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 (SR) 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$^{3834}$ → Arg. Using high affinity $[3H]$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) 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$^{615}$ → 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, 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 containing a homologous mutation to that identified in porcine MHS muscle. Six mutations have been localized in the N-terminal sequence of RYR1 and 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 approaches (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$^{2434}$ → 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 Alu/NI was from New England Biolabs (Schwalbach, Germany). A DNA preparation kit was obtained from MWG-Biotech (Ebersberg, Germany). (9,21-$[3H]$N)Ryanodine was purchased from DuPont NEN (Bad Homburg, Germany). Ryanodine was purchased 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 $[3H]$ryanodine binding were purchased from Schleicher & Schüll (Dassel, Germany).

**Patient Characterization—**Skeletal muscle biopsies (Musculus vas tus 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$^{2434}$ → 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 prepared from a few stranded DNA. The PCR was performed with the Taq polymerase (Pharmacia). The DNA was digested with Alu/NI with or without Alu/NI. The results were analyzed by electrophoresis on a 2% agarose gel.
isolated from 10 ml of blood from MHN and MHS individuals using a DNA preparation kit (MWG-Biotech). For analysis of mutation G730A predicting the Gly5257C to Arg substitution, flanking primers as designed from the published sequence (14) (Ex5R forward 5'-TTCCCTGCTCACTGGTTCG-3' and Ex45R reverse 5'-GGGGTCTCACATGGGGT-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 MgCl2, 0.1% 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 AluNI.

Preparation of SR Membranes—MHS muscle samples obtained by biopsy from the M. vastus lateralis were collected from five individuals carrying the RYR1 mutation Gly5257C → 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. MHS samples were collected from five individuals heterozygous for the RYR1 Gly5257C → Arg mutation. To investigate the presence of modulating factors, the following protease inhibitors were included in the incubation medium as indicated in the figure legends. Unbound SR vesicles (at a protein concentration of 10 µg/ml) were incubated with indicated concentrations of [3H]ryanodine in a medium containing 100 mM KCl, 100 mM EGTA, 20 mM Na-PIPES, 200 µM Pefabloc, pH 6.8, for 3 h at 37 °C. Varying concentrations of Ca2+, 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 products (14 µg) through Schleicher & Schuell GP515 filters pre-soaked 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.

**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 Gly5257C → Arg mutation. To investigate the presence of the mutation in this pedigree, the 128-bp region spanning the G730A mutation was amplified and subjected to restriction enzyme analysis. The base exchange results in the creation of a new AluNI 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 MHN. 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 [3H]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 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 [3H]ryanodine affinity of isolated SR vesicles from MHN and MHS muscle was determined in the presence of an activating Ca2+ 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 (Kd = 47.0 ± 3.7 nM, n = 4) was approximately 1.5-fold lower compared with MHS vesicles (Kd = 31.7 ± 3.9 nM, n = 2). No significant differences between MHN and MHS vesicles were found for the maximal activation of [3H]ryanodine binding (for Bmax(MHN), 1.37 ± 0.12 pmol/mg protein (n = 4) versus 1.44 ± 0.05 pmol/mg protein (n = 2) for Bmax(MHS)).

The binding of [3H]ryanodine is greatly influenced by ligands of RYR1 that activate or inhibit SR Ca2+ 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 (Arg1285 → Cys) porcine muscle (reviewed in Ref. 23).

Fig. 4 shows the dependence of high affinity [3H]ryanodine binding on cytoplasmic Ca2+ concentration. Ca2+ activated [3H]ryanodine binding more potently in MHS vesicles, but the maximum binding was reached at 10 µM Ca2+ for both vesicle types. Whereas the threshold of activation for MHN vesicles was around 1 µM Ca2+ (pCa = 6), binding to MHS vesicles was distinctly activated by this Ca2+ concentration. The largest differences for activating Ca2+ 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 2.8 µM, whereas the EC50 for binding to MHS vesicles was 3-fold lower (1.2 µM). In parallel, MHS vesicles were approximately 2-fold less sensitive for inhibiting Ca2+ concentrations (for MHN, IC50 = 135 µM (n = 6), and for MHS, IC50 = 282 µM (n = 3)).

Calmodulin (CaM) inhibits SR Ca2+ release when the release channel (RYR1) is previously activated by Ca2+ (24–30). For the experiments described here, [3H]ryanodine binding was initially activated by 10 µM Ca2+. CaM inhibited Ca2+-acti-
vated binding in a concentration-dependent manner (Fig. 5). Contrary to the porcine Arg615 → Cys mutation (30), the human Gly2434 → Arg mutation resulted in 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 Ca²⁺ concentration of 0.1 μM, which is below the threshold of activation for both vesicle types.

