Functional Studies of RYR1 Mutations in the Skeletal Muscle Ryanodine Receptor Using Human RYR1 Complementary DNA

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ABSTRACT

Background: Malignant hyperthermia is associated with mutations within the gene encoding the skeletal muscle ryanodine receptor, the calcium channel that releases Ca\(^{2+}\) from sarcoplasmic reticulum stores triggering muscle contraction, and other metabolic activities. More than 200 variants have been identified in the ryanodine receptor, but only some of these have been shown to functionally affect the calcium channel. To implement genetic testing for malignant hyperthermia, variants must be shown to alter the function of the channel. A number of different ex vivo methods can be used to demonstrate functionality, as long as cells from human patients can be obtained and cultured from at least two unrelated families. Because malignant hyperthermia is an uncommon disorder and many variants seem to be private, including the newly identified H4833Y mutation, these approaches are limited.

Methods: The authors cloned the human skeletal muscle ryanodine receptor complementary DNA and expressed both normal and mutated forms in HEK-293 cells and carried out functional analysis using ryanodine binding assays in the presence of a specific agonist, 4-chloro-m-cresol, and the antagonist Mg\(^{2+}\).

Results: Transiently expressed human ryanodine receptor proteins colocalized with an endoplasmic reticulum marker in HEK-293 cells. Ryanodine binding assays confirmed that mutations causing malignant hyperthermia resulted in a hypopersensitive channel, while those causing central core disease resulted in a hyposensitive channel.

Conclusions: The functional assays validate recombinant human skeletal muscle ryanodine receptor for analysis of variants and add an additional mutation (H4833Y) to the repertoire of mutations that can be used for the genetic diagnosis of malignant hyperthermia.

What We Already Know about This Topic
- Malignant hyperthermia reflects alterations in function of the ryanodine receptor, in many cases due to specific mutations of this ion channel
- An ex vivo analysis of human ryanodine receptors has not been constructed to examine the impact of mutations known to cause malignant hyperthermia

What This Article Tells Us That Is New
- Human complementary DNA was expressed in cells in vitro and mutations which cause malignant hyperthermia resulted in a hypersensitive channel, whereas those causing central core disease resulted in a hyposensitive channel

MALIGNANT hyperthermia (MH; Mammalian Inheritance in Man #145600) is a potentially fatal human skeletal muscle disorder linked to mutations within the skeletal muscle ryanodine receptor gene RYR1 (Mammalian Inheritance in Man #180901).\(^1\)–\(^3\) MH is a pharmacogenetic disorder that leads to a hypermetabolic crisis including muscle rigidity, tachycardia, acidosis, rhabdomyolysis, and rapid temperature increase.\(^4\)–\(^5\) An MH reaction is triggered by volatile anesthetics and depolarizing muscle relaxants. Central core disease (CCD; Mammalian Inheritance in Man #117000) is a congenital myopathy characterized by a structural defect in muscle fibers and muscle weakness and is allelic to MH.\(^6\)

The RYR1 protein is located on the sarcoplasmic reticulum membrane in skeletal muscle cells and functions as a calcium channel releasing Ca\(^{2+}\) from the sarcoplasmic reticulum to sarcoplasm, leading to muscle contraction and to other metabolic activities. More than 200 RYR1 variants that cosegregate with MH and/or CCD have been reported to date,\(^7\)–\(^12\) and current interests are now focused on the functional effects of these variants to both understand the molec-
ular basis of MH and CCD and to increase the repertoire of mutations that can be diagnostically used. According to the European Malignant Hyperthermia Group guidelines, only those variants that have been functionally characterized can be diagnostically used, otherwise the physiologic in vitro contracture test of excised muscle tissue must be used for clinical diagnosis. Cultured muscle cells or B lymphocytes from affected patients carrying an RYR1 variant linked to MH have shown an abnormal response to agonists including 4-chloro-m-cresol (4-CmC). For these to be accepted by the European Malignant Hyperthermia Group, they must be carried out using cells derived from at least two families, precluding many variants being accepted as having diagnostic potential.

