Measurement of Resting Cytosolic Ca\textsuperscript{2+} Concentrations and Ca\textsuperscript{2+} Store Size in HEK-293 Cells Transfected with Malignant Hyperthermia or Central Core Disease Mutant Ca\textsuperscript{2+} Release Channels*

Jiefei Tong†‡§¶, Tommie V. McCarthy†, and David H. MacLennan‡§**

From the †Banting and Best Department of Medical Research and §Department of Biochemistry, University of Toronto, Toronto, Ontario M5G 1L6, Canada and ‡Department of Biochemistry, University College Cork, Cork, Ireland

Malignant hyperthermia (MH) and central core disease (CCD) mutations were introduced into full-length rabbit Ca\textsuperscript{2+} release channel (RYR1) cDNA, which was then expressed transiently in HEK-293 cells. Resting Ca\textsuperscript{2+} concentrations were higher in HEK-293 cells expressing homotetrameric CCD mutant RyR1 than in cells expressing homotetrameric MH mutant RyR1. Cells expressing homotetrameric CCD or MH mutant RyR1 exhibited lower maximal peak amplitudes of caffeine-induced Ca\textsuperscript{2+} release than cells expressing wild type RyR1, suggesting that MH and CCD mutants might be "leaky." In cells expressing homotetrameric wild type or mutant RyR1, the amplitude of 10 mM caffeine-induced Ca\textsuperscript{2+} release was correlated significantly with the amplitude of carbachol- or thapsigargin-induced Ca\textsuperscript{2+} release, indicating that maximal drug-induced Ca\textsuperscript{2+} release depends on the size of the endoplasmic reticulum Ca\textsuperscript{2+} store. The content of endogenous sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase isoform 2b (SERCA2b), measured by enzyme-linked immunosorbent assay, 45Ca\textsuperscript{2+} uptake, and confocal microscopy, was increased in HEK-293 cells expressing wild type or mutant RyR1, supporting the view that endoplasmic reticulum Ca\textsuperscript{2+} storage capacity is increased as a compensatory response to an enhanced Ca\textsuperscript{2+} leak. When heterotetrameric (1:1) combinations of MH/CCD mutant and wild type RyR1 were expressed together with SERCA1 to rameric (1:1) combinations of MH/CCD mutant and wild type RyR1, suggesting that MH and CCD mutants might be "leaky." In cells expressing homotetrameric wild type RyR1, indicating that caffeine hypersensitivity observed with a variety of MH/CCD mutant RyR1 proteins is not dependent on extracellular Ca\textsuperscript{2+} concentration.

Malignant hyperthermia (MH)\textsuperscript{3} is an autosomal dominant

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† Supported by a studentship from the Medical Research Council of Canada.
¶ To whom correspondence should be addressed: Banting and Best Dept. of Medical Research, University of Toronto, Charles H. Best Institute, 112 College St., Toronto, Ontario, Canada M5G 1L6. Tel.: 416-978-5008; Fax: 416-978-8528; E-mail: david.maclennan@utoronto.ca.
§ The abbreviations used are: MH, malignant hyperthermia; CCD, central core disease; ER, endoplasmic reticulum; SERCA, sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; GFP, green fluorescent protein; RyR, ryanodine receptor; IP\textsubscript{3}, inositol 1,4,5-trisphosphate.
a homogenous genetic background, and using a fluorescent (Fura-2) imaging assay to measure resting Ca\(^{2+}\) concentrations in these transfected cells. In an earlier study (22), we showed that 15 MH or CCD (MH/CCD) mutant homotetramers expressed in HEK-293 cells were more sensitive to caffeine and halothane than wild type RyR1. In addition, the response observed for the mutant channels expressed in HEK-293 cells correlated closely with the response observed in contracture tests of muscle samples from humans bearing the same mutations (22). In human MH or CCD individuals, however, heterozygosity is most common. In the present study, we have measured resting Ca\(^{2+}\) concentrations in HEK-293 cells expressing the various mutations, analyzed the relationship between caffeine-induced Ca\(^{2+}\) release and intracellular Ca\(^{2+}\) stores, tested for an increase in sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase isof orm 2b (SERCA2b) synthesis as a compensatory factor in cells expressing wild type or mutant RyR1, tested the caffeine sensitivity of heterotetrameric MH or CCD mutants expressed in the presence or absence of SERCA1, and tested the influence of extracellular Ca\(^{2+}\) concentration on caffeine responses of MH or CCD mutant channels.

**EXPERIMENTAL PROCEDURES**

**Materials**—Enzymes for DNA manipulation were obtained from Boehringer Mannheim, New England Biolabs, Promega, and Ame rsham Pharmacia Biotech. Tissue culture reagents were purchased from Life Technologies, Inc. Monoclonal antibody 34C (27) was a kind gift from Dr. Judith Airey (University of Nevada, Reno). Fura-2 acetoxy methyl ester (Fura-2/AM) and pluronic F-127 were from Molecular Probes. Caffeine was from Sigma. Halothane was from Fluka. Carbachol and thapsigargin were from Calbiochem. 4\(^{6}\)Ca\(^{2+}\)/Cl\(_2\) (10–40 mCi/mg Ca\(^{2+}\)) was obtained from Amersham Pharmacia Biotech. All other chemicals were of reagent (or highest available) grade.

**Construction of MH/CCD Mutants in RYR1 Cassettes**—The cloning, expression, and construction of the full-length rabbit skeletal muscle ryanodine receptor (RyR1) cDNA cassettes were described previously (22, 28). The construction and expression of single MH/CCD mutant RyR1 cDNAs were also described previously (22).

