Caffeine and Halothane Sensitivity of Intracellular Ca\textsuperscript{2+} Release Is Altered by 15 Calcium Release Channel (Ryanodine Receptor) Mutations Associated with Malignant Hyperthermia and/or Central Core Disease\textsuperscript{*}

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Malignant hyperthermia (MH) and central core disease (CCD) are autosomal dominant disorders of skeletal muscle in which a potentially fatal hypermetabolic crisis can be triggered by commonly used anesthetic agents. To date, 17 mutations in the human \textit{RYR1} gene encoding the Ca\textsuperscript{2+} release channel of skeletal muscle sarcoplasmic reticulum (the ryanodine receptor) have been associated with MH and/or CCD. Although many of these mutations have been linked to MH and/or CCD, with high lod (log of the odds favoring linkage versus nonlinkage) scores, others have been found in single, small families. Independent biochemical evidence for a causal role for these mutations in MH is available for only two mutants. Mutations corresponding to the human MH mutations were made in a full-length rabbit \textit{RYR1} cDNA, and wild type and mutant cDNAs were transfected into HEK-293 cells. After about 48 h, intact cells were loaded with the fluorescent Ca\textsuperscript{2+} indicator, fura-2, and intracellular Ca\textsuperscript{2+} release, induced by caffeine or halothane, was measured by photometry. Ca\textsuperscript{2+} release in cells expressing MH or CCD mutant ryanodine receptors was invariably significantly more sensitive to low concentrations of caffeine and halothane than Ca\textsuperscript{2+} release in cells expressing wild type receptors or receptors mutated in other regions of the molecule. Linear regression analysis showed that there is a strong correlation ($r = 0.95$, $p < 0.001$) between caffeine sensitivity of different \textit{RYR1} mutants measured by the cellular Ca\textsuperscript{2+} photometry assay and by the \textit{in vitro} caffeine halothane contracture test (IVCT). The correlation was weaker, however, for halothane ($r = 0.49$, $p > 0.05$). Abnormal sensitivity in the Ca\textsuperscript{2+} photometry assay provides supporting evidence for a causal role in MH for each of 15 single amino acid mutations in the ryanodine receptor. The study demonstrates the usefulness of the cellular Ca\textsuperscript{2+} photometry assay in the assessment of the sensitivity to caffeine and halothane of specific ryanodine receptor mutants.

Malignant hyperthermia (MH)\textsuperscript{1} is an autosomal dominant muscle disorder in which genetically susceptible individuals among populations of humans and domestic animals respond to the administration of potent inhalational anesthetics and depolarizing skeletal muscle relaxants with high fever and skeletal muscle rigidity (1–3). Central core disease (CCD) is a rare, non-progressive myopathy, presenting in infancy and characterized by hypotonia and proximal muscle weakness (4). An important feature of CCD is its close association with MH susceptibility (5).

Although diagnosis of CCD is made on the basis of the lack of oxidative enzymatic activity in central regions of skeletal muscle fibers (6), the only accepted diagnostic test for MH susceptibility in humans is the North American caffeine halothane contracture test (CHCT) (7) or its European counterpart, the \textit{in vitro} contracture test (IVCT) (8). These tests are based on the hypersensitivity of contracture, induced in muscle strips obtained by biopsy, in the presence of caffeine or halothane.

Early genetic linkage studies showed that a single amino acid mutation, Arg\textsuperscript{615} → Cys (R615C), in the skeletal muscle ryanodine receptor gene (\textit{RYR1}) is tightly linked to MH in swine (8, 9). Further genetic and biochemical data have supported the view that this mutation is causal of MH in swine and humans (2). The corresponding mutation (R614C) has been linked to MH in some human families (11, 12), but in other families, discordance has been reported (13, 14). Discordance raises questions concerning the causal nature of such mutations, but other reasons for discordance include the possibility that two MH mutations might be segregating in one family or that the \textit{in vitro} contracture test for MH susceptibility fails to provide a phenotypic diagnosis of MH with sufficient accuracy for genetic analysis (15). Analysis of the CHCT as a diagnostic test for MH indicates that it can achieve 97% sensitivity and 79% specificity,\textsuperscript{2} whereas the IVCT can achieve 99% sensitivity and 93.6% specificity (17). The IVCT protocol permits an equivocal diagnosis (MHE) if the muscle biopsy responds to either caffeine or halothane, but not both.