Current studies using recombinant RYR1 for the functional analysis of point variants have used rabbit RYR1 complementary DNA (cDNA). Recombinant human RYR1 has not been used for functional studies to date, and although the amino acid sequences of human and rabbit RYR1 are similar, they are not identical. Therefore, the use of mutant forms of recombinant human RYR1 may provide a more relevant system to study the function of the channel. In this study, the human RYR1 cDNA was cloned and expressed in HEK-293 cells to analyze the functional effects of variants that cause MH or CCD.

We constructed six mutant RYR1 cDNA clones: R163C, G248R, T4826I, and H4833Y, each associated with MH, and I4898T and G4899R, which are associated with CCD. The two N-terminal mutations R163C and G248R are common variants identified in several MH-susceptible families. The R163C mutation has also been identified in patients with CCD. The two C-terminal variants T4826I and H4833Y have been recently identified in MH-susceptible New Zealand Maori families. Both of these variants have been reported only in patients with MH. More importantly, the H4833Y variant has been reported in only one extended New Zealand family, and although lymphoblastoid cells isolated from affected family members showed hypersensitivity to 4-CmC, this analysis was insufficient to class the H4833Y variant as “diagnostic.” The other two C-terminal mutations, I4898T and G4899R, have been identified only in patients with CCD. This study focuses on analysis of human RYR1 carrying these variants in response to a RYR1-specific agonist and antagonist when expressed in HEK-293 cells to validate the use of human recombinant RYR1 in functional analysis.

Materials and Methods

Construction of Human RYR1 cDNA Mutants

The wild-type (WT) human RYR1 cDNA was produced from messenger RNA using reverse transcription–polymerase chain reaction and cloned in the mammalian expression vector pcDNA3.1 (+) (Invitrogen, Carlsbad, CA). Site-directed mutagenesis was then carried out as described previously. Inserts in the completed vectors were completely sequenced on both strands to confirm the absence of any unwanted polymerase chain reaction–induced errors. Details of the cloning strategy and primers used may be obtained from the corresponding author on request.

Expression of WT and Mutant RYR1 Proteins

WT and mutant RYR1 cDNAs were transiently expressed in HEK-293 cells maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37°C under 5% CO2. Transient transfection was performed at 90% confluence using FuGENE HD (Hoffmann-LaRoche Ltd., Basel, Switzerland) according to the manufacturer’s instructions. Western blotting and immunolocalization were performed with monoclonal antibody 34C (Sigma-Aldrich, St. Louis, MO) using standard protocols. The 34C antibodies were detected using horseradish peroxidase-conjugated antimouse secondary immunoglobulin G (Sigma-Aldrich) or fluorescein isothiocyanate-labeled goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), respectively. Protein disulfide isomerase was also stained as an endoplasmic reticulum marker using protein disulfide isomerase primary (Sigma-Aldrich) (1:1,000) and tetramethylrhodamine isothiocyanate-labeled goat anti-rabbit secondary antibodies (1:200) (Jackson ImmunoResearch Laboratories). 4’,6-Diamidino-2-phenylindole was also used for staining of nuclei at 1 μg/ml.

3H]Ryanodine Binding Assay

Cells gently harvested from tissue culture flasks in phosphate-buffered saline were pelleted and solubilized in 1% 3-[3-cholamidopropyl]dimethylammonio]propanesulfonic acid buffer containing 150 mM NaCl, 100 mM Tris-Cl, pH 8.0, and 5 mg/ml 1-α-phosphatidylcholine as described previously. Solubilized proteins were stored at −80°C until use. A measure of 100 μg of crude proteins was incubated in buffer containing 25 mM piperoxone-1,4-bis(2-ethanesulfonic acid), 1 mM KCI, 0.5 mM EGTA, 100 mM free Ca2+, protease inhibitors (Complete Mini, EDTA-Free; Roche Diagnostics, Indianapolis, IN), pH 7.3, with 1 nM [3H]ryanodine (PerkinElmer, Waltham, MA) and various concentrations of 4-CmC for 3 h. The concentrations of free Ca2+ in the binding solution were calculated using the computer software WINMAXC§ (v2.50, Stanford University, Pacific Grove, CA). Binding reactions were quenched by rapid filtration through ADVANTEC GB-140 glass fiber filters (ADVANTEC MFS, Dublin, CA). The filters were washed twice with 10 ml of ice-cold washing buffer containing 20 mM Tris, 250 mM KCl, 15 mM NaCl, and pH 7.1 and were soaked in 1 ml of scintillation fluid containing 0.4% (w/v) 2,5-di(phenyl)-1,3-oxazole, 0.01% (w/v) 1,4-bis (5-phenyloxazol-2-yl)benzene, and 30% Triton X-100 in toluene overnight. The radioactivity remaining on the filters was determined by liquid scintillation counting to quantify bound [3H]ryanodine. For Mg2+ inhibition and equilib-

