Ca\textsuperscript{2+} Signaling in HEK-293 and Skeletal Muscle Cells Expressing Recombinant Ryanodine Receptors Harboring Malignant Hyperthermia and Central Core Disease Mutations*

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Malignant hyperthermia (MH) and central core disease (CCD) are caused by mutations in the \textit{RYR1} gene encoding the skeletal muscle isoform of the ryanodine receptor (RyR1), a homotetrameric Ca\textsuperscript{2+} release channel. Rabbit RyR1 mutant cDNAs carrying mutations corresponding to those in human RyR1 that cause MH and CCD were expressed in HEK-293 cells, which do not have endogenous RyR, and in primary cultures of rat skeletal muscle, which express rat RyR1. Analysis of intracellular Ca\textsuperscript{2+} pools was performed using aequorin probes targeted to the lumen of the endo/sarcoplasmic reticulum (ER/SR), to the mitochondrial matrix, or to the cytosol. Mutations associated with MH caused alterations in intracellular Ca\textsuperscript{2+} homeostasis different from those associated with CCD. Measurements of luminal ER/SR Ca\textsuperscript{2+} revealed that the mutant channels in all cases, but the leak was particularly pronounced in CCD mutants. Cytosolic and mitochondrial Ca\textsuperscript{2+} transients induced by caffeine stimulation were drastically augmented in the MH mutant, slightly reduced in one CCD mutant (Y523S) and completely abolished in another (I4898T). The results suggest that local Ca\textsuperscript{2+} derangements of different degrees account for the specific cellular phenotypes of the two disorders.

The contraction/relaxation cycle of skeletal muscles is prominent among the processes controlled by Ca\textsuperscript{2+} (1). The rapid release of Ca\textsuperscript{2+} from the sarcoplasmic reticulum (SR) through the ryanodine receptors induces the contraction of myofibrils; Ca\textsuperscript{2+} reuptake into the SR lumen by the Ca\textsuperscript{2+} ATPase (SERCA pump) induces relaxation. The correct functioning of the contraction/relaxation cycle demands a precise balance between Ca\textsuperscript{2+} release and re-uptake. Disturbances of this balance, such as those that result from mutations in the proteins involved in the uptake and release processes, lead to muscle diseases, including Brody disease, malignant hyperthermia (MH), central core disease (CCD), and cardiomyopathy (2–6).

CCD is an autosomal dominant myopathy characterized by hypotonia during infancy, proximal muscle weakness, delayed motor development, and reduced muscle bulk. MH is a pharmacogenetic disorder of skeletal muscle triggered in susceptible individuals by inhalational anesthetics and depolarizing skeletal muscle relaxants. Susceptible individuals respond with skeletal muscle rigidity, tachycardia, unstable and rising blood pressure, and eventually dramatic hyperthermia. More than 80 different mutations in the \textit{RYR1} gene have so far been associated with MH and/or CCD (2, 7, 8). They may either be point mutations or small, in-frame deletions, mostly clustered in three separate regions, between amino acids 35 and 614 (MH/CCD region 1), 2129 and 2458 (MH/CCD region 2), and 4214 and 4914 (MH/CCD region 3). Although both MH and CCD mutations are found in all three regions, CCD mutations predominate in region 3, which contains the channel pore (9–12). The MH and CCD clinical phenotypes differ, in line with the greater damage to the receptor induced by the CCD mutations (2, 13–15). Many CCD patients are MH susceptible, but most MH patients do not display symptoms of central core disease. Some CCD patients, particularly those carrying region 3 mutations, are not MH susceptible (15).

Studies in cellular models and in muscle biopsies have clarified important questions on the molecular mechanisms leading to the phenotypes of the two disorders. MH and some CCD mutant channels display increased RyR1 sensitivity to caffeine and halothane, suggesting that the defect may impair the channel gating properties (14, 16–19). Some CCD mutations have been found to generate constitutively active channels, which would reduce the size of the SR Ca\textsuperscript{2+} pool (14, 15, 20). This has led to the "leaky channel" hypothesis for the formation of central cores (2, 13). Other experiments have defined a second class of CCD mutations that do not respond to ligand activation (15) and are not responsive to orthograde activation by the dihydropyridine receptor, but retain the retrograde ability to activate the latter channel function (21). The loss of orthograde activation led to their designation as excitation-contraction...
(EC)-uncoupling mutants (21). Conflicting results, in particular on the ability of EC-uncoupling mutants to elevate resting Ca\(^{2+}\) concentration, have been attributed to differences in experimental conditions, including the cell type employed (8).

The proposed reduction in the size of the SR Ca\(^{2+}\) store that would accompany a leaky channel has not yet been documented directly. The clarification of this and other points was the primary aim of this study. We have analyzed the size of the Ca\(^{2+}\) stores and the amplitudes and kinetics of Ca\(^{2+}\) transients in HEK-293 cells and in primary cultures of rat skeletal myotubes transfected with expression plasmids containing rabbit RyR1 mutant cDNAs: mutation R615C as an example of a channel with increased sensitivity to ligands, causing MH; mutation Y523S as an example of a leaky channel causing CCD; and mutation I4898T (corresponding to the human mutant Y523S) as an example of a leaky channel with increased sensitivity to ligands, causing MH; mutation I4898T (corresponding to the human mutant Y523S) as an example of a leaky channel causing CCD; and mutation I4898T (corresponding to the human mutant Y523S) as an example of a leaky channel causing CCD.