\textsuperscript{1}The abbreviations used are: MH, malignant hyperthermia; CCD, central core disease; IVCT, \textit{in vitro} caffeine halothane contracture test; CHCT, caffeine halothane contracture test.

\textsuperscript{2}Allen, G. C., Larach, M. G., and Kunselman, A. R., (1997) Anesthesiology, in press.
To date, 17 point mutations have been described in human RYR1. Amino acid residue 4 (Gly4) in the rabbit RYR1 sequence, expressed in this study, is deleted in the human RYR1 sequence, accounting for discrepancies in numbering of these mutations in the two species. Twelve mutations have been linked to human MH only (C35R, G248R, G341R, R552W, R614C, R614L, R2163C, V2168M, G2435R, R2456C and R2458H), and five have been linked to human CCD plus MH (R163C, I403M, Y522S, R2163H, and R2436H) in several human families (11, 18–25, 27). These mutations clearly fall in two distinct clusters in the linear sequence of RYR1 lying between residues 35 and 614 and between residues 2163 and 2458.

Of these mutations, only the human R614C mutation has been characterized thoroughly in the form of the corresponding R615C mutation in swine. In this case, gating has been confirmed to be more sensitive to caffeine and halothane than the normal Ca2+ release channel in tests utilizing vesicles (28), single channel assays (29, 30), and cellular Ca2+ photometry (31, 32). The G2434R mutation has been shown to have higher normal [3H]ryanodine binding in human MH muscle, an indication of its greater open probability (33). For the remaining twelve mutations in the Ca2+ sensitivities of wild type and mutated channels to caffeine and halothane. The mutations into a rabbit that would associate them in a functional way with MH and/or method of demonstrating defects in these and other mutations in two members of a single, small CCD pedigree (20), could represent rare polymorphisms rather than causal mutations. A method of demonstrating defects in these and other mutations that would associate them in a functional way with MH and/or CCD is an important goal.

In this study, we have introduced each of 15 known MH mutations into a rabbit RYR1 cDNA, expressed each mutation in HEK-293 cells, and measured the intracellular responses of wild type and mutated channels to caffeine and halothane. The caffeine and halothane sensitivities of RYR1 mutants, measured in the Ca2+ photometry assay and by the clinical IVCT, were compared. The study shows that each mutant protein is more sensitive to caffeine and halothane than the wild type channel. A strong correlation was confirmed to be more sensitive to caffeine and halothane than the wild type channel. A strong correlation was confirmed by sequence analysis, it was necessary to develop a series of unique restriction endonuclease sites and the introduction of five new restriction endonuclease sites (pBS-RYR1) was subcloned into pBS1 to yield plasmid pBS1-H. Two restriction endonuclease sites, BstI (3798) and MluI (5355), were introduced into pBS1-H to yield pBS-BHM. The XhoI (6800/XhoI (12317) fragment from pBS-RYR1 was subcloned into Bluescript SK II (+) to yield pBS-X. Two restriction endonuclease sites, SpeI (6822) and AvaII (9804), were then introduced into pBS-X to yield pBS-XSA.

The restriction endonuclease site mutations, only the human R614C mutation has been described previously (35–37). Inasmuch as all of our mutations are RYR1 mutants, measured in the Ca2+ photometry assay and by the clinical IVCT, it was necessary to develop a series of unique restriction endonuclease sites and the introduction of five new restriction endonuclease sites (pBS-RYR1) was subcloned into pBS1 to yield plasmid pBS1-H. Two restriction endonuclease sites, BstI (3798) and MluI (5355), were introduced into pBS1-H to yield pBS-BHM. The XhoI (6800/XhoI (12317) fragment from pBS-RYR1 was subcloned into Bluescript SK II (+) to yield pBS-X. Two restriction endonuclease sites, SpeI (6822) and AvaII (9804), were then introduced into pBS-X to yield pBS-XSA.