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Human RYR1 cDNA in Study of Malignant Hyperthermia

Results

Detection of Recombinant RYR1 Proteins

WT and six mutant RYR1 proteins were expressed in HEK-293 cells after transient transfection using cloned RYR1 cDNAs. All expressed RYR1 proteins were separated and detected by western blotting (data not shown). Expressed RYR1 proteins were also detected using immunofluorescence to confirm colocalization of the RYR1 proteins with the endoplasmic reticulum in HEK-293 cells (data not shown). No differences in expression or localization were detected between WT and mutants in either immunofluorescence or western blotting.

Functional Properties of Mutant RYR1 Proteins

The [3H]ryanodine binding assays were performed using solubilized cell extracts in 3-[3-cholamidopropyl]dimethylammonio]propanesulfonic acid buffer. Concentration-response curves for 4-CmC revealed that MH-linked RYR1 mutants (R163C, G248R, T4826I, and H4833Y) were more sensitive than WT to 4-CmC (fig. 1). Sigmoidal fitted curves generated from mutant RYR1 were shifted to the left from WT, showing a lower EC50 for 4-CmC activation than WT (table 1). Two C-terminal RYR1 mutants linked to MH, T4826I and H4833Y, showed strong sensitivity to 4-CmC and significantly lower EC50 values (P < 0.05) compared with WT. T4826I and H4833Y mutants also showed significant (P < 0.05) ryanodine binding even without 4-CmC (fig. 1), indicating that they may cause the channel to be leaky. In contrast, the two RYR1 mutants linked to CCD, I4898T and G4899R, did not show any response at any concentration of 4-CmC including more than 500 μM (data not shown). This observation suggests that RYR1 with either

![Fig. 1. [3H]Ryanodine binding activated by 4-CmC. Sigmoidal curves showed increased sensitivity of malignant hyperthermia (MH)-linked RYR1 mutants to 4-CmC. Binding reactions were performed with 100 nm free Ca2+, 1 nm [3H]ryanodine, and 0–500 μM 4-CmC. Each data point represents the mean ± SD of six separate data points. Sigmoidal fitted curves were drawn using OriginPro 8 sr6 (OriginLab Corporation, Northhampton, MA) by using the DoseResp function with four parameters.](image)

Table 1. EC50 for Ca2+ Activation, IC50 for Mg2+ Inhibition, Kd and Bmax of [3H]ryanodine Binding of Wild-type and Mutant RYR1 Proteins