The work has shown that RyR1 mutations responsible for MH and CCD cause different alterations in the size of the Ca\(^{2+}\) stores and in Ca\(^{2+}\) transients.

The study was extended to primary cultures of skeletal muscle cells, where the expression of mutant RyR1 cDNAs and the Ca\(^{2+}\) content of the ER/SR had to be reduced drastically before the reconstitution of functional ERaEQ and sErAEG. To this end, the cells were incubated for 1 h at 4°C in KRB (125 mM NaCl, 5 mM KCl, 1 mM Na\(_2\)PO\(_4\), 20 mM HEPES, pH 7.4, 37°C, supplemented with 5 μL coelenterazine n, the Ca\(^{2+}\) ionophore ionomycin (5 μM), and 600 μM EGTA. After incubation, the cells were washed extensively with KRB supplemented with 2% bovine serum albumin and 1 mM EGTA, and transferred to the chamber of a dedicated luminometer, where they were perfused with KRB supplemented with 100 μM EGTA. EGTA was then replaced with 1 mM CaCl\(_2\) and monitoring was continued until a steady state [Ca\(^{2+}\)] level was reached. Transfected cytAEQ and m3AEQ were reconstituted by incubating the cells for 3–6 h with 5 μL coelenterazine wild type in Dulbecco’s modified Eagle’s medium supplemented with 1% FCS, 2 mM glutamine, penicillin (60 μg/mL)/streptomycin (120 μg/mL), and placed in 10-cm Petri dishes at a density of 10\(^6\) cells/dish to decrease the number of fibroblasts in the culture. After 1 h of incubation at 37°C, non-adherent cells were collected and seeded at a density of 2.5 × 10\(^4\) cells per 15-mm coverslip, in 2% gelatin.

The next day the cells were washed with DMEM supplemented with 10% FCS, 2 mM glutamine, penicillin (60 μg/mL)/streptomycin (120 μg/mL). Transfections with the different plasmids were carried out on the third day of culture using the FuGENE VI reagent (Roche Applied Science, Monza, Italy) in DMEM supplemented with 2% horse serum to induce myoblast fusion. Experiments were then performed on day 6 of culture.

**Aequorin Measurements**—Aequorin was used as a 

**Experimental Procedures**

**Cell Culture and Transfection**—HEK-293 cells were grown in minimum essential medium (MEM, Cellbio, Italy), supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and penicillin (60 μg/mL)/streptomycin (120 μg/mL) in 75-cm\(^2\) Falcon flasks. Prior to transfection, the cells were seeded onto 13-mm glass coverslips and allowed to grow to 50% confluence. At this stage, transfection with 3 μg of plasmid DNA (or 1.5:1.5 μg in the co-transfection experiments) was carried out as described previously (25). Aequorin measurements and immunocytochemistry were performed 36 h later.

Primary cultures of rat skeletal muscle cells were prepared from newborn rats, as described previously (26). Briefly, posterior limb muscles were removed and washed in PBS (140 mM NaCl, 2 mM KCl, 1.5 mM KH\(_2\)PO\(_4\), 8 mM Na\(_2\)HPO\(_4\), pH 7.4). Primary cultures were initiated from satellite cells obtained by four successive treatments of skeletal muscles with 0.125% trypsin in PBS. The first harvest, containing mostly fibroblasts and endothelial cells, was discarded. The remaining cell suspension was filtered and centrifuged for 10 min at 1200 rpm. Cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS, 2 mM glutamine, penicillin (60 μg/mL)/streptomycin (120 μg/mL), and plated in 1-cm Petri dishes at a density of 10\(^6\) cells/dish to decrease the number of fibroblasts in the culture. After 1 h of incubation at 37°C, non-adherent cells were collected and seeded at a density of 2.5 × 10\(^4\) cells per 15-mm coverslip, in 2% gelatin.
Measurement of Cytosolic Resting Ca\(^{2+}\) in HEK-293 Cells Transfected with pcDNA or RyR1 Constructs—HEK-293 cells were loaded with 4 \(\mu\)M Fura-2/AM 48 h after transfection, placed on the stage of an inverted microscope. RyR-transfected cells were identified by co-transfection with an EGFFP expression plasmid or following stimulation with 10 mM caffeine. The two protocols yielded the same results. Cells responding to caffeine were regarded as RyR1-transfected and those not responding were regarded as untransfected. Cells were then washed to allow them to return to resting Ca\(^{2+}\) levels before imaging experiments were carried out, as described previously (14). Changes in Fura-2 fluorescence, presented as the ratio of fluorescence at 340/380 nm, were recorded with a CCD camera and converted into cytosolic Ca\(^{2+}\) concentration. Expression of RyR1 and mutants probed with 34C antibody to RyR1 showed that all three proteins were expressed in comparable amounts. In this and in earlier studies (14, 15), RyR1-transfected cells were identified by their response to caffeine, but this technique could not be used to identify cells transfected with mutant I4898T, which does not respond to caffeine. Accordingly, cells were imaged at random, with the result that two populations were clearly distinguishable. The first, most abundant population provided images that matched the cells transfected with pcDNA only; the second, smaller population had an elevated cytosolic Ca\(^{2+}\) concentration. Data from the second population were pooled to obtain the resting Ca\(^{2+}\) concentration presented for I4898T.