**Cell Culture and DNA Transfection**—Culture and transfection of HEK-293 cells using the calcium phosphate precipitation method of Chen and Okayama (29) were carried out as described earlier (22). Ten µg of plasmid DNA were used to transfect 2 × 10\(^6\) cells/60-mm plate. Control cells were treated in the same way, but with no DNA or with expression vector DNA only.

**Fluorescence Measurements**—Ca\(^{2+}\) photometry and Ca\(^{2+}\) imaging assays were used to measure Ca\(^{2+}\) concentration changes in transfected HEK-293 cells as described previously (22). A Photon Technologies Inc. microfluorimetry system was used in photometric assays to measure caffeine-induced changes in Fura-2/AM fluorescence resulting from Ca\(^{2+}\) release through the different ryanodine receptor proteins expressed in HEK-293 cells. Dose-response curves were generated and normalized to the maximal Ca\(^{2+}\) release response observed at 10 mM caffeine for both the caffeine and the halothane responses.

**Microsome Preparation**, Ca\(^{2+}\) Uptake, and Enzyme-linked Immunoabsorbent Assay (ELISA)—Microsomes were prepared and assayed for Ca\(^{2+}\) transport activity, and data were analyzed as described previously (31). Aliquots of 50 µl of the microsome preparation (1 mg/ml) were analyzed by ELISA, as described previously (32). A primary mouse monoclonal anti-SERCA2 ATPase antibody (IgG2a, Affinity Bioreagents Inc.) was used to detect SERCA2 in microsomes.

**Immunofluorescence Labeling and Laser Scanning Confocal Microscopy**—HEK-293 cells transfected with RyR1 cDNA were cultured on coverslips. They were fixed for 20 min in 3.7% formaldehyde at room temperature, washed once with PBS, and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Cells were then incubated with IgG2a (Affinity Bioreagents Inc.) in 1:100 dilution for 1 h, washed 3 times with PBS, followed by tetramethylrhodamine isothiocyanate-conjugated anti-mouse secondary antibody in 1:100 dilution for 1 h, and washed 3 times with PBS. All antibodies were diluted in PBS containing 0.1% Triton X-100. Coverslips were mounted on microscope slides in ImmunoFloure Mounting Medium obtained from ICN.

Cells were observed and analyzed with a Nikon Optiphot II microscope equipped with epifluorescence illumination, and a Bio-Rad MRC 600 confocal laser scanning microscope system. Immunofluorescent images were recorded on a zip disk and analyzed using NIH Image 5.19 software.

**Statistical Methods**—All data are expressed as mean ± S.E. Linear regression analysis was performed using Origin software (Microcal Software Ltd., Northampton, MA). An unpaired Student’s t test was used for statistical comparisons of mean values between samples. A value of p < 0.05 was taken to indicate statistical significance.

**RESULTS**

**Comparison of Resting Ca\(^{2+}\) Concentration and Caffeine-induced Ca\(^{2+}\) Release among Wild Type, MH Mutant, and CCD Mutant RyR1 Proteins**—In this study, we used Ca\(^{2+}\) photometry and Ca\(^{2+}\) imaging to record the Ca\(^{2+}\) release properties of a number of RyR1 mutants expressed in HEK-293 cells. Ca\(^{2+}\) photometry, which is fast and accurate, provides spatially averaged measurements of the fluorescence used to monitor Ca\(^{2+}\) release. Ca\(^{2+}\) imaging provides spatially resolved measurements and was used to acquire images at 2.5 s/frame, permitting us to analyze events in individual cells in response to caffeine.

We used Ca\(^{2+}\) imaging to detect caffeine-induced Ca\(^{2+}\) release in HEK-293 cells transiently transfected with RyR1 cDNA, thereby distinguishing transfected cells from untransfected cells. Ca\(^{2+}\) photometry indicates that Ca\(^{2+}\) release is a rare event in vector-transfected or nontransfected HEK-293 cells which is lost in the background fluorescence when a cluster of 50 or more cells is analyzed. In single cell imaging of pcDNA vector-transfected cells, 6 out of 200 cells responded to 10 mM caffeine, increasing cytosolic Ca\(^{2+}\) concentrations to an average of 180 ± 54 nM (n = 6) and establishing a background release rate of about 3%. In wild type or MH/CCD mutant RyR1-transfected cells, however, 40–60% of isolated cells, where higher DNA transfection was observed, responded to 10 mM caffeine, a rate about 13–20-fold higher than background. Accordingly, caffeine-responsive cells in our Ca\(^{2+}\) imaging studies were regarded as RyR1-transfected cells, and an error rate of 3% was considered to be acceptable.

