THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 15, Issue of April 15, pp. 15380–15389, 2005
Printed in U.S.A.

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

Received for publication, September 10, 2004, and in revised form, February 2, 2005
Published, JBC Papers in Press, February 2, 2005, DOI 10.1074/jbc.M410421200

Marisa Brini‡§¶, Sabrina Manni‡**, Nicola Pierobon‡, Guo Guang Du‖, Parveen Sharma‖, David H. MacLennan‖, and Ernesto Carafoli‡****

*From the ‡Department of Biochemistry and Center for the Study of Biomembranes of the National Research Council (CNR), University of Padova, Viale G. Colombo 3, 35121 Padova, Italy and the **Venetian Institute of Molecular Medicine (VIMM), Via Orus 2, 35129 Padova, Italy, the §Department of Experimental Veterinary Sciences, Viale dell'Università 16, Agripolis, Legnaro Padova, Italy, and the ‖Banting and Best Department of Medical Research, Charles H. Best Institute, University of Toronto, Toronto, Ontario M5G 1L6, Canada*

Malignant hyperthermia (MH) and central core disease (CCD) are caused by mutations in the *RYR1* gene encoding the skeletal muscle isoform of the ryanodine receptor (RyR1), a homotetrameric Ca^{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^{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^{2+} homeostasis different from those associated with CCD. Measurements of luminal ER/SR Ca^{2+} revealed that the mutations generated leaky channels in all cases, but the leak was particularly pronounced in CCD mutants. Cytosolic and mitochondrial Ca^{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^{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^{2+} (1). The rapid release of Ca^{2+} from the sarcoplasmic reticulum (SR)[1] through the ryanodine receptors induces the contraction of myofibrils; Ca^{2+} re-uptake into the SR lumen by the Ca^{2+} ATPase (SERCA pump) induces relaxation. The correct functioning of the contraction/relaxation cycle demands a precise balance between Ca^{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 *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^{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

* This work was supported by the Telethon Foundation of Italy (Grant GP0193Y01, to E. C.), by contributions from the Italian Ministry of University and Scientific Research (PRIN 2001, 2002, 2003; FIRB 2001; to M. B. and E. C.), by the National Research Council of Italy (Target Project on Biotechnology and Agency 2000), by the Human Frontier Science Program Organization, by Grants MT-3399 and MOP-49493 from the Canadian Institutes of Health Research, by the Neuromuscular Research Partnership (to D. H. M.), and by a postdoctoral fellowship (to P. S.) from the Heart and Stroke Foundation of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Biochemistry, University of Padova, Viale G. Colombo, 3, 35121 Padova Italy. Tel.: 39-049-8276167; Fax: 39-049-8276125; E-mail: brini@mail.bio.unipd.it.

[1] The abbreviations used are: SR, sarcoplasmic reticulum; RyR1, ryanodine receptor; MH, malignant hyperthermia; ER, endoplasmic reticulum; CCD, central core disease; HEK, human embryonic kidney cell; FCS, fetal calf serum; PBS, phosphate-buffered saline; wt, wild-type; EC, 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 mutation I4897T reported in Ref. 15) as an example of an EC-uncoupled channel causing CCD. While the R615C and Y523S mutations are located in the regulatory cytoplasmic N-terminal portion, the I4898T mutation, which causes an unusually severe and highly penetrant form of CCD (15), is located in a proposed pore-forming sequence in the C-terminal transmembrane region and is likely to affect the functional properties of the Ca^{2+} release channel directly. HEK-293 cells lack endogenous RyR (22) and thus permit the study of homozygous recombinant RyR1 mutants. This has facilitated the detection of differences between normal and abnormal channels, which may not have been detectable in native skeletal muscle cells.

HEK-293 cells were transiently co-transfected with wild-type or mutant RyR1 cDNAs and the Ca^{2+}-sensitive photoprotein aequorin targeted to the cytoplasm, or, using appropriate lead sequences, to the mitochondrial matrix or to the lumen of the endoplasmic reticulum (ER). Ca^{2+} concentrations were measured in resting cells and in cells stimulated with histamine, a 1,4,5-trisphosphate ($InsP_3$)- generating agonist, or with caffeine, which induces Ca^{2+} release through the transfected RyR1. The work has shown that RyR1 mutations responsible for MH and CCD cause different alterations in the size of Ca^{2+} stores and in Ca^{2+} transients.

The study was extended to primary cultures of skeletal muscle, where the expression of mutant RyR1 failed to affect Ca^{2+} transients, possibly because of the formation of heterotetrameric, mutated/native forms of RyR1, that could have resulted in the attenuation of the defects. The ER/SR Ca^{2+} concentration in these cells was also monitored with aequorin chimeras, targeted to the entire ER lumen, (erAEQ, Ref. 23) or preferentially to the terminal cisternae of the SR (a chimera of aequorin fused with calsequestrin, srAEQ, Ref. 24). The experiments revealed heterogeneity in luminal Ca^{2+} concentration in the different ER/SR portions. The severity of MH and CCD disorders could be linked to local differences in the size of the Ca^{2+} stores rather than to their general depletion. The resulting local derangements of Ca^{2+} homeostasis may have significant consequences for muscle physiology, but could be undetected in global measurements of cytosolic Ca^{2+}.