**RESULTS**

Expression and Cellular Localization of Ryanodine Receptors—Prior to monitoring [Ca\(^{2+}\)]c the correct sorting of recombinant RyR1 to the ER membrane was verified. RyR1 and the three mutants, R615C, Y523S, and I4898T, cloned in the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA) (15, 18), were transfected into HEK-293 cells and stained 36 h later with a mouse monoclonal antibody (34C) against the ryanodine receptor. Observation in the epifluorescence microscope showed no difference in transfection efficiency between wild-type and mutant forms of RyR1: the percentage of transfected cells in all cases was between 20 and 30%. Fig. 1, panel A, shows that the antibody yielded a strong signal with a delicate reticular distribution throughout the cytoplasm (a typical staining pattern for ER proteins), showing that the transfected RyR1 had been targeted correctly. The fluorescence intensity was similar for all four forms of RyR1, suggesting that the level of expression of each protein was comparable. No signal was obtained in untransfected HEK-293 cells, as reported previously (15, 18) (data not shown).

The receptors were also expressed transiently in primary cultures of rat skeletal muscle cells. Fig. 1, panel B, shows that the immunostaining yielded a typical punctate SR/ER pattern in these cells as well. No obvious differences in the fluorescence signals were observed in the four different samples, supporting the conclusion that expression levels were similar. Since it proved extremely difficult to estimate the level of expression of exogenous RyR1, the percentage of positively transfected cells was evaluated from the level of aequorin co-expression. Immunocytochemistry analysis using a commercial monoclonal antibody against the HA1 epitope tag added to aequorin revealed that the percentage of positive cells was about 20%. Unfortunately, Western blotting analysis indicated approximately equivalent intensities of RyR1 bands (not shown), in control and transfected myotubes. To quantify the level of expression of exogenous RyR1 receptors in myotubes we also performed an analysis of the immunofluorescent signals on control and transfected myotubes. The average fluorescence intensity per cell area was quantified with respect to control cells (not transfected, or transfected with the empty plasmid (mock)) and is reported in Fig. 1B, panel F. The analysis was carried out as described under “Experimental Procedures”: it indicated equivalent intensities in control and transfected myotubes. Evidently, the increase of recombinant RyR1 expression over the endogenous level was below the level that would have made it detectable by Western blotting analysis and by quantification of the average fluorescent intensity per cell area.

Endoplasmic Reticulum Ca\(^{2+}\) in Cells Overexpressing RyR1 and RyR1 Mutants—We used two different aequorin probes to measure ER/SR Ca\(^{2+}\) content in HEK-293 cells and in rat primary cultures of skeletal myotubes. Resting ER Ca\(^{2+}\) concentration, [Ca\(^{2+}\)]ER, was measured directly using a low affinity aequorin targeted to the ER (eraEQ, Ref. 23), but in the case of muscle cells, a specially targeted aequorin/calsequestrin chimera (srAEQ, Ref. 24) was used to monitor Ca\(^{2+}\) in the lumen of the terminal cisternae of the SR, [Ca\(^{2+}\)]L, where calsequestrin and the ryanodine receptor are located. Cells were co-transfected with eraEQ/srAEQ and RyR1 cDNAs. The efficiency of transfection of skeletal muscle myotubes increased if the transfection was carried out before induction of myoblast fusion (24). In all experiments, the measurements of [Ca\(^{2+}\)]c were performed after the application of the Ca\(^{2+}\) depletion protocols described under “Experimental Procedures.” Functional aequorin was reconstituted with a modified prosthetic group, coelenterazine n, that decreased the Ca\(^{2+}\) affinity of the photoprotein, thus permitting measurement of the high concentration of Ca\(^{2+}\) in the ER/SR lumen (28, 29). After aequorin reconstitution in a Ca\(^{2+}\)-free medium, cells were transferred to the luminometer chamber and refilling of the ER/SR Ca\(^{2+}\) stores was initiated by supplementing the perfusion medium with a concentration of Ca\(^{2+}\) approximating that of the extracellular space (1 \(\mu\)M). About 1 min after Ca\(^{2+}\) addition, [Ca\(^{2+}\)]ER/SR reached a steady state level that is assumed to correspond to that of the ER/SR lumens and that is quantified in Fig. 2. The ER Ca\(^{2+}\) content in HEK-293 cells was not reduced by expression of wt RyR1 and may be reduced but slightly by the expression of the R615C mutant. However, the ER Ca\(^{2+}\) content was reduced by about 20–25% by the expression of the Y523S and I4898T CCD mutants (Fig. 2, panel C). The mean values differed significantly for the CCD RyR1 mutants with respect to control cells and to cells transfected with the wt RyR1 protein.