Resting cytoplasmic Ca\(^{2+}\) concentrations and 10 mM caffeine-induced Ca\(^{2+}\) release for wild type and 15 MH/CCD mutant RyR1 proteins were measured in single RyR1-transfected cells. The resting cytoplasmic Ca\(^{2+}\) concentration in untransfected HEK-293 cells was 97 ± 5 nM. Transfection with wild type RyR1 raised the resting cytoplasmic Ca\(^{2+}\) concentration to 112 ± 11 nM (Fig. 1A). The resting cytoplasmic Ca\(^{2+}\) concentration in HEK-293 cells transfected with each of the 10 MH mutants tested (C36R, G249R, G342R, R553W, R615C, R615L, R2163C, G2435R, R2458C, and R2458H) also raised the resting cytoplasmic Ca\(^{2+}\) concentration to values ranging from 103 ± 7 to 119 ± 7 nM with an average value of 110 ± 2 nM (Fig. 1A). There were no significant differences in resting Ca\(^{2+}\) concentrations between wild type and any of the MH mutant forms of RyR1. The resting Ca\(^{2+}\) concentration (Fig. 1A) for each of the 5 CCD mutants tested (R164C, 1404M, Y523S, R2163H, and R2436H) was higher than for any of the 10 MH mutants and the average resting Ca\(^{2+}\) concentration for the 5 CCD mutants, 142 ± 9 nM, was significantly higher (p < 0.001) than the average resting Ca\(^{2+}\) concentration for the 10 MH mutants.
however, when resting Ca\(^{2+}\) concentrations for individual CCD mutants were compared with wild type RYR1, significant differences were found for only 2 of the 5 individual CCD mutants tested, Y523S and R2163H (p < 0.05 for Y523S and p < 0.001 for R2163H).

Average caffeine-induced Ca\(^{2+}\) release and maximal 340/380 nm ratio change values for MH mutants were 590 ± 107 nM (Fig. 1B) and 0.36 ± 0.03 (Fig. 1C) (n = 10), respectively, and the comparable values for CCD mutants were 495 ± 60 nM (Fig. 1B) and 0.28 ± 0.06 (Fig. 1C) (n = 5). The caffeine-induced Ca\(^{2+}\) release values for MH mutants was not significantly different from the comparable values for CCD mutants. It should be noted, however, that many individual MH and CCD mutants had a significantly lower response to caffeine, measured by both Ca\(^{2+}\) photometry and Ca\(^{2+}\) imaging, than wild type RYR1 (Fig. 1). Maximal 340/380 nm ratio changes were significantly lower for the Y523S (p < 0.001) and R2163H (p < 0.001) mutants (Fig. 1B), which had higher resting Ca\(^{2+}\) concentrations (Fig. 1A) than wild type RyR1. These results are consistent with the view that CCD mutants might be more leaky than MH mutants, accounting for higher resting Ca\(^{2+}\) concentrations and lower Ca\(^{2+}\)- releasable stores, and that MH mutants might also be more leaky than wild type, accounting for lower concentrations of Ca\(^{2+}\) in caffeine-releasable stores.

To test whether resting Ca\(^{2+}\) concentrations might determine caffeine sensitivity and influence maximal caffeine-induced Ca\(^{2+}\) release, a linear correlation analysis was carried out. Resting Ca\(^{2+}\) concentrations had no linear correlation with caffeine ED\(_{50}\) values (r = −0.31, p = 0.24) or with maximal caffeine-induced Ca\(^{2+}\) release (r = −0.31, p = 0.24). Surprisingly, a linear correlation was observed between ED\(_{50}\) and maximal caffeine-induced Ca\(^{2+}\) release (r = 0.63, p < 0.05) and between ED\(_{50}\) and maximal 340/380 nm ratio change (r = 0.67, p < 0.05, data not shown). A linear correlation analysis was also carried out between maximal caffeine-induced Ca\(^{2+}\) release for 9 MH/CCD mutants obtained in this study and caffeine-induced muscle tension or caffeine threshold obtained through in vitro contracture test in an earlier study (19). A linear correlation was observed between maximal caffeine-induced Ca\(^{2+}\) release and caffeine-induced muscle tension (r = −0.83, p < 0.05) and between maximal caffeine-induced Ca\(^{2+}\) release and caffeine threshold (r = 0.83, p < 0.05).

**Relationship among Caffeine-, Carbachol-, and Thapsigargin-induced Ca\(^{2+}\) Release in Transfected Cells**—To test whether releasable ER Ca\(^{2+}\) stores were really lower in MH/CCD mutant-transfected cells, thapsigargin and carbachol were used to gate Ca\(^{2+}\) release, determined by fluorescence measurement of the amount of Ca\(^{2+}\) released. Thapsigargin increases intracellular Ca\(^{2+}\) concentration by irreversible blocking of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) molecules. Thapsigargin-induced Ca\(^{2+}\) release, therefore, represents the rapid, passive depletion of the internal Ca\(^{2+}\) store (33). Carbachol increases intracellular Ca\(^{2+}\) concentration by activation of the phospholipase C pathway through an endogenous muscarinic receptor found in HEK-293 cells, resulting in elevation of intracellular IP\(_3\) and activation of the IP\(_3\) receptor (34). Thus low ER Ca\(^{2+}\) stores would respond with low Ca\(^{2+}\) release following the application of either of these agents.

Figs. 2, A–C, shows that there was a close linear correlation
for Ca²⁺ release induced by any of these three reagents in 27 RYR1 cDNA-transfected cells. These cells were challenged sequentially by 10 mM caffeine, 20 μM carbachol (IP₃ receptor), and 1.5 μM thapsigargin (SERCA). In cells transfected with different MH/CCD mutant cDNAs to form either homotetrameric or heterotetrameric channels, caffeine-induced Ca²⁺ release was closely correlated with thapsigargin-induced Ca²⁺ release (Fig. 2D). This close correlation among caffeine-, carbachol-, and thapsigargin-induced Ca²⁺ release indicates that maximal caffeine-induced Ca²⁺ release reflects the size of the ER Ca²⁺ store. The lower maximal caffeine-induced Ca²⁺ release in cells transfected with MH/CCD mutants is likely to be due to a lower Ca²⁺ store, which, in turn, is likely to reflect Ca²⁺ leakage through MH/CCD mutant channels.