Similar to Ca²⁺, caffeine stimulated [³H]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 [³H]ryanodine binding to porcine SR vesicles (Table I). The calculated EC₅₀ 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 [3H]ryanodine binding by caffeine and 4-CmC.](https://doi.org/10.1074/jbc.M109.088571) The activating effect of caffeine (left) and 4-CmC (right) was investigated in the presence of 0.1 μM Ca2+ and 12 nM [3H]ryanodine. Data were fitted according to the Hill equation: EC50/nHill (caffeine): MHN, 24.9 mM/1.8 (n = 3), and MHS, 9.5 mM/1.8 (n = 3); EC50/nHill (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 Arg615 → Cys with the human Gly2434 → Arg mutation | [3H]Ryanodine binding | [3H]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 EC50 values were approximately 20-fold lower. MHS vesicles were about 3-fold more sensitive (EC50 = 190 μM (n = 3)) compared with MHN (EC50 = 535 μM (n = 8)). The largest differences in activation were observed at 4-CmC concentrations between 200 and 500 μM. |
|---|---|---|
| Arg615 → Cys (porcine RYR1)* | Gly2434 → Arg (human RYR1) |
| High affinity [3H]ryanodine binding | High affinity [3H]ryanodine binding |
| MHN, Ka = 43.3 nM | MHN, Ka = 47.0 nM |
| MHS, Ka = 11.1 nM | MHS, Ka = 31.7 nM |
| Ca2+ dependence | Ca2+ dependence |
| MHN, EC50 = 2.60 μM | MHN, EC50 = 3.55 μM |
| MHS, EC50 = 0.96 μM | MHS, EC50 = 1.20 μM |
| MHN, IC50 = 270 μM | MHN, IC50 = 135 μM |
| MHS, IC50 = 560 μM | MHS, IC50 = 282 μM |
| Caffeine dependence | Caffeine dependence |
| MHN, EC50 = 10.7 mM | MHN, EC50 = 24.9 mM |
| MHS, EC50 = 3.7 mM | MHS, EC50 = 9.5 mM |
| 4-Chloro-m-cresol dependence | 4-Chloro-m-cresol dependence |
| MHN, EC50 = 395 μM | MHN, EC50 = 535 μM |
| MHS, EC50 = 193 μM | MHS, EC50 = 190 μM |

* Data were taken from Ref. 16.

[3H]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 EC50 values were approximately 20-fold lower. MHS vesicles were about 3-fold more sensitive (EC50 = 190 μM (n = 3)) compared with MHN (EC50 = 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 Ca2+. SR Ca2+ release is mediated by the high molecular weight ligand-gated Ca2+ 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). Hydrophaty 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 pore 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 Ca2+ from SR, which is probably due to an altered function of the mutant Ca2+ release channel. Six of these mutations (Arg163 → Cys (MH, CCD), Gly248 → Arg (MH), Gly244 → Arg (MH), Ile403 → Met (MH, CCD), Tyr522 → Ser (MH, CCD), and Arg614 → Cys (MH)) have been identified in the N-terminal part of the receptor and two (Gly2434 → Arg (MH) and Arg2435 → His (MH, CCD)) in the central part. Although the functional effects of a RYR1 mutation in the N-terminal part of the receptor (Arg614 → 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 Gly2434 → 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 [3H]ryanodine binding to isolated SR vesicles to study the effect of this mutation on SR Ca2+ release. Binding of [3H]ryanodine reflects the functional state of the SR Ca2+ release channel because ligands that have been shown to activate or inhibit the Ca2+ release channel modulate [3H]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 [3H]ryanodine binding to porcine and human SR vesicles of MHN and MHS muscle samples carrying the point mutations Arg615 → Cys and Gly2434 → Arg, respectively. The data are derived from experiments that were carried out under exactly the same conditions for the Arg615 → Cys and Gly2434 → Arg mutation.
porcine vesicles, both human MHN and MHS vesicles were less in a typically biphasic manner. Compared with human vesicles, both human MHN and MHS vesicles were less sensitive to activating and more sensitive to inhibiting Ca²⁺ concentrations. We also observed a distinct lower sensitivity of human SR vesicles for caffeine, while 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 EC₅₀ values to lower and, for inhibiting Ca²⁺, 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 Gly²⁴³⁴ → Arg mutation (30, 44, 45). The mutant porcine receptor has been found more sensitive to activating CaM concentrations in the absence of Ca²⁺ (30). We also investigated this effect on the human mutation. CaM activation of [³²P]ryanodine binding to human SR vesicles in the absence on Ca²⁺, 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 Arg₆¹₅ → Cys and the human Gly²⁴³⁴ → 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 Ca²⁺ release channel into a hypersensitive state.

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