Experiments were then performed to determine whether the RyR1 mutant proteins caused changes in the size of the Ca\(^{2+}\) store in the more native environment of muscle cells. The measurements revealed significant differences in the level of Ca\(^{2+}\) monitored with the two different aequorin probes. In agreement with the results of previous work (29) the level indicated by the eraEQ probe in control cells, which monitored the combined space of the longitudinal ER/SR and of the terminal cisternae, was about 200 \(\mu\)M (Fig. 2, panel B). It did not differ significantly in control myotubes transfected only with eraEQ, in myotubes overexpressing wt RyR1 or in myotubes overexpressing the RyR1 mutants. Monitoring [Ca\(^{2+}\)]c in the terminal cisternae of SR with srAEQ revealed a significantly higher value (about 350 \(\mu\)M) (Fig. 2, panel A) and in control myotubes (as already reported) and in myotubes expressing wt RyR1. The overexpression of the three RyR1 mutants decreased [Ca\(^{2+}\)]c substantially, i.e. by about 15% in myotubes overexpressing the MH mutant and by 25–30% in those overexpressing the CCD mutants.

Cytosolic and Mitochondrial Ca\(^{2+}\) in HEK-293 Cells Overexpressing RyR1—The analyses described above measured the effects of overexpressing RyR1 proteins on resting ER/SR Ca\(^{2+}\) levels and were carried out under conditions which would not activate Ca\(^{2+}\) release channels. It was, therefore, of interest to measure cytosolic Ca\(^{2+}\) concentrations under the same conditions and, furthermore, under conditions where Ca\(^{2+}\) release was stimulated by caffeine and histamine. Caffeine acts directly on the ryanodine receptor, whereas histamine increases
cytosolic Ca$^{2+}$ by activating a signaling pathway that elevates intracellular InsP$_3$ thus activating the InsP$_3$ receptor.

To measure resting cytosolic Ca$^{2+}$, HEK-293 cells transfected transiently with the CCD RyR1 mutants were loaded with Fura-2 and analyzed in Ca$^{2+}$ imaging experiments. Fig. 3 shows that the low resting cytosolic Ca$^{2+}$ concentration in cells transfected with vector only was increased by the expression of wt RyR1. This may be caused by an increase of the number of Ca$^{2+}$

**FIG. 1.** Immunolocalization of wt RyR1, and the R615C, Y523S, and I4898T mutants, in transiently transfected HEK-293 cells. Panel A, bars = 40 μm. Immunolocalization of RyR1 in control myotubes (panel A), in myotubes transiently expressing wt RyR1 (panel B), or the three mutants R615C (panel C), Y523S (panel D), and I4898T (panel E). Panel B, bars = 15 μm. Immunocytochemistry was carried out using a commercial monoclonal antibody, which recognizes mainly the skeletal muscle isoform of the ryanodine receptor (RyR1). Staining was revealed by a fluorescein isothiocyanate-conjugated antibody. Panel F, average fluorescence intensities per cell areas (n) calculated on parallel batches of transfected myotubes and reported as arbitrary units. Bars indicates S.D. Staining was revealed by the Alexa Fluor 488-conjugated antibody and by exposing the cells to a fixed a excitation time.
release channels within the ER induced by the overexpression. Resting [Ca\textsuperscript{2+}] levels following expression of the I4898T CCD mutant were similar to those measured in cells transfected with wt RyR1, but resting [Ca\textsuperscript{2+}] values following expression of the Y523S CCD mutant were increased significantly over the levels seen following transfection with pcDNA only or with wt RyR1 or the I4898T mutant. The R615C MH mutant was shown previously not to induce significant changes in resting Ca\textsuperscript{2+} values in HEK-293 cells, when compared with either non-transfected cells or cells transfected with wt RyR1 (14).

To measure cytosolic [Ca\textsuperscript{2+}] transients HEK-293 cells were transfected with cytosolic aequorin (cytAEQ, Ref. 27) or co-transfected with cytAEQ and wt RyR1 or mutant RyR1 cDNAs. After aequorin reconstitution, cells were incubated in a modified KRB medium supplemented with 1 mM CaCl\textsubscript{2} and challenged after about 1 min with 20 mM caffeine. They were then washed for 2 min with KRB supplemented with 1 mM CaCl\textsubscript{2} and challenged with 100 \textmu M histamine. Fig. 4 (panel A) shows that cells transfected only with cytAEQ failed to respond to caffeine, as expected from the absence of endogenous ryanodine receptors. However, caffeine elicited a response in cells transfected with RyR1 cDNAs (panels B, C, and D). The average values reported in Table I show that the response was the same in cells transfected with wt RyR1 and with CCD mutant Y523S, was augmented in those cells transfected with the MH mutant R615C, and was absent in cells transfected with the CCD mutant I4898T.

Fig. 4 also shows that histamine-induced Ca\textsuperscript{2+} transients (for average values see Table I), did not differ significantly between wt RyR1 and the RyR1 mutants. The reduction in Ca\textsuperscript{2+} transients in cells transfected with RyR1 proteins were likely to be caused by the incomplete replenishment of the Ca\textsuperscript{2+} stores after they had been emptied by caffeine stimulation. Experiments in which histamine was applied without previous stimulation with caffeine also showed that the Ca\textsuperscript{2+} transients in control cells and in cells expressing the RyR1 proteins were identical (data not shown).