Comparison of Ca²⁺ Stores and SERCA2b Content among Untransfected and RYR1 cDNA-transfected Cells—To compare ER Ca²⁺ stores among untransfected and RYR1 cDNA-transfected cells, carbachol-induced Ca²⁺ release was measured by Ca²⁺ imaging and was regarded as an indicator of the size of the ER Ca²⁺ store. The lower maximal carbachol-induced Ca²⁺ release in HEK-293 cells transfected with CCD mutant Y523S is likely to be due to a lower Ca²⁺ store, which, in turn, is likely to reflect Ca²⁺ leakage through MH/CCD mutant channels.

Fig. 2. Correlation among drug-induced Ca²⁺ release, measured by Ca²⁺ imaging, in HEK-293 cells transfected with wild type RYR1 (A–C) and individual MH/CCD mutants (D). The linear analysis method was used to analyze the following: the correlation between Ca²⁺ release induced by carbachol, an agonist of an endogenous muscarinic receptor which releases IP₃, and acts on an IP₃ receptor, and by caffeine, a drug which acts directly on RyR1 (A); the correlation between Ca²⁺ release induced by thapsigargin, an irreversible SERCA inhibitor, and by caffeine (B); the correlation between Ca²⁺ release induced by carbachol and thapsigargin (C); and the correlation between mean thapsigargin- and caffeine-induced Ca²⁺ release values in MH/CCD mutant homotetramers and heterotetramers (D).

The enhancement of the Ca²⁺ store by transfection of wild type RyR1 led us to test the hypothesis that cells transfected with channels that increase release from ER Ca²⁺ stores may overexpress endogenous SERCA2b in an attempt to reestablish Ca²⁺ homeostasis by lowering elevated resting Ca²⁺ concentrations to normal (Fig. 1). To determine whether cells transfected with wild type or mutant RYR1 had the capacity to create a larger Ca²⁺ store, SERCA2b contents were measured by ELISA (Fig. 4A), and Ca²⁺ uptake was measured in microsomes extracted from transfected cells (Fig. 4B). When HEK-293 cells were transfected with wild type RYR1, the SERCA2b content was increased to 119% (ELISA) or 120% (Ca²⁺ uptake). Transfection with MH mutant R615L led to an increase in SERCA2b content to 117% (ELISA) or 120% (Ca²⁺ uptake), whereas transfection with CCD mutant Y523S led to an increase in SERCA2b content to 127% (ELISA) or 123% (Ca²⁺ uptake). Although transfection efficiency approaches 50% for non-confluent cells, overall transfection efficiency is only about 25%. Therefore, the real increase in SERCA2b expression in transfected cells would be about 4-fold higher, approaching a doubling of SERCA2b content.

Immunofluorescent labeling and confocal microscopy were used in a third measurement of the enhancement of SERCA2b content in single cells transfected with mutant RyR1. Because only mouse anti-RyR1 and mouse anti-SERCA2 monoclonal antibodies are available, green fluorescent protein (GFP) vector (CLONTECH) and rabbit RyR1 mutant Y523S were cotransfected into HEK-293 cells in a 1:9 molar ratio. GFP fluores-
It is assumed that the activity of SERCA pumps is coordinated with the activity of RyR \( \text{Ca}^{2+} \) release channels in healthy skeletal muscle to regulate cytosolic \( \text{Ca}^{2+} \) concentrations. Thus the 2-fold enhancement of SERCA2 synthesis that we observed in cells expressing MH/CCD mutants could be explained logically as a compensatory event. Nevertheless, enhanced endogenous SERCA2b synthesis was not sufficient to compensate for the higher resting \( \text{Ca}^{2+} \) concentrations attributed to leaky mutant channels R615L or Y523S (Fig. 1). Since it is unlikely that compensatory SERCA synthesis had reached equilibrium in these cells over the 48-h period between transfection and analysis, we attempted to increase SERCA synthesis at a more rapid rate by transfection with SERCA1 under conditions where high levels of activity are observed within 48 h (35). Under these conditions, SERCA1 was functional, since it increased the rate of \( \text{Ca}^{2+} \) removal from the cytoplasm following caffeine-induced \( \text{Ca}^{2+} \) release and lowered the elevated base line of \( \text{Ca}^{2+} \) fluorescence resulting from repeated caffeine stimulation of cells cotransfected with wild type or MH/CCD mutant RyR1 (not shown).

Surprisingly, coexpression of mutants R615L or Y523S with SERCA1 raised resting \( \text{Ca}^{2+} \) concentrations (Fig. 5C). The coexpression of SERCA1 with Y523S, or with Y523S plus wild type RyR1, or with R615L, or with R615L plus wild type RyR1 decreased caffeine sensitivity (Fig. 5A), increased resting \( \text{Ca}^{2+} \) concentration (Fig. 5C), and increased maximal \( \text{Ca}^{2+} \) release for both RyR1 mutants (Fig. 5B and D). These results suggest that SERCA1 can increase \( \text{Ca}^{2+} \) removal rates and \( \text{Ca}^{2+} \) stores but that increased flux through the mutant channels in the ER, even under conditions where \( \text{Ca}^{2+} \) uptake is greatly increased, ultimately results in an increase in resting \( \text{Ca}^{2+} \) concentrations.