The five endogenous restriction endonuclease sites are marked above the long horizontal bar in Fig. 1B, and the five restriction endonuclease sites introduced into RYR1 cDNA by site-directed mutagenesis are marked beneath the long horizontal bar. The 5′ XbaI site and the 3′ HindIII sites were unique sites introduced into the clone earlier (36) (Fig. 1B). To work with these cassettes, two new vectors, pBS1 and pBS2, were also constructed by the introduction of additional restriction endonuclease sites into the multiple cloning site between BamHI and SmaI I in plBluescript II KS (+) (Stratagene). The multiple cloning site in pBS1 contains BamHI-BgII-StuI-AatII-BspGI-MH-PspI-SmaI and, in pBS2, contains BamHI-NdeI-MluI-Aat II-BstBI-SpI- SpeI-BamHI-NheI-SmaI restriction endonuclease sites.

In the introduction of the new restriction endonuclease mutations, the HpaI (16865/HpaI (7155) fragment from RYR1 cDNA (pBS-RYR1) was subcloned into pBS1 to yield plasmid pBS1-H. Two restriction endonuclease sites, BstI (3798) and MluI (5355), were introduced into pBS1-H to yield pBS-BHM. The XhoI (6800/XhoI (12317) fragment from pBS-RYR1 was subcloned into Bluescript SK II (+) to yield pBS-X. Two restriction endonuclease sites, SpeI (6822) and AvaII (9804), were then introduced into pBS-X to yield pBS-XSA.

The NdeI (11290/ClaI (14427) fragment from pBS-RYR1 was subcloned into Bluescript SK II (+) to yield pBS-X. Two restriction endonuclease sites, SpeI (6822) and AvaII (9804), were then introduced into pBS-X to yield pBS-XSA. The NdeI (12920/SacI (13015) fragment from pBS-RYR1 was subcloned into pBS1 to yield pBS-BNS. A restriction endonuclease site, Nhel (12675), was introduced into pBS-BNS. The yield pBS-BNNS, the five newly introduced restriction endonuclease sites, were subcloned back into pBS-RYR1 to yield pBS-RYR1c in the Bluescript KS vector. The 11 cassettes were subcloned from pBS-RYR1c into pBluescript KS (+), pBS1, and pBS2 to yield pBS-RYR1c–11 as follows: XbaI (Bluescript)/SalI (677), SalI/BspI611 (2349), BspI611/BstBI (3788), BstBI/MluI (5355), MluISpeI (6822), SpeI/SpeI (6841), SpeI/AatII (9804), AatII/NdeI (12920), NdeI/Nhel (12675), Nhel/ClaI (14427), and ClaI/HindIII (Bluescript). The full-length RYR1 cDNA, with introduced restriction endonuclease sites (pBS-RYR1c) (Fig. 1B), could not be cloned into the Pmt2 vector, which has EcoRI, XbaI, SalI, and EcoRI cloning sites. Accordingly, PBS-RYR1c was digested with HindIII at a unique site, blunt-ended, and digested with XhoI to obtain a full-length XhoI-blunt fragment. This was cloned into the mammalian expression vector pMT2 (a gift of Dr. R. Kaufman, Genetics Institute, Boston, MA) after blunt-ending the SalI site and digesting the XbaI site. Some mutant clones were introduced into the XbaI/HindIII site of an expression vector pCDNA3(−) (Invitrogen, San Diego, CA).