The experiments in Fig. 4 show that the transients generated in MH and CCD mutants (panels B and C) returned to the
basal level faster than those generated in cells transfected with wt RyR1. This could have been due to compensatory mechanisms, such as the induced expression of SERCA or PMCA-type Ca\textsuperscript{2+} pumps, as observed by Tong et al. (14), or to the existence of regions of local depletion within the Ca\textsuperscript{2+} stores. Estimates of the area under the curves covering the caffeine-induced transients indicated that the total amount of released Ca\textsuperscript{2+} was the same in RyR1- and R615C-transfected cells, (64.63 ± 6.34, n = 12 and 66.62 ± 7.48, n = 12, respectively), but the dynamic of the release was clearly different. Presumably, the increase in peak amplitude in R615C transfected cells compensates for the faster return to basal level. In the case of cells expressing the Y523S RyR1 mutant, the total amount of Ca\textsuperscript{2+} released was reduced (area, 47.37 ± 7.51, n = 9), in line with the suggestion that this mutant greatly increased the leakiness of the store and in line with the observed increase in cytosolic resting Ca\textsuperscript{2+} (Fig. 3). Estimates of the area under the curves covering the histamine-induced transients indicated that Ca\textsuperscript{2+} leak may also occur through the R615C RyR1 mutant. In fact the area was significantly reduced in the cells expressing this mutant (58.18 ± 8.87, n = 12 versus 68.28 ± 8.66, n = 14 in RyR1-transfected cells).

Next, experiments were performed to explore whether possible differences in the Ca\textsuperscript{2+} releasing properties of the RyR1 mutants could be amplified by specific monitoring of [Ca\textsuperscript{2+}] at the mouth of the Ca\textsuperscript{2+} release channels (transfected RyR1 or endogenous InsP\textsubscript{3}R channels). Previous work has shown that activated Ca\textsuperscript{2+} release from the endoplasmic reticulum generates microdomains of high [Ca\textsuperscript{2+}]\textsubscript{c}, which are sensed and accumulated by vicinal mitochondria (30, 31). Thus, to monitor Ca\textsuperscript{2+} closer to the mouth of the release channels, mitochondrial targeted aequorin was used. In the experiments presented in Fig. 5, HEK-293 cells were transfected with mitochondrial aequorin (mtAEQ, 32) or co-transfected with mtAEQ and RyR1 cDNAs. As expected, the stimulation with 20 mM caffeine induced a transient increase of [Ca\textsuperscript{2+}]\textsubscript{c} in cells transfected with wt RyR1 (peak value about 3.5 \mu M, see Table II). As expected, HEK-293 cells transfected only with mtAEQ exhibited no increase in [Ca\textsuperscript{2+}]\textsubscript{c} when caffeine was applied. In cells expressing the R615C MH mutant, the height of the mitochondrial peak was about 5.6 \mu M, which was significantly higher than in cells transfected with wt RyR1. By contrast, the peak value in cells expressing the Y523S CCD mutant was about 2.8 \mu M, which was significantly lower than that observed in wt RyR1-transfected cells. As expected from the lack of caffeine response previously observed, the I4898T CCD mutant showed no increase in [Ca\textsuperscript{2+}]\textsubscript{c}. Results obtained with mitochondria thus matched those obtained with [Ca\textsuperscript{2+}]\textsubscript{c}.

Fig. 5 also shows the mitochondrial Ca\textsuperscript{2+} uptake triggered by stimulation with histamine. After stimulation with caffeine,
and washing with KRB medium supplemented with 1 mM CaCl$_2$ to permit refilling of the ER, cells were challenged with histamine to induce Ca$^{2+}$ release. The peak value of [Ca$^{2+}$]$_{m}$ was about 5 μM in cells transfected only with mtAEQ, and did not differ significantly in cells expressing wt RyR1 or the I4898T CCD mutant. In cells overexpressing the R615C and Y523S mutants, the height of the mitochondrial peak was significantly lower (about 4 μM for R615C and 3 μM for Y523S) than in cells overexpressing wt RyR1. These data are consistent with localized ER Ca$^{2+}$ leakiness. While the augmented release of Ca$^{2+}$ through the R615C MH mutant of RyR1 indicated increased susceptibility to Ca$^{2+}$ release, the reduced mitochondrial [Ca$^{2+}$] transient following InsP$_3$ channel opening indicated that the ER Ca$^{2+}$ content was reduced, at least locally. This would agree with the measurements of ER/SR [Ca$^{2+}$] in HEK-293 and skeletal muscle cells.

**Cytoplasmic and Mitochondrial Ca$^{2+}$ in Primary Cultures of Skeletal Muscle Myotubes**—Primary cultures of skeletal muscle myotubes were transfected with cytAEQ/mtAEQ or co-transfected with cytAEQ/mtAEQ and RyR1 cDNAs. After aequorin reconstitution and incubation in the modified KRB medium containing 1 mM CaCl$_2$, [Ca$^{2+}$]$_{c}$ and [Ca$^{2+}$]$_{m}$ were monitored after plasma membrane depolarization with high K$^+$, following the addition of caffeine, or after stimulation of the acetylcholine nicotinic receptor. To this aim the perfusion medium was replaced rapidly with a KRB medium containing either iso-osmotic KCl instead of NaCl, 20 mM caffeine, or 500 μM carbachol in the presence of 10 μM atropine. Between stimuli, cells were washed with the KRB medium containing 1 mM CaCl$_2$. Large rises in [Ca$^{2+}$]$_{c}$ and [Ca$^{2+}$]$_{m}$ were observed in control myotubes subjected to the three treatments. The peak values are summarized in Tables III and IV, which also show the peak values of the [Ca$^{2+}$]$_{c}$ and [Ca$^{2+}$]$_{m}$ transients in myotubes overexpressing the wt RyR1 and the RyR1 mutants.