We used \( \text{Ca}^{2+} \) imaging to test caffeine-induced \( \text{Ca}^{2+} \) release in MH/CCD mutant heterotetramers. We found that the heterotetrameric R615L mutant plus SERCA1 and the heterotetrameric mutant Y523S plus SERCA1 were significantly more sensitive to low concentrations of caffeine (0.5 and 1 \( \text{mM} \)) and halothane (0.1 and 0.25 \( \text{mM} \)) than wild type RyR1 plus SERCA1 (not shown). The MH/CCD mutant heterotetramers plus SERCA1 released more \( \text{Ca}^{2+} \) when challenged by low concentrations of caffeine and halothane than wild type RyR1 plus SERCA1 (not shown).

**Influence of Extracellular \( \text{Ca}^{2+} \) Levels on Caffeine Sensitivity of Wild Type and MH/CCD Mutant RyR1**—All of the results described above were obtained from HEK-293 cells incubated in a medium containing 2 mM \( \text{Ca}^{2+} \). To confirm that caffeine-induced \( \text{Ca}^{2+} \) release in RyR1-transfected cells was caused by \( \text{Ca}^{2+} \) release from ER \( \text{Ca}^{2+} \) stores, caffeine-induced \( \text{Ca}^{2+} \) release in RyR1-transfected cells was measured in \( \text{Ca}^{2+} \)-free medium. \( \text{Ca}^{2+} \) release, induced by caffeine or carbachol and measured by \( \text{Ca}^{2+} \) imaging in RyR1-transfected cells, was reduced dramatically, and resting \( \text{Ca}^{2+} \) concentrations in both transfected and untransfected cells were much lower in a \( \text{Ca}^{2+} \)-free medium than in \( \text{Ca}^{2+} \)-containing medium (not shown). These results indicate that extracellular \( \text{Ca}^{2+} \) has a significant influence on intracellular \( \text{Ca}^{2+} \) concentration.

In early experiments, we found that caffeine-induced \( \text{Ca}^{2+} \) release could be abolished by the addition of thapsigargin in \( \text{Ca}^{2+} \)-containing medium (not shown), indicating that caffeine-induced \( \text{Ca}^{2+} \) release depends on the ER \( \text{Ca}^{2+} \) store. In later studies, RyR1 cDNA-transfected cells were incubated in \( \text{Ca}^{2+} \)-free medium with Fura-2/AM for 30 min, and caffeine-induced \( \text{Ca}^{2+} \) release was measured in \( \text{Ca}^{2+} \)-free medium, then in \( \text{Ca}^{2+} \)-containing medium, and, finally, in \( \text{Ca}^{2+} \)-free medium.
In Ca\textsuperscript{2+}-free medium, caffeine responses were reduced dramatically after the third caffeine stimulation (Fig. 6A). In Ca\textsuperscript{2+}-containing medium (not shown), caffeine stimulation of Ca\textsuperscript{2+} release could be induced repeatedly. The addition of Ca\textsuperscript{2+}-containing medium (2 mM Ca\textsuperscript{2+}) resulted in an increase in resting Ca\textsuperscript{2+} concentration and an increase in the amplitude of caffeine-induced Ca\textsuperscript{2+} release (Fig. 6A). When the cells were returned to Ca\textsuperscript{2+}-free medium, the caffeine response was reduced dramatically. Similar results were obtained for carbachol responses in untransfected cells (not shown). These results confirm that extracellular Ca\textsuperscript{2+} concentrations have a profound influence on cellular Ca\textsuperscript{2+} homeostasis.

When we measured caffeine dose-response curves for wild type and Y523S and R615L mutants in Ca\textsuperscript{2+}-free medium, we

(Fig. 6A). In Ca\textsuperscript{2+}-free medium, caffeine responses were reduced dramatically after the third caffeine stimulation (Fig. 6A). In Ca\textsuperscript{2+}-containing medium (not shown), caffeine stimulation of Ca\textsuperscript{2+} release could be induced repeatedly. The addition of Ca\textsuperscript{2+}-containing medium (2 mM Ca\textsuperscript{2+}) resulted in an increase in resting Ca\textsuperscript{2+} concentration and an increase in the amplitude of caffeine-induced Ca\textsuperscript{2+} release (Fig. 6A). When the cells were returned to Ca\textsuperscript{2+}-free medium, the caffeine response was reduced dramatically. Similar results were obtained for carbachol responses in untransfected cells (not shown). These results confirm that extracellular Ca\textsuperscript{2+} concentrations have a profound influence on cellular Ca\textsuperscript{2+} homeostasis.

When we measured caffeine dose-response curves for wild type and Y523S and R615L mutants in Ca\textsuperscript{2+}-free medium, we
observed that the mutant channels were more sensitive than wild type to caffeine (Fig. 6B), just as they were in Ca\(^{2+}\)-containing medium. The caffeine ED_{50} values for RyR1, Y523S, and R615L in Ca\(^{2+}\)-free media were 8.1, 0.99, and 2.64 mM, respectively. These values are 2 to 4 times higher than the Ca\(^{2+}\) sensitivity of RyR1.

The differences in the results of our experiments and those of Querfurth et al. (36) are most likely to be due to the way in which experiments were carried out. Querfurth et al. (36) could not observe caffeine-induced Ca\(^{2+}\) release when they allowed buffer changes to flow over the cells (the conditions of our experiments). In order to see Ca\(^{2+}\) release, they had to interrupt flow and then remove and replace buffer to achieve instant 15 mM caffeine. Thus re-perfusion of high caffeine seemed to sensitize the endogenous RyR activity, even though the resulting spike of Ca\(^{2+}\) release was actually slower than that which we observed. Even if there were re-perfusion artifacts in the Ca\(^{2+}\)-imaging protocol of Querfurth et al. (36) that could account for the exaggerated Ca\(^{2+}\) release attributed to endogenous RyR, they would not be relevant to our studies, since we used a different protocol that, maximally, triggered only rare cases of Ca\(^{2+}\) release in untransfected cells.