<table>
<thead>
<tr>
<th>Mutations</th>
<th>EC50 for Ca2+ Activation (μM)</th>
<th>IC50 for Mg2+ Inhibition (nm)</th>
<th>Kd (nm)</th>
<th>Bmax (fmol/mg)</th>
</tr>
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<tbody>
<tr>
<td>Wild type</td>
<td>252.77 ± 34.62 (6)</td>
<td>2.81 ± 0.83 (6)</td>
<td>8.87 ± 3.16 (4)</td>
<td>49.76 ± 5.46 (4)</td>
</tr>
<tr>
<td>R163C</td>
<td>205.31 ± 30.58 (6)*</td>
<td>3.87 ± 0.62 (6)*</td>
<td>7.37 ± 3.33 (4)</td>
<td>42.28 ± 7.45 (4)</td>
</tr>
<tr>
<td>G248R</td>
<td>211.28 ± 14.70 (6)*</td>
<td>5.21 ± 1.94 (6)*</td>
<td>6.76 ± 2.23 (4)</td>
<td>55.73 ± 5.79 (4)</td>
</tr>
<tr>
<td>T4826I</td>
<td>73.77 ± 39.83 (6)†</td>
<td>9.84 ± 4.52 (6)*</td>
<td>4.55 ± 1.34 (4)</td>
<td>65.42 ± 6.20 (4)*</td>
</tr>
<tr>
<td>H4833Y</td>
<td>95.28 ± 31.72 (6)*</td>
<td>6.56 ± 2.06 (6)*</td>
<td>4.04 ± 0.94 (4)*</td>
<td>60.12 ± 2.28 (4)*</td>
</tr>
<tr>
<td>I4898T</td>
<td>0 (4)*</td>
<td>Not applicable</td>
<td>9.31 ± 2.06 (4)</td>
<td>27.47 ± 2.95 (4)*</td>
</tr>
<tr>
<td>G4899R</td>
<td>0 (4)*</td>
<td>Not applicable</td>
<td>10.32 ± 4.06 (4)</td>
<td>28.45 ± 5.55 (4)*</td>
</tr>
</tbody>
</table>

Results are shown as mean ± SD at each concentration for 4-CmC activation and Mg2+ inhibition. Sigmoidal curve fitting, EC50 and IC50 calculations were carried out for these six data sets individually. Student t test was performed with each data set for each mutant compared independently to wild type. Sigmoidal curve fitting was performed using OriginPro 8 sr6 (OriginLab Corporation, Northhampton, MA) by using the DoseResp function with four parameters. For assays used to calculate Kd (dissociation constant) and Bmax (maximal binding capacity), which were performed only four times, the Mann–Whitney U test was used. Significance values apply to each mutant compared with wild type.

n = 6; *P < 0.05, †P < 0.001 in two-sample t test; n = 4; *P < 0.05 in Mann–Whitney U test.
of these variants may be hyposensitive to 4-CmC, and the channel could not be opened. Binding reactions were also performed with various concentrations of the RYR1 antagonist Mg\textsuperscript{2+}. RYR1 proteins were activated by 10 \mu M free Ca\textsuperscript{2+}, and sigmoidal fitted curves were used to calculate sensitivity to Mg\textsuperscript{2+} inhibition (IC\textsubscript{50}). All RYR1 mutants linked to MH were less sensitive to Mg\textsuperscript{2+} inhibition than WT (table 1). The equilibrium binding parameters, dissociation constant \(K_d\), and maximal binding capacity \(B_{\text{max}}\) were obtained by reactions with various concentrations of \([\text{3H}]\text{ryanodine}\) and calculated using Scatchard analysis (table 1). \(K_d\) and \(B_{\text{max}}\) for the two N-terminal RYR1 MH-linked mutants were not significantly different from WT RYR1, although they did show significantly different responses against 4-CmC and Mg\textsuperscript{2+}. The two C-terminal RYR1 mutants linked to MH showed a higher affinity and binding capacity for \([\text{3H}]\text{ryanodine}\). Although the \(B_{\text{max}}\) values of both mutants were statistically significantly (\(P < 0.05\)) different from WT RYR1, only the H4833Y variant had a statistically significant higher affinity for \([\text{3H}]\text{ryanodine}\), largely due to the small sample number. In contrast, the two RYR1 mutants linked to CCD, I4898T and G4899R, showed significantly lower \(B_{\text{max}}\) values compared with WT (\(P < 0.05\)), although binding affinity was not significantly different, suggesting a decrease in \([\text{3H}]\text{ryanodine}\) binding site accessibility.

**Discussion**

The aim of this study was to construct the human RYR1 cDNA and assess its potential in the study of functional effects of RYR1 variants linked to either MH or CCD. We show in this study for the first time using recombinant human RYR1 that two N-terminal MH-linked RYR1 mutants, R163C and G248R, show hypersensitivity to 4-CmC and decreased sensitivity to Mg\textsuperscript{2+} inhibition. These N-terminal mutants did not show significantly different \(K_d\) and \(B_{\text{max}}\) for \([\text{3H}]\text{ryanodine}\) compared with WT, and these data support those from previous studies using recombinant rabbit RYR1.