The amplitude of the cytosolic and mitochondrial Ca$^{2+}$ transients induced by high KCl, by caffeine, or by carbachol were not affected by expression of the RyR1 mutants. This may reflect the formation of heterotetrameric channels composed of wt and mutant subunits, which would also explain why differences were observed in HEK-293 cells, but not in muscle cells. In myotubes overexpressing wt RyR1, the heights of the [Ca$^{2+}$]$_{c}$ and [Ca$^{2+}$]$_{m}$ peaks induced by caffeine were significantly higher than in control cells (1.43 ± 0.63 μM, n = 6 versus 0.81 ± 0.35 μM, n = 7 for [Ca$^{2+}$]$_{c}$, and 7.53 ± 1.87 μM, n = 8 versus 3.40 ± 0.60 μM, n = 6 for [Ca$^{2+}$]$_{m}$). The finding could be rationalized by suggesting that the number of native channels expressed by transfected cells was in-
TABLE III
Cytosolic [Ca\(^{2+}\)] in primary cultures of myotubes transfected with the different forms of RyR

|            | Caffeine [Ca\(^{2+}\)] | Carbachol [Ca\(^{2+}\)] |
|------------|------------------------|-------------------------|
| KCl [Ca\(^{2+}\) ] | μM                     | μM                      | μM                     |
| Control    | 1.62 ± 0.27 (n = 8)    | 0.81 ± 0.35 (n = 7)     | 2.54 ± 0.30 (n = 4)    |
| wt RyR1    | 1.68 ± 0.33 (n = 7)    | 1.43 ± 0.62 (n = 6)     | 2.47 ± 0.48 (n = 5)    |
| R615C      | 1.77 ± 0.27 (n = 9)    | 0.95 ± 0.29 (n = 9)     | 2.85 ± 0.28 (n = 6)    |
| Y523S      | 1.67 ± 0.25 (n = 9)    | 0.88 ± 0.32 (n = 9)     | 2.80 ± 0.69 (n = 6)    |
| I4898T     | 1.66 ± 0.20 (n = 9)    | 0.93 ± 0.23 (n = 9)     | 2.46 ± 0.38 (n = 3)    |

a Significant differences at p < 0.05.

TABLE IV
Mitochondrial [Ca\(^{2+}\)] in primary cultures of skeletal muscle myotubes transfected with the different forms of RyR

|            | Caffeine [Ca\(^{2+}\)] | Carbachol [Ca\(^{2+}\)] |
|------------|------------------------|-------------------------|
| KCl [Ca\(^{2+}\) ] | μM                     | μM                      | μM                     |
| Control    | 7.66 ± 1.82 (n = 9)    | 3.35 ± 0.92 (n = 9)     | 7.89 ± 1.04 (n = 7)    |
| wt RyR1    | 8.53 ± 1.88 (n = 11)   | 6.90 ± 1.81 (n = 13)    | 9.41 ± 2.52 (n = 12)   |
| R615C      | 9.33 ± 2.29 (n = 9)    | 3.79 ± 1.50 (n = 10)    | 11.33 ± 0.92 (n = 6)   |
| Y523S      | 8.95 ± 2.52 (n = 11)   | 3.38 ± 0.65 (n = 11)    | 10.09 ± 1.96 (n = 9)   |
| I4898T     | 7.11 ± 1.64 (n = 12)   | 4.04 ± 1.24 (n = 10)    | 9.00 ± 2.44 (n = 12)   |

a Significant differences at p < 0.005.

increased, contributing to augmented Ca\(^{2+}\) release from the stores. This effect was particularly evident when the Ca\(^{2+}\) transients were produced exclusively by the opening of RyR1 channels following caffeine stimulation.

DISCUSSION

The aim of this study was to evaluate the impact of RyR1 mutations that produce MH and CCD in humans on cellular Ca\(^{2+}\) homeostasis. Earlier work in which wt and mutant forms of RyR1 were expressed in heterologous and homologous cells focused on measurements of caffeine and halothane sensitivity, on the amplitude of Ca\(^{2+}\) release from the SR, on resting Ca\(^{2+}\) levels and on retrograde and orthograde interactions between mutant forms of RyR1 and DHPR (8, 14–21, 33, 34). The conclusion that has emerged from these studies is that MH and some CCD mutations are more sensitive to caffeine and halothane than wt RyR1. Because caffeine enhances the sensitivity of RyR1 to the most important activating ligand, Ca\(^{2+}\) itself, it is not surprising that there should also be consensus that MH and some CCD RyR1 mutants are more leaky than wt RyR1 channels, since they are activated at Ca\(^{2+}\) levels nearer to basal. There is also a general consensus that many CCD mutants represent a more severe disruption of the Ca\(^{2+}\) release channel than MH mutations and that leaky RyR1 channels, resulting in elevated cytosolic Ca\(^{2+}\), are responsible for the structural defects defined as “central cores” in skeletal muscle fibers from CCD mutants (2, 13). There is, however, a second class of CCD mutations that do not appear to induce RyR1 leakiness to Ca\(^{2+}\). These mutant channels have a much lower affinity for Ca\(^{2+}\) (35) and, accordingly, are resistant to activation by physiological levels of Ca\(^{2+}\), caffeine or to the interaction with the DHPR. Their ability to block excitation-contraction coupling has led to their designation as EC-uncoupling (21).