Oligonucleotide-directed Mutagenesis—Short fragments were removed from pBS-RYR1c–1, pBS-RYR1c–2, or pBS-RYR1c–6, as indicated by the small bar in Fig. 1C, and ligated into the polylinker region of Bluescript, pBS1, or PBS2 for site-specific mutagenesis by oligonucleotide-directed mutagenesis (39). The integrity of the mutated segment of the cDNA insert was checked by sequencing the entire insert by the dideoxyxynucleotide chain termination method of Sanger et al. (40). Fragments containing mutations were subcloned back into their original context in pBS-RYR1c or pMT2 vectors for expression in HEK-293 cells. Mutations R164C, G249R, G342R, 1404M, Y522S, and R615C were subcloned into the original pMT2–RYR1 vector, and mutations G2435R and R2436H were subcloned into the pMT–RYR1c vector. Mutations C36R, R553W, R615L, R2163C, R2163H, R2458C, or R2458H were subcloned into pCDNA(−) vector. The expression of wild type cDNA in all these vectors yielded virtually identical expression levels and activities.

Cell Culture and DNA Transfection—HEK-293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 0.1 mM minimal Eagle’s medium nonessential amino acids, 4 mM L-glutamine, 100 units of penicillin/ml, 100 mg of streptomycin/ml, 4.5 g of glucose/liter, and 10% fetal calf serum, at 37 °C under 5% CO2. DNA transfection was carried out by the calcium phosphate precipitation method as described by Chen and Okayama (41). Ten μg of plasmid DNA were used to transfect 2 × 106 cells/60-mm plate. Control cells were treated in the same way, but with no DNA or with expression vector DNA.

Immunocytochemical Staining—Immunocytochemical staining was carried out as described by Chen et al. (36). Monoclonal antibody 34C (a kind gift from Dr. Judith Airey) and alkaline phosphatase-conjugated anti-mouse IgG were used to detect expressed ryanodine receptor protein in HEK-293 cells. 5-Bromo-4-chloro-3-indolyl phosphate and p-nitrotritrazolium chloride blue (Sigma) were used in the development of the color reaction.

Immunoblotting—Immunoblotting was performed on total proteins collected from one 60-mm plate of whole cells using detergent solution,
statistical significance.

Results

Construction and Expression of Mutant RYR1 cDNAs—The cloning, sequencing, and functional expression of RYR1 cDNA have been reported previously (35–37). Fig. 1A shows the approximate positions of 15 mutations in the human ryanodine receptor protein which have been linked to MH (11, 18–25, 27, 46). Fig. 1B shows the location of these 15 mutations in RYR1 cDNA, relative to the 11 cassettes that were created in the cDNA to facilitate excision and mutagenesis. Fig. 1C shows the subcloned fragments that were used for site-directed mutagenesis. The figure illustrates the way in which the cDNA was reduced in size for mutant construction and rebuilt to form full-length cDNA.

The transient expression of a fully functional RYR1 cDNA in HEK-293 cells and characterization of the product have been described (37). We used this transient expression system in this study. Fig. 2 shows immunoblotting of representative regions of expression vector-transfected (A) and RYR1 cDNA-transfected (B) HEK-293 cells stained with a mouse monoclonal antibody (34C) against the ryanodine receptor. The epitope for this antibody lies between amino acids Asn2756 and Glu 2803 near the body of the large cassettes as indicated. After mutagenesis, the small, mutant-containing cassettes were sequenced and built back into a full-length cDNA.

Western blotting after SDS-PAGE of whole cell extracts of transfected and non-transfected HEK-293 cells (Fig. 2C) were incubated first with monoclonal antibody 34C at a dilution of 1:5000 and then with horseradish peroxidase-conjugated anti-mouse secondary IgG at a dilution of 1:5000 in a solution containing 10 mM Tris-HCl, pH 7.5, 1% BSA, 150 mM NaCl, and 0.1% Tween-20. Finally, the blots were incubated with the SuperSignal ultra chemiluminescent substrate (Pierce) and exposed to a BioMax film (Eastman Kodak Co.).