Functional Differences in MH and MH/CCD Mutations—In earlier publications (7, 9, 10), we proposed that there might be spontaneous Ca\(^{2+}\) leakage through MH mutant channels but that compensatory mechanisms would lead to rapid re-establishment of Ca\(^{2+}\) homeostasis. Thus muscle hypertrophy in MH swine might result from spontaneous Ca\(^{2+}\) release from an abnormal Ca\(^{2+}\) release channel leading to spontaneous muscle contraction. We also proposed that CCD mutations might lead to more serious spontaneous Ca\(^{2+}\) release, which would disrupt Ca\(^{2+}\) homeostasis in the core of the cell but not in the periphery where re-establishment of Ca\(^{2+}\) homeostasis would be aided by plasma membrane Ca\(^{2+}\)-ATPases or Na\(^+\)/Ca\(^{2+}\) exchangers in the plasma membrane (9, 10).

In this study, in which we present single cell imaging of pcDNA-transfected HEK-293 cells, we also measured the background of endogenous Ca\(^{2+}\) release in untransfected and vector-transfected cells. We found that 6 out of 200 cells responded to 10 mM caffeine, increasing cytosolic Ca\(^{2+}\) concentrations to an average of 180 ± 54 mM and establishing our background response rate at 3%. In wild type or mutant RyR1-transfected cells, 40–60% of cells responded to 10 mM caffeine, a rate 13–20-fold higher than background, increasing cytosolic Ca\(^{2+}\) concentrations well above 500 nM (Fig. 1A). The differences in the results of our experiments and those of Querfurth et al. (36) are most likely to be due to the way in which experiments were carried out. Querfurth et al. (36) could not observe caffeine-induced Ca\(^{2+}\) release when they allowed buffer changes to flow over the cells (the conditions of our experiments). In order to see Ca\(^{2+}\) release, they had to interrupt flow and then remove and replace buffer to achieve instant 15 mM caffeine. Thus re-perfusion of high caffeine seemed to sensitize the endogenous RyR activity, even though the resulting spike of Ca\(^{2+}\) release was actually slower than that which we observed. Even if there were re-perfusion artifacts in the Ca\(^{2+}\)-imaging protocol of Querfurth et al. (36) that could account for the exaggerated Ca\(^{2+}\) release attributed to endogenous RyR, they would not be relevant to our studies, since we used a different protocol that, maximally, triggered only rare cases of Ca\(^{2+}\) release in untransfected cells.
In this study, we observed higher resting Ca\textsuperscript{2+} concentrations in HEK-293 cells transfected with wild type RYR1, suggesting that even the normal expressed channel might increase the permeability of the ER Ca\textsuperscript{2+} store. This might explain the difficulty that we have encountered in attempts to obtain a stable HEK-293 cell line expressing RYR1. When the cells were transfected with RYR1 carrying MH mutations, resting cytosolic Ca\textsuperscript{2+} concentrations were raised over that of wild type RyR1-transfected cells in some cases (Fig. 1), but average values were not significantly different from wild type. Of greater interest was the observation that the average resting cytosolic Ca\textsuperscript{2+} concentration was elevated significantly over wild type and over MH mutant RyR1 proteins for the five CCD mutant RyR1 proteins expressed in HEK-293 cells. For the individual CCD mutants, Y523S and R2163H, resting cytosolic Ca\textsuperscript{2+} concentrations were elevated significantly over wild type RyR1.

Maximal caffeine-induced Ca\textsuperscript{2+} release, measured by both Ca\textsuperscript{2+} photometry and Ca\textsuperscript{2+} imaging, was lower in cells transfected with individual MH/CCD mutants than with wild type RYR1. We measured the size of the ER Ca\textsuperscript{2+} store that was releasable by three different triggers acting through three different mechanisms as follows: caffeine, which releases Ca\textsuperscript{2+} through RyR; thapsigargin, which inhibits SERCA2, thereby preventing re-uptake of Ca\textsuperscript{2+} lost through passive leaks; and carbachol, which releases Ca\textsuperscript{2+} indirectly through the IP\textsubscript{3} receptor. The close correlation of caffeine-, carbachol-, and thapsigargin-induced Ca\textsuperscript{2+} release indicates that the maximal caffeine-induced Ca\textsuperscript{2+} release is proportional to the size of the ER Ca\textsuperscript{2+} store. We noted a large variation in amplitude of Ca\textsuperscript{2+} release in different cells to the same releasing agents (caffeine, carbachol, and thapsigargin) (Fig. 2). This variation was not caused by DNA transfection, because untransfected cells also showed a large variation in their response to carbachol and thapsigargin. It is more likely that variation was based on factors such as age or cell cycle stage. Despite this variation among individual cells, we were able to obtain clear correlations from measurements of sizes of ER Ca\textsuperscript{2+} stores in response to different triggering agents. Accordingly, leakage from these stores through an abnormal RyR1 would be predicted to lower the store available for caffeine-induced Ca\textsuperscript{2+} release and, at the same time, to increase cytosolic Ca\textsuperscript{2+} concentrations, as observed in Fig. 1. These observations suggest that CCD and MH mutant channels are more leaky than wild type RYR1 channels.