On the basis of our data, it is suggested that MH disorders are related mainly to acute Ca^{2+} derangements involving an augmented Ca^{2+} transient generated by MH mutant RyR1 channel opening, while CCD disorders are related to the chronic depletion of intracellular Ca^{2+} stores, with possible elevation of resting cytosolic Ca^{2+}.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK-293 cells were grown in minimum essential medium (MEM, Celbio, Italy), supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and penicillin (60 μg/μl)/streptomycin (120 μg/μl) in 75-cm^2 Falcon flasks. Prior to transfection, 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_2PO_4, 8 mM Na_2HPO_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/μl)/streptomycin (120 μg/μl), and plated 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 2.5×10^5 cells onto 13-mm coverslips, coated with 2% gelatin. The next day the cells were washed with DMEM supplemented with 10% FCS, 2 mM glutamine, penicillin (60 μg/μl)/streptomycin (120 μg/μl). 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.

Immunolocalization and Western Blot Analysis—HEK-293 cells were processed for immunofluorescence 36 h after transfection by fixation in 3.7% formaldehyde in PBS for 20 min, washing three times with PBS, followed by incubation for 10 min in PBS supplemented with 50 mM NH_4Cl. Membranes were permeabilized during a 5-min incubation with 0.1% Triton X-100 in PBS, followed by a 1-h wash with 1% gelatin (type IV, from calf skin) in PBS. The coverslip was then processed for RyR1 staining with a commercial monoclonal antibody, 34C, which recognizes mainly the RyR1 isoform (Affinity Bioreagents, Golden, CO) at a 1:100 dilution in PBS. Staining was then carried out with a fluorescein isothiocyanate-labeled anti-mouse secondary antibody (1:50 dilution in PBS; Dako, Glostrup, Denmark) or with the Alexa Fluor 488-conjugated anti-mouse secondary antibody (1:50 dilution in PBS; Molecular Probes, Eugene, OR). After each incubation, cells were washed four times with PBS. The same protocol was also applied for the immunostaining of RyR1 in cultures of rat skeletal muscle cells, but in this case the cells were fixed for 30 min and permeabilization was carried out using 1% Triton X-100. Fluorescence was analyzed with a Zeiss Axiovert microscope equipped with a 12-bit digital cooled camera (Micromax-1300Y, Princeton Instruments Inc., Trenton, NJ). Images were acquired using Metamorph software (Universal Imaging Corporation, West Chester, PA). To quantify the fluorescence signals the cells were illuminated for 50 ms, and images were acquired, stored, and successively analyzed using Metamorph software. The average fluorescence intensity per cell area was calculated on at least 60 different areas selected on different coverslips containing processed batches in parallel of control and transfected myotubes to reduce the variability. Average fluorescence was expressed as arbitrary fluorescence units. *n* indicates the number of cell areas analyzed. Western blot analysis of RyR constructs expression was carried out as described previously (14).

Aequorin Measurements—The Ca^{2+} content of the ER/SR had to be reduced drastically before the reconstitution of functional erAEQ and srAEQ. To this end, the cells were incubated for 1 h at 4 °C in KRB (125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM HEPES, pH 7.4, 37 °C), supplemented with 5 μM 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 delegated 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 mtAEQ were reconstituted by incubating the cells for 1–3 h with 5 μM coelenterazine wild type in Dulbecco's modified Eagle's medium supplemented with 1% FCS, at 37 °C in a 5% CO_2 atmosphere.

Cells were stimulated by the addition of 100 μM histamine, 20 mM caffeine, or 500 μM carbachol, and 10 μM atropine in KRB. Depolarization stimulus of muscle cells was obtained by replacing NaCl with KRB containing iso-osmotic KCl.

Experiments were terminated by lysing the cells with 100 μM digitonin in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in H_2O), to discharge the remaining aequorin pool. The light signal was measured and calibrated into [Ca^{2+}] values using a computer algorithm based on the Ca^{2+} response curve of wild-type and mutant aequorins, as described (23, 27).

Measurement of Cytosolic Resting Ca^{2+} in HEK-293 Cells Transfected with pcDNA or RyR1 Constructs—HEK-293 cells were loaded with 4 μM 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 EGFP 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.

Statistical Analysis—Data are reported as mean ± S.D. Statistical differences were evaluated by Student's 2-tailed t test for unpaired samples. A p value <0.05 was considered statistically significant.