Fig. 1. Construction of mutations associated with MH or CCD in rabbit skeletal muscle RYR1 cDNA. A, the linear amino acid sequence of the ryanodine receptor is indicated by a solid horizontal line in which the NH2 and COOH termini and amino acid numbers are marked. The approximate positions in which the MH mutations are clustered are depicted as boxes above the horizontal line. B, the construction of 11 DNA cassettes of about 1500 base pairs each, used for mutagenesis of RYR1 cDNA. Five unique restriction sites found in the cDNA are indicated above the horizontal line, and five more unique restriction endonuclease sites built into the cDNA are indicated below the line. The 5′ XbaI site and the 3′ HindIII site are also unique. C, cassettes used for mutant constructs were derived by further cleavage of the large cassettes as indicated. After mutagenesis, the small, mutant-containing cassettes were sequenced and built back into a full-length cDNA.

Fig. 2. Expression of the wild type and 15 mutant skeletal muscle RYR1 cDNAs in HEK-293 cells. HEK-293 cells transfected with vector alone (A) or RYR1 cDNA (B) were subjected to immunocytochemical staining for ryanodine receptor expression. Cells were fixed and permeabilized about 48 h after transfection. Expressed ryanodine receptors were detected by immunochemical staining using monoclonal antibody 34C and secondary alkaline-phosphate-conjugated anti-mouse IgG. C, immunoblots of HEK-293 cell extracts containing about 100 μg of protein were separated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The samples were probed with monoclonal antibody 34C and secondary horseradish peroxidase-conjugated anti-mouse IgG. Monoclonal antibody 34C is a mouse IgG that detects a determinant in the ryanodine receptor which is clustered near residues 2756 to 2803 of the human ryanodine receptor protein.
showed that there were no obvious differences in the levels of expression or of molecular size between wild type and mutant ryanodine receptors.

In control experiments, HEK-293 cells were transfected with different amounts of RYR1 cDNA (e.g., 1 µg of RYR1 cDNA plus 3 µg of vector or 4 µg of RYR1 cDNA/35-mm plate). The expression of RYR1 protein, monitored by Western blotting, differed, but no significant difference was observed in transfection efficiency, monitored by immunostaining, or in caffeine response, monitored by the cellular Ca$^{2+}$ photometry, indicating that transfection efficiency was not improved by higher cDNA levels and that the number of expressed channels required for a full caffeine response in an individual cell was reached at low cDNA concentrations.

**Photometric Assay of Ca$^{2+}$ Release in Response to Caffeine and Halothane**—Fig. 3A shows representative fluorometric re-
FIG. 4. Comparisons and correlations of the effect of caffeine and halothane on wild type and MH or CCD mutant Ca\(^{2+}\) release channels. With the exception of mutant C36R, all MH mutants were significantly more sensitive to caffeine (mean $ED_{50} = 0.81 \pm 0.15$ mM) than wild type $RYR1$ ($ED_{50} = 1.4 \pm 0.26$ mM) (A), and all MH mutants were significantly more sensitive to halothane (mean $ED_{50} = 0.30 \pm 0.10$ mM) than wild type $RYR1$ ($ED_{50} = 0.74 \pm 0.20$ mM) (B). Comparison of the threshold concentration of caffeine (C) or halothane (D) for activation of muscle contracture in the IVCT shows that nine mutant $RYR1$ were more sensitive to caffeine (mean threshold = 1.2 $\pm$ 0.4 mM) and halothane (mean threshold = 0.83 $\pm$ 0.25% v/v) than wild type $RYR1$. The linear analysis method was used to analyze the correlation between the effects of caffeine and halothane obtained by the cellular Ca\(^{2+}\) photometry assay (E) and the IVCT (F) and to analyze the correlation between the effect of caffeine (O) and halothane (H) obtained by the two assays. One vertical dashed line indicates the mean value for wild type, and the other indicates the mean value for all mutants. All data are expressed as mean $\pm$ S.E.
responses to incremental additions of caffeine, and Fig. 3B shows representative fluorometric response to incremental doses of halothane for vector-transfected HEK-293 cells and for cells transfected with the wild type Ca\textsuperscript{2+} release channel, the MH mutant channel G2435R and the CCD mutant channel R2436H. Traces from representative experiments in which fura-2 fluorescence from about 50 HEK-293 cells were monitored after the addition of increasing concentrations of caffeine are shown in Fig. 3A. Under resting conditions, there was no significant difference in the fluorescence ratio measured in vector-transfected HEK-293 cells and in RYR1 cDNA-transfected HEK-293 cells. Caffeine from 1 to 10 mM caused a small increase in base-line fluorescence in vector-transfected HEK-293 cells, but no clear [Ca\textsuperscript{2+}]\textsubscript{i} transients were observed. Peak heights in the fluorescence ratio measuring Ca\textsuperscript{2+} release in HEK-293 cells transfected with the G2435R and R2436H mutant cDNAs indicated that these mutants were sensitive to lower concentrations of caffeine (0.25–1 mM) and halothane (0.1–0.5 mM) than HEK-293 cells transfected with wild type RYR1 cDNA. Removal of caffeine by washout allowed the Ca\textsuperscript{2+} concentration to return to near resting levels over a period of about 2 min. Peak heights were measured for each increment in caffeine and halothane concentration and normalized to the peak height for maximal Ca\textsuperscript{2+} release in the case of caffeine, or to the response obtained with 10 mM caffeine in the case of halothane. Caffeine ED\textsubscript{50} values, calculated from the dose-response curves generated in this way for wild type, G2435R, and R2436H mutant channels, are presented in Fig. 3C. They were 1.4, 0.84, and 0.89 mM, respectively.