In published studies, the degree of RyR1 leakiness, and the size of the ER Ca\(^{2+}\) store have been evaluated indirectly by measuring resting cytosolic Ca\(^{2+}\) and the amplitude of Ca\(^{2+}\) release by physiological stimuli or following inhibition of the SERCA pump. These approaches have intrinsic limitations: when measuring Ca\(^{2+}\) in the bulk cytosol, transient and/or localized differences in resting cytosolic Ca\(^{2+}\) may be obscured by an increased activity of Ca\(^{2+}\) pumps and exchangers that may hasten Ca\(^{2+}\) removal. When monitoring Ca\(^{2+}\) release from Ca\(^{2+}\) stores, stable, non-mobilized Ca\(^{2+}\) pools, and possible microheterogeneity in the Ca\(^{2+}\) content of the stores may be missed. Until now, no clear demonstration that defects in RyR1 channels cause a reduction in the Ca\(^{2+}\) content of the stores has been provided.

In the present study, ER/SR Ca\(^{2+}\) concentrations were measured directly in HEK-293 cells and in primary cultures of rat skeletal muscle myotubes expressing wild-type and RyR1 mutants. The work on HEK-293 cells has revealed that the two CCD mutants induced a 20–25% reduction in the ER Ca\(^{2+}\) content, in line with the suggestion that both mutations generate constitutively leaky channels. By contrast, no differences were observed in the resting ER Ca\(^{2+}\) content in HEK-293 cells expressing the MH mutant, R615C. The experiments in muscle cells, in which recombinant, heterotetrameric RyR1 channels could form, produced results that underline the complexity of Ca\(^{2+}\) dysregulation in these disorders. The aequorin chimera, srAEQ, confined to the terminal cisternae, revealed a reduction in the size of the Ca\(^{2+}\) store in myotubes transfected with RyR1 mutants. By contrast, the erAEQ chimera, which also reflected the Ca\(^{2+}\) content in the longitudinal SR showed that global ER/SR Ca\(^{2+}\) was not reduced by the expression of the three RyR1 mutants. Thus, Ca\(^{2+}\)-dysregulation could be restricted to specific areas of the Ca\(^{2+}\) store.

This may suggest that localized differences in Ca\(^{2+}\) handling of the ER/SR are sufficient for the generation of the pathological phenotype and that the severity of the disease could be related to the degree of Ca\(^{2+}\) store depletion and/or to the depletion of a specialized portion of the Ca\(^{2+}\) store. It is of interest to note that the degree of reduction was more pronounced in the two CCD mutants (25–30%) than in the MH mutant (15%). The results of expression of the R615C mutant were different in HEK-293 cells and in myotubes. This could be related to the expression of homotetrameric mutated channels in HEK-293 cells versus the heterotetrameric channels which could form in myotubes. It is also important that Ca\(^{2+}\) leakiness in the native muscle environment is confined to junctional stores. The lower histamine-induced mitochondrial [Ca\(^{2+}\)] transient produced by the R615C mutant with respect to control and wt RyR1-trans-
fected cells, might have been related to the reduction in ER [Ca\textsuperscript{2+}] in a specialized/restricted ER region.

Our results with the I4898T mutant deserve special mention. Initial studies on this mutant (15) showed that the homotetrameric channel expressed in HEK-293 cells did not mediate caffeine-induced Ca\textsuperscript{2+} release, suggesting that it was no longer activated by caffeine. Moreover, the homotetrameric channel failed to bind \textsuperscript{3}H]ryanodine, indicating a low probability of opening. In HEK-293 cells expressing the heterotetrameric channel (i.e. cells co-transfected with wild-type and the I4898T RyR1 mutant) plus the SERCA1 pump, the amplitudes of caffeine- and thapsigargin-induced Ca\textsuperscript{2+} release were reduced. Caffeine sensitivity was not altered, but resting Ca\textsuperscript{2+} levels were increased. Accordingly, it was suggested that the I4898T mutant behaved like a leaky channels (15). Our current experiments have confirmed the suggestion, since a reduction of the ER Ca\textsuperscript{2+} content was found in HEK-293 cells and in the terminal cisternae of skeletal muscle myotubes (see Fig. 2).

In subsequent studies by Avila et al. (21), excitation-contraction coupling was reconstituted in RyR1-null (dyspedic) mouse myoblasts by expressing wild-type and mutated forms of RyR1. Under these conditions, the I4898T mutant was unable to reconstitute EC-coupling or to raise resting Ca\textsuperscript{2+} levels. The I4898T and other mutants in the same group were thus called EC-uncoupled mutants. Peculiarities in the activation of the I4898T mutant channel were also underlined by studies of single channel function (9–12).