Resting cytosolic Ca\textsuperscript{2+} concentrations were not correlated with caffeine sensitivity or with maximal caffeine-induced Ca\textsuperscript{2+} release. This lack of correlation is consistent with clinical observations and with our previous results (22), which showed no differences in caffeine and halothane sensitivity between MH and CCD mutants. The results suggest that resting Ca\textsuperscript{2+} concentrations do not have a major influence on the caffeine sensitivity of MH/CCD mutants.

Caffeine ED\textsubscript{50} values for Ca\textsuperscript{2+} release through MH/CCD mutant proteins were linearly correlated with maximal caffeine responses, and the maximal caffeine-induced Ca\textsuperscript{2+} release was also linearly correlated with clinical caffeine thresholds, indicating that higher ER Ca\textsuperscript{2+} stores inhibit caffeine responses. In single-channel measurements with rabbit RYR1, an increase in luminal Ca\textsuperscript{2+} concentration, from micromolar to millimolar, has been shown to decrease single channel activity (37–39). Since high luminal Ca\textsuperscript{2+} lowers channel open probability, it may raise caffeine ED\textsubscript{50}. The corollary is that low luminal concentrations may increase caffeine ED\textsubscript{50}. On the other hand, lower Ca\textsuperscript{2+} concentration in the sarcoplasmic reticulum lumen may provide a compensatory mechanism in MH/CCD skeletal muscle cells. Whether halothane will trigger an MH reaction may depend not only on the sensitivity of MH/CCD mutants to halothane but also on the balance between the decreased inhibitory effect of luminal Ca\textsuperscript{2+} concentration and the reduced net Ca\textsuperscript{2+} efflux from the sarcoplasmic reticulum. This may explain why some individuals who carry MH mutations do not show higher sensitivity to caffeine and halothane.

The observation of lower caffeine-induced Ca\textsuperscript{2+} release in HEK-293 cells transfected with MH/CCD mutants is not consistent with clinical observations. However, in vivo, MH individuals rarely have homozygous MH/CCD mutations, and an increase in the number of SERCA pumps could compensate for enhanced Ca\textsuperscript{2+} release. We tested whether MH/CCD mutants had higher caffeine-induced Ca\textsuperscript{2+} release when they were coexpressed with wild type RyR1 and SERCA1. The caffeine sensitivity of the heterotetrameric MH/CCD mutants was between that of the homotetrameric MH/CCD mutants and wild type RyR1 (Fig. 2), but maximal 340/380 nm ratio changes in MH/CCD heterotetrameric mutants were higher than those in homotetrameric MH/CCD mutants. RyR1 isolated from pigs heterozygous for the R614C MH mutation demonstrated intermediate values for the rate of Ca\textsuperscript{2+} release and the affinity for [3H]ryanodine (40–44). Maximal Ca\textsuperscript{2+} release responses were similar in heterotetrameric MH/CCD mutants and wild type homotetramers, but the heterotetrameric MH/CCD mutants plus SERCA1 were more sensitive to low concentrations of caffeine and halothane than the cells transfected with wild type RyR1 plus SERCA1 (Fig. 5). Overall, these results indicate that coexpression of SERCA1 increases the ER Ca\textsuperscript{2+} store and that expression of heterotetrameric MH/CCD mutants reduces the abnormal leak of homotetrameric MH/CCD mutant channels.

Compensation in RYR1-transfected Cells—Our overall observations concerning the expression of wild type, MH, and CCD mutant RyR1 in HEK-293 cells can be interpreted as a coherent pattern of events. The expression of wild type RyR1 raises the resting Ca\textsuperscript{2+} concentration (Fig. 1A), increases the size of the Ca\textsuperscript{2+} store (Fig. 3), and increases both SERCA2b content and activity (Fig. 4). These observations can be explained on the basis of an increased permeability of the ER Ca\textsuperscript{2+} store which is compensated for by Ca\textsuperscript{2+}-induced synthesis of SERCA2b, with a consequent enlargement of the Ca\textsuperscript{2+} store. Despite this attempt at re-establishment of Ca\textsuperscript{2+} homeostasis, the enhanced flux of Ca\textsuperscript{2+} through the ER still results in a higher resting Ca\textsuperscript{2+} concentration. Expression of MH or CCD mutant RyR1 channels also raises resting Ca\textsuperscript{2+} concentrations and enhances synthesis of SERCA2b, leading to a higher potential for Ca\textsuperscript{2+} storage. This potential was not realized, however, since the carbachol-releasable stores for MH and CCD mutants were seen to be depleted (Fig. 3). This suggests that the attempt to re-establish Ca\textsuperscript{2+} homeostasis for MH and CCD mutants was less successful, possibly because the flux through even more permeable channels would require even higher synthesis of SERCA2b. In attempts to determine whether full compensation could ever be achieved, we coexpressed SERCA1 with homoygous and heterozygous mutant channels (Fig. 5). We found that coexpression of higher levels of SERCA1 did not reduce resting Ca\textsuperscript{2+} concentrations but did increase carbachol-releasable Ca\textsuperscript{2+} stores, particularly for RyR1 heterozygotes (Fig. 5). These experiments provide new insights into the way in which diseases arising from defects in Ca\textsuperscript{2+} regulatory proteins progress and are compensated. Clearly compensation is a much more complex process involving Ca\textsuperscript{2+} regulatory proteins in the sarcoplasmic reticulum, the plasma membrane, and mitochondria (7, 9, 10), and the contributions of these systems to disturbances in Ca\textsuperscript{2+} homeostasis will have to be investigated in future studies.
Our current observations provide support for the hypothesis (7, 10) that MH and CCD mutants have enhanced permeability and that compensatory mechanisms such as enhanced SERCA synthesis are brought into play to restore Ca\(^{2+}\) homeostasis. The synthesis of SERCA1 is enhanced in myoblasts by elevated Ca\(^{2+}\) (45, 46). Our results confirm that SERCA2b expression is enhanced in transfected HEK-293 cells and show that the most leaky CCD mutant channel (Y523S) induces the highest expression of endogenous SERCA2 (Fig. 4). The correlation between higher permeability and enhancement of Ca\(^{2+}\) stores, however, was not perfect, however. We observed only slightly higher synthesis of SERCA2b for the MH and CCD mutants than for wild type RyR1. This may simply reflect the fact that our observations were made only 48 h after transfection, a period too short for equilibrium to be established. One of the striking morphological features of CCD muscle is a proliferation of the sarcotubular system in the core (47). The development of such cores occurs over a period of months or even years, so that compensatory mechanisms may require a long time to reach equilibrium.