The traces in Fig. 3B show that vector-transfected HEK-293 cells did not respond to halothane concentrations below 5 mM, but did respond weakly to 10 mM halothane. In HEK-293 cells transfected with wild type channels, weak responses were observed at 0.1 and 0.25 mM halothane, but cells transfected with MH mutant channels (G2435R) and CCD mutant channels (R2436H) gave robust responses to 0.1 and 0.25 mM halothane. Inasmuch as vector-transfected HEK-293 cells all responded to halothane concentrations to return to near resting levels over a period of about 2 min. Peak heights were measured for each increment in caffeine and halothane concentration and normalized to the peak height for maximal Ca\textsuperscript{2+} release in the case of caffeine, or to the response obtained with 10 mM caffeine in the case of halothane. Caffeine ED\textsubscript{50} values, calculated from the dose-response curves generated in this way for wild type, G2435R, and R2436H mutant channels, are presented in Fig. 3C. They were 1.4, 0.84, and 0.89 mM, respectively.

In Fig. 4A, mean ED\textsubscript{50} values for caffeine activation of Ca\textsuperscript{2+} release are presented for wild type and 15 mutant Ca\textsuperscript{2+} release channel proteins expressed in HEK-293 cells. In Fig. 4B, mean ED\textsubscript{50} values are presented for halothane activation of Ca\textsuperscript{2+} release through the same channels. Of the 15 mutations linked to MH, Ca\textsuperscript{2+} release in 14 was significantly more sensitive to lower concentrations of caffeine (Fig. 4A) and halothane (Fig. 4B) than was the wild type channel, when analyzed by cellular Ca\textsuperscript{2+} photometry. The exception was the mutant C36R.

