Table I compares the sensitivities of various SR modulators on [^{3}H]ryanodine binding to porcine and human SR vesicles of MHN and MHS muscle samples carrying the point mutations $Arg^{615} \rightarrow Cys$ and $Gly^{2434} \rightarrow Arg$, respectively. The data are derived from experiments that were carried out under exactly the same conditions for both tissues (16) and that have been described as optimal for visualizing differences in [^{3}H]ryanodine binding to MHS and MHS porcine SR vesicles (42). In contrast to the material obtained from humans who were heterozygous for the $Gly^{2434} \rightarrow Arg$ mutation, porcine muscle was obtained from pigs that were homozygous for the $Arg^{615} \rightarrow Cys$ mutation. [^{3}H]Ryanodine binding to SR vesicles derived from porcine muscle heterozygous for the mutation have not been characterized in detail.

Porcine MHS SR vesicles exerted an approximately 4-fold higher affinity for [^{3}H]ryanodine compared with MHN vesicles. This difference is less pronounced for the human mutation. The smaller difference could be either explained by the different locations of the mutations in the amino acid sequence, or it could be due to the fact that the human muscle is heterozygous for the mutation. A heterozygous RYR1 mutation should result in five different populations of RYR1 tetramers: homotetramers consisting of only normal or mutant subunits, respectively, and heterotetramers consisting of one to three mutant sub-

units. Single channel measurements with heterozygous porcine Ca^{2+} release channels showed different classes of activities that might be associated to different populations of tetramers (43). In the functional assay used here, we measured the averaged effects of different tetramer populations. The observed lower [^{3}H]ryanodine affinity of the heterozygous human MHS SR vesicles compared with the homozygous pig vesicles may be due to a contribution of heterotetramers exhibiting lower affinity.

Ca^{2+} activated [^{3}H]ryanodine binding to both porcine and human vesicles in a typically biphasic manner. Compared with porcine vesicles, both human MHN and MHS vesicles were less sensitive to activating and more sensitive to inhibiting Ca^{2+} concentrations. We also observed a distinct lower sensitivity of human SR vesicles for caffeine, whereas 4-CmC activated binding to porcine and human SR in a similar concentration range. In all cases, however, both the human and the porcine mutation induced a similar shift in the EC_{50} values to lower and, for inhibiting Ca^{2+}, to higher concentrations.

Differences in the functional consequences of both mutations were observed for the inhibitory effect of CaM in that the mutant human RYR1 was less sensitive to inhibiting CaM concentrations compared with the MHN receptor (Fig. 5). A similar tendency has also been described for the mutant porcine receptor (30). These differences in inhibition, however, were not found to be significant. The distinct lower sensitivity of the human MHS receptor can be explained by the close vicinity of CaM binding sites to the $Gly^{2434} \rightarrow Arg$ mutation (30, 44, 45). The mutant porcine receptor has been found more sensitive to activating CaM concentrations in the absence of Ca^{2+} (30). We also investigated this effect on the human mutation. CaM activation of [^{3}H]ryanodine binding to human SR vesicles in the absence on Ca^{2+}, however, was so low that it was not possible to visualize differences in activation between MHN and MHS vesicles.

In conclusion, our data show that the porcine $Arg^{615} \rightarrow Cys$ and the human $Gly^{2434} \rightarrow Arg$ mutation induce similar shifts in sensitivities of RYR1 toward some major endogenous ligands and to compounds that are utilized in the diagnosis of MH. It might be tempting to speculate that in the three-dimensional conformation the N-terminal and central part of RYR1 are in close vicinity. The mutations in this area may be acting in a similar manner in transferring the Ca^{2+} release channel into a hypersensitive state.

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