Two C-terminal MH-linked RYR1 variants, T4826I and H4833Y, were hypersensitive to 4-CmC compared with WT and compared with the two N-terminal mutants, R163C and G248R. The T4826I mutant also showed significantly decreased inhibition by Mg\textsuperscript{2+} compared with the other mutants. These data support previous studies using rabbit cDNA for the T4826I mutation.\textsuperscript{21} The more recently identified MH variant, H4833Y, showed results similar to those of the T4826I mutation, and hence, these two C-terminal variants may cause similar functional effects on RYR1 proteins in patients’ skeletal muscle cells. They also showed higher affinity and maximal binding capacity to \([\text{3H}]\text{ryanodine}\), suggesting that the function of RYR1 mutants varies depending on the mutation carried in the protein. The N-terminal R163C and G248R mutations affect sensitivity of the channel to agonists, whereas the C-terminal T4826I and H4833Y variants affect sensitivity and binding properties of the protein to ryanodine. The T4826I and H4833Y variants also showed significantly higher binding than WT in the absence of 4-CmC. More than 10% of \([\text{3H}]\text{ryanodine}\) binding without 4-CmC suggests that these mutants may form a leaky channel. The \([\text{3H}]\text{ryanodine}\) binding assay shows that the channel is opened because ryanodine binds to the open state of the channel. Although the opened channel should result in Ca\textsuperscript{2+} release to the cytosol, measurement of resting intracellular Ca\textsuperscript{2+} concentrations is required to confirm that these mutations cause leaky channels.

One hypothesis used to describe the molecular defect underlying CCD is that some RYR1 mutations cause the channel to be leaky. Using rabbit RYR1 cDNA RYR1 mutants linked to CCD including Y523S (Y522S in humans) has shown high resting cytosolic Ca\textsuperscript{2+} concentrations and reduced agonist-dependent Ca\textsuperscript{2+} release.\textsuperscript{22, 23} Reduced Ca\textsuperscript{2+} release during excitation–contraction coupling is believed to cause muscle weakness, which is one of the most common clinical symptoms of CCD. Some RYR1 mutants such as Y522S may be leaky and hyposensitive leading to CCD, and some mutants such as T4826I and H4833Y may result in a “compensated” leaky channel leading to only MH.\textsuperscript{24} Anderson et al.\textsuperscript{14} using B-lymphoblastoid cells have shown that the heterozygous H4833Y variant leads to a hypersensitive but not leaky RYR1, whereas our current results suggest that the homozygous H4833Y mutant is both hyposensitive and leaky. The difference could be due to the different systems used, but, nevertheless, these results confirm that the H4833Y variant disrupts channel function and hence should be considered diagnostic of MH susceptibility.

The other hypothesis used to account for CCD pathology is that the channel is hyposensitive and thus excitation–contraction is uncoupled \textit{in vivo}. In this study, we analyzed two common CCD mutations, I4898T and G4899R, which have been identified in CCD families. These mutants showed no response to any concentration of 4-CmC, and this hyposensitive observation is identical to results from a previous study using immortalized B cells from affected patients.\textsuperscript{25} Our data also show that these mutants had reduced binding capacity for \([\text{3H}]\text{ryanodine}\), indicating that they may have lost their ability to open the channel. This could be due to a structural change in the RYR1 tetramer that removes or occludes a ryanodine binding site.

In conclusion, although we have used an essentially homozygous rather than heterozygous system, our results suggest that this heterologous system has the potential to produce data of physiologic relevance and is able to distinguish between different functional effects of mutations in different regions of RYR1. MH mutations showed a higher response, whereas CCD mutants did not respond to 4-CmC. Our observations support previous studies and suggest that the human RYR1 cDNA clone will be a useful tool in the further investigation of genotype/phenotype correlations for both MH and CCD, in particular in calcium release assays as a more direct measure of channel function. Other private variants will be able to be intro-
duced into the WT human RYR1 cDNA, for functional analysis, and thus variants to be classified as diagnostic will not be limited to only those that occur in more than one unrelated kindred.

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References


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