Comparisons between ED\textsubscript{50} Values for Calcium Mobilization and Thresholds for IVCT Responses—IVCT data for nine different RYR1 mutations have been collected, making it possible to compare our test results with the IVCT data for the nine different mutations (Fig. 4). Correlations between caffeine and halothane responses measured by cellular Ca\textsuperscript{2+} photometry and by IVCT were subjected to linear analysis. There was a correlation (coefficient of correlation = 0.79, p < 0.05) between ED\textsubscript{50} values for caffeine and halothane, measured by cellular Ca\textsuperscript{2+} photometry (Fig. 4E), but there was no clear correlation between the caffeine and halothane threshold measured by IVCT (Fig. 4F). This result, indicating that a muscle biopsy from a MHS individual might have a strong response to caffeine but not to halothane, must be considered cautiously, however, because a single data point, the IVCT halothane response to the R2458C mutation, is an outlier. This mutation was relatively insensitive to caffeine in both assays and to halothane in the cellular Ca\textsuperscript{2+} photometry assay, but was highly sensitive to halothane in the IVCT (Fig. 4). There was a strong correlation (r = 0.95, p < 0.001) between the IVCT caffeine threshold and the caffeine ED\textsubscript{50} measured in the cellular Ca\textsuperscript{2+} photometry assay (Fig. 4G), but not between the IVCT halothane threshold and halothane ED\textsubscript{50} (Fig. 4H), measured by cellular Ca\textsuperscript{2+} photometry.

**DISCUSSION**

In this study, we demonstrate the use of HEK-293 cells for the functional expression and analysis of wild type and MH or CCD mutant Ca\textsuperscript{2+} release channels. In our earliest studies, we encountered difficulty in the characterization of the expressed Arg\textsuperscript{615} \rightarrow Cys RYR1 mutant using cellular Ca\textsuperscript{2+} photometry, because of a high background of Ca\textsuperscript{2+} release in several different cell lines. This problem was later solved by Otsu et al. (31), who used C2C12 cells as host and demonstrated that the mutant Ca\textsuperscript{2+} release channel is more sensitive than wild type to both caffeine and halothane, and by Treves et al. (32), who used COS-1 cells to show that the mutant Ca\textsuperscript{2+} release channel is more sensitive to 4-chloro-m-cresol than wild type.

The single channel properties of the recombinant Ca\textsuperscript{2+} release channel, expressed in HEK-293 cells under the exact conditions used in this study, have been shown, in extensive analyses, to be virtually identical to the native channel (37). Although endogenous forms of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release channels, sensitive to ryanodine, have been reported in HEK-293 cells (47), we have been unable to detect any endogenous skeletal muscle ryanodine receptor in HEK-293 cells using several ryanodine receptor-specific antibodies, including 34C, in immunostaining and immunoblotting of HEK-293 cell extracts (37). We have, however, noted the occasional burst of Ca\textsuperscript{2+} release while imaging single HEK-293 cells. Accordingly, it was important to carry out photometry with a large number of HEK-293 cells so that spurious bursts of Ca\textsuperscript{2+} release were minimized by averaging with unresponsive cells. The larger signal also facilitated the detection of a response to the addition of very low concentrations of caffeine or halothane to RYR1-transfected cells. Dose-response curves were readily generated from the continuous photometric recording.

Among the problems that we have considered in the refinement of this assay system was the question of whether differences in transfection efficiency might affect our results. Optimal transfection conditions were determined on the basis of measurement of maximal transfection efficiency and photometric response. If photometric measurements were carried out on different regions of the same coverslip, peak caffeine and halothane responses did vary from one region of the slide to another, on occasion up to 3-fold. However, ED\textsubscript{50} values for caffeine and halothane responses did not change, because curves from which ED\textsubscript{50} values were derived were calculated after normalization to maximal release values obtained with 10 mM caffeine in the same field.

The question of whether the volatility of halothane could affect dose-response results was considered. Liquid halothane, diluted to 500 mM in dimethylsulfoxide (DMSO), was diluted further in aqueous assay solutions to achieve the specific concentrations of halothane used in cellular Ca\textsuperscript{2+} photometry. The same diluted halothane solution was tested at 40-min intervals on HEK-293 cells transfected with RYR1 cDNA. Inasmuch as no changes were observed in the amplitude of the halothane-induced contracture, we conclude that evaporation of halo-
than is not a serious problem in our cellular Ca\textsuperscript{2+} photometry assay.

In other, unlabeled experiments involving mutations unrelated to MH or CCD, in other regions of RYR1, we have not observed any increased sensitivity to caffeine and halothane.