The ability of the heterotetrameric mutant found in CCD patients to elevate resting Ca\textsuperscript{2+} levels remains an open question, since no direct measurements of resting cytosolic Ca\textsuperscript{2+} concentrations have been carried out in the muscles of CCD patients. Nevertheless, the development of a central core in these muscles is completely consistent with elevated levels of Ca\textsuperscript{2+} within the core region (2, 13). The results reported here suggest that the I4898T and, presumably other EC-uncoupled mutants are, indeed, leaky. The homotetrameric I4898T mutant channels were insensitive to caffeine, showing that the mutation had altered channel function. Nevertheless, the expression of the mutant protein reduced the ER Ca\textsuperscript{2+} content of HEK-293 cells, and the SR Ca\textsuperscript{2+} content of skeletal myotubes.

These results suggest that the mutant channels have abnormal permeability to Ca\textsuperscript{2+} under resting conditions, but this reduction was not reflected in the increase of resting cytosolic Ca\textsuperscript{2+}, as I4898T RyR1 channels produced cytosolic Ca\textsuperscript{2+} values which were similar to those observed for wt RyR1 channels (Fig. 3). However, when the I4898T RyR1 mutant was expressed in the heterotetrameric form together with wt RyR1 and SERCA1a (to compensate for the possible reduction in the ER Ca\textsuperscript{2+} store), it produced an enhanced resting Ca\textsuperscript{2+} level in comparison to cells transfected with wt RyR1 and SERCA1a (15). Recent reports have demonstrated that the I4898T mutant RyR1 channel can be opened by a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release mechanism, but with very low sensitivity to Ca\textsuperscript{2+} (35). If localized Ca\textsuperscript{2+} elevation were to occur in the proximity of mutated RyR1 channels by an unknown mechanism in living CCD muscle cells, the open probability of the channels would be increased leading to partial depletion of the ER Ca\textsuperscript{2+} stores. Taken together, these observations show that the I4898T mutant is not simply a closed channel, but its properties are not yet fully understood.

The effects described could be synergistic, rationalizing the severity of CCD in patients carrying the I4898T mutation. The chronic depletion of ER/SR Ca\textsuperscript{2+} stores could have deleterious consequences on the contraction mechanism of muscle cells. In addition, the insufficient voltage gating of Ca\textsuperscript{2+} release observed in myotubes co-expressing wt RyR1 and the I4898T RyR1 mutant (21) would impair Ca\textsuperscript{2+} release and could be responsible for the reduction of muscle strength.

The direction of the derangement of cytosolic and mitochondrial Ca\textsuperscript{2+} transients was opposite between the MH and CCD mutations, and could account for the specific cellular phenotype of the two disorders. Transfection of the R615C RyR1 mutant into HEK-293 cells augmented the amplitude of the cytosolic and mitochondrial Ca\textsuperscript{2+} transients following cell stimulation. By contrast, the mitochondrial Ca\textsuperscript{2+} transients were reduced in cells expressing the Y523S CCD mutation, reflecting a pronounced reduction in the Ca\textsuperscript{2+} content of intracellular stores. The increase in the amplitude of the [Ca\textsuperscript{2+}]\textsubscript{em} transient in the R615C mutant would have the effect of increasing the mitochondrial metabolic rate, including ATP production, while the increase in the [Ca\textsuperscript{2+}] transient would be accounted for the contracture typical of MH patients. Similarly, the reduction in the [Ca\textsuperscript{2+}]\textsubscript{em} transient in cells transfected with the Y523S mutant could result in ATP depletion and thus in damage to the contractile apparatus, explaining the hypotonia, the proximal muscle weakness and the eventual degeneration of the fiber core. Possibly, chronic defects in Ca\textsuperscript{2+} homeostasis (protracted store depletion) would cause CCD, whereas acute defects (enhanced Ca\textsuperscript{2+} release following RyR1 channel opening) would induce the MH disorders.

Surprisingly, the results in primary cultures of skeletal muscles showed no differences in mitochondrial and cytosolic [Ca\textsuperscript{2+}] transients upon caffeine stimulation between cells transfected with the mutant RyR1 and recombinant aequorin probes, or with recombinant aequorin probes only. Myotubes transfected with wt RyR1 displayed an increase in cytosolic and mitochondrial [Ca\textsuperscript{2+}] transients following caffeine stimulation, but not following a depolarizing stimulus induced by KCl or nicotinic receptor stimulation. The combination of endogenous and wild-type-expressed RyR1 channels would increase the number of functional channels in the ER/SR membrane, perhaps accounting for the augmented Ca\textsuperscript{2+} release induced by channel activators. The fact that the RyR1 mutations had no effect on the size of the ER/SR Ca\textsuperscript{2+} store may have been caused by the formation of heterotetrameric channels that would obscure the differences observed in HEK-293 cells, or to the fact that localized Ca\textsuperscript{2+} depletion in terminal cisternae could evoke only localized Ca\textsuperscript{2+} handling alterations which may have gone underdetected, but still be sufficient to generate the pathological phenotype.

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