In Fig. 7, we illustrate the Ca\(^{2+}\) concentration changes that we have observed in our studies of the size of the ER Ca\(^{2+}\) stores and resting cytosolic Ca\(^{2+}\) concentrations in cells transfected with wild type, MH, or CCD mutant RyR1. The expression of wild type RyR1 in HEK-293 cells increases resting cytosolic Ca\(^{2+}\) concentrations, probably by the enhanced permeability of ER Ca\(^{2+}\) stores. In an effort to compensate for increased Ca\(^{2+}\) release to restore Ca\(^{2+}\) homeostasis, the HEK-293 cells express more SERCA2b, increasing the potential for more Ca\(^{2+}\) storage. Transfection with MH mutant RyR1 increases permeability of the ER Ca\(^{2+}\) store even more, so that ER Ca\(^{2+}\) stores begin to be depleted. Resting cytosolic Ca\(^{2+}\) concentrations are higher, and ER Ca\(^{2+}\) stores are lower in CCD mutant RyR1-transfected cells, because of the high permeability of the CCD mutant RyR1 proteins.

**Influence of Extracellular Ca\(^{2+}\) Concentration on Intracellular Ca\(^{2+}\) Homeostasis**—Extracellular Ca\(^{2+}\) concentrations were not considered when we compared caffeine or halothane sensitivities among wild type, MH, and CCD mutant RyR1 because we were measuring $K_{\text{activation}}$ properties of different mutant proteins. It became a concern, however, when the size of the ER Ca\(^{2+}\) store was measured. Under the conditions of these experiments, we found that extracellular Ca\(^{2+}\) has a profound effect on caffeine-induced Ca\(^{2+}\) release and ER Ca\(^{2+}\) stores (Fig. 6). It is impossible to obtain an accurate RyR1 caffeine ED$_{50}$ in a Ca\(^{2+}\)-free medium, because cytosolic Ca\(^{2+}\) is lost to extracellular spaces after it is released from the ER. Accordingly, caffeine ED$_{50}$ measured in Ca\(^{2+}\)-free medium reflected not only the sensitivity of the RyR1 channel but also the size of the ER Ca\(^{2+}\) store in the transfected cells. The lower caffeine sensitivity in Ca\(^{2+}\)-free medium than in Ca\(^{2+}\)-containing medium indicates that resting Ca\(^{2+}\) concentrations can influence RyR1 caffeine sensitivity.

In this study, we have identified at least three factors that influence the RyR1 caffeine response. These are the sensitivity of RyR1 proteins, the size of the releasable ER Ca\(^{2+}\) store, and the resting Ca\(^{2+}\) concentration. Several laboratories have shown that MH mutants are more sensitive to a variety of channel activators, including Ca\(^{2+}\), ATP, caffeine, and halothane, than wild type (9, 22, 38, 40–44) and are less sensitive to Mg\(^{2+}\) (48). Thus the main cause of MH is the hypersensitivity of RyR1 mutant proteins to very basic stimulus, but the occurrence of MH in humans may also be influenced by the size of the Ca\(^{2+}\) store and resting Ca\(^{2+}\) concentration which may be modulated by a system of regulatory proteins in skeletal muscle.

The present results were obtained with HEK-293 cells transfected with wild type and MH/CCD mutant RyR1 constructs, and the data were obtained 48 h after transfection. HEK-293-transfected cells differ from skeletal muscle cells in that they lack many of the proteins that may modulate myoplasmic Ca\(^{2+}\) concentrations. The advantage of the HEK-293 cell expression system is that it permits the expression of homozygous and
heterozygous MH/CCD mutants in a homogenous genetic background. This has facilitated the detection of functional differences between normal and abnormal channels, which may not be detectable in native skeletal muscle samples. It is difficult or impossible to obtain substantial quantities of human MH mutant muscle, and most human samples are from heterozygotes where it is more difficult to detect small changes in RyR1 function. Moreover, differences between MH/CCD and normal samples might be too small to be detected due to the compensatory effects of other Ca\(^{2+}\) regulatory systems unique to skeletal muscle cells.

The absence of potential Ca\(^{2+}\) regulatory systems in HEK-293 cells is a disadvantage that must be weighed against the long term compensatory effects that are probably missing in transiently transfected HEK-293 cells. Despite the differences in the assay systems, it is reasonable to believe that MH/CCD mutants, which are more leaky in HEK-293 cells, would be of interest, if cell lines could be established, to observe the long term compensatory effects of other Ca\(^{2+}\) regulatory systems.

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