We have analyzed the ligand gating characteristics of 15 mutations created in rabbit skeletal muscle ryanodine receptor, which are associated with MH or CCD in the corresponding human Ca\textsuperscript{2+} release channel. Our data demonstrate that 14 of the mutant ryanodine receptor proteins respond significantly to abnormally low concentrations of caffeine and halothane. The abnormal Ca\textsuperscript{2+} transients in transfected HEK-293 cells are most likely to be due to intrinsic differences in the gating properties of the mutated RYR1.

Comparison of the caffeine responses for the various RYR1 mutations obtained by cellular Ca\textsuperscript{2+} photometry and by the muscle biopsy IVCT shows that there was an excellent correlation between both responses (Fig. 4). The correlation is surprisingly good, because the caffeine data were obtained from different laboratories across Europe over a period of several years. The close correlation that we observed demonstrates that the cellular Ca\textsuperscript{2+} photometry system described should be considered a valuable predictor of RYR1 MH mutations.

In the cellular Ca\textsuperscript{2+} photometry assay, a significant correlation was observed between sensitivity to caffeine and sensitivity to halothane (Fig. 4E). However, the correlation between the halothane response in the cellular Ca\textsuperscript{2+} photometry assay and the halothane response in the IVCT was not significant (Fig. 4H). This is not surprising, inasmuch as a poor correlation also exists between the caffeine and halothane responses obtained for the IVCT (Fig. 4P).

The poor correlation between the halothane responses in the cellular Ca\textsuperscript{2+} photometry assay and in the IVCT could result from the different nature of the cellular and muscle biopsy tests. Alternatively, such results could arise from experimental deviation in halothane responses in the IVCT. There are three main points that argue in favor of the latter explanation. (i) A good correlation was observed between the halothane and caffeine response in the cellular assay but not in the IVCT; (ii) in the IVCT, there was a poor correlation between halothane threshold and tension values; and (iii) the incidence of people responding abnormally to halothane, but not to caffeine, in the IVCT (MHE/h) differs across different centers in Europe. Thus, it is possible that the halothane response of different RYR1 mutations may be more accurately measured in the cellular assay than in the IVCT.

The IVCT responses shown were collected from heterozygous individuals, whereas the cellular assay system would be expected to mimic a homozygous response. Interestingly, the ED\textsubscript{50} values for caffeine and halothane are generally half the threshold values for caffeine and halothane in the IVCT. This further supports the notion that the cellular assay gives a good representation of the IVCT response. The results of this study add to a growing body of biochemical and genetic evidence that mutations in the RYR1 gene are causal of malignant hyperthermia.

A serious problem in the diagnosis of MH by \textit{in vitro} contracture testing is that the tests can attain close to 100% specificity, but with no more than 93% sensitivity (17). The 10–20% false positives and the few false negatives that are observed with the test are not likely to be due to technical error. It is much more likely that they arise because the contracture test results record the interplay of multiple gene products in the muscle strips which are assayed. Moreover, it is clear that muscle cells have the capacity to compensate for genetic changes that might otherwise be lethal (16, 26, 48). Accordingly, CHCT results in a family will reflect not only the effect of the RYR1 mutation causing MH, but the different genetic background of each individual, the level of “compensation” achieved in that individual, gender differences (more MH actions are recorded in males than in females) and age differences (MH susceptibility peaks in the second to fourth decades) (1).

Because of these diagnostic difficulties, it is important to consider the possibility that the CHCT should be replaced, wherever possible, by DNA based tests for MH. Such tests would easily achieve 100% accuracy. One hindrance, however, has been to define which RYR1 mutations are, in fact, causal of MH and which are merely polymorphisms. It is clear now that mutations in the RYR1 gene causing MH, but the different genetic background provided by HEK-293 cells. If any mutation is shown to result in higher sensitivity than wild type to the Ca\textsuperscript{2+} releasing effects of caffeine and halothane, then any individual carrying such a mutation should be considered to be MH-susceptible and treated accordingly during administration of anesthetics.

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