RESULTS

Expression and Cellular Localization of Ryanodine Receptors—Prior to monitoring $[Ca^{2+}]$ 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 RyR 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. 1*B*, *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, $[Ca^{2+}]_{SR}$, 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 $[Ca^{2+}]$ 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 mM). 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 lumen 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 MH 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 A*). 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 μM (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 of $[Ca^{2+}]$ in the terminal cisternae of SR with srAEQ revealed a significantly higher value (about 350 μM; Fig. 2, *panel C*) in control myotubes (as already reported) and in myotubes expressing wt RyR1. The overexpression of the three RyR1 mutants decreased $[Ca^{2+}]$ 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

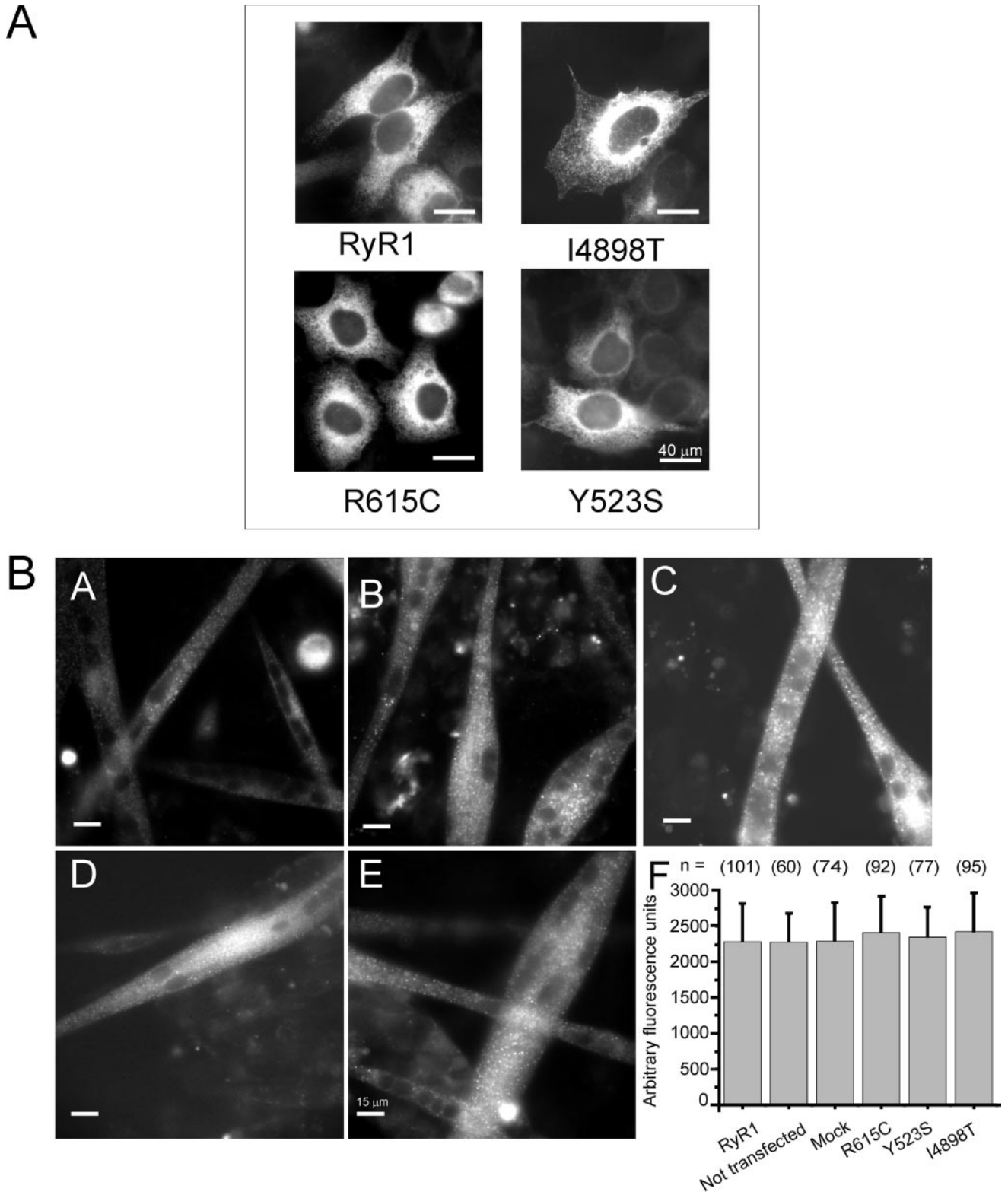

FIG. 1. **Immunolocalization of wt RyR1, and the R615C, Y523S, and I4898T mutants, in transiently transfected HEK-293 cells.** *Panel A*, *bars* = 40 μm. Immunolocalization of RyR1 in control myotubes (*panel A*), in myotubes transiently expressing wt RyR1 (*panel B*), or the three mutants R615C (*panel 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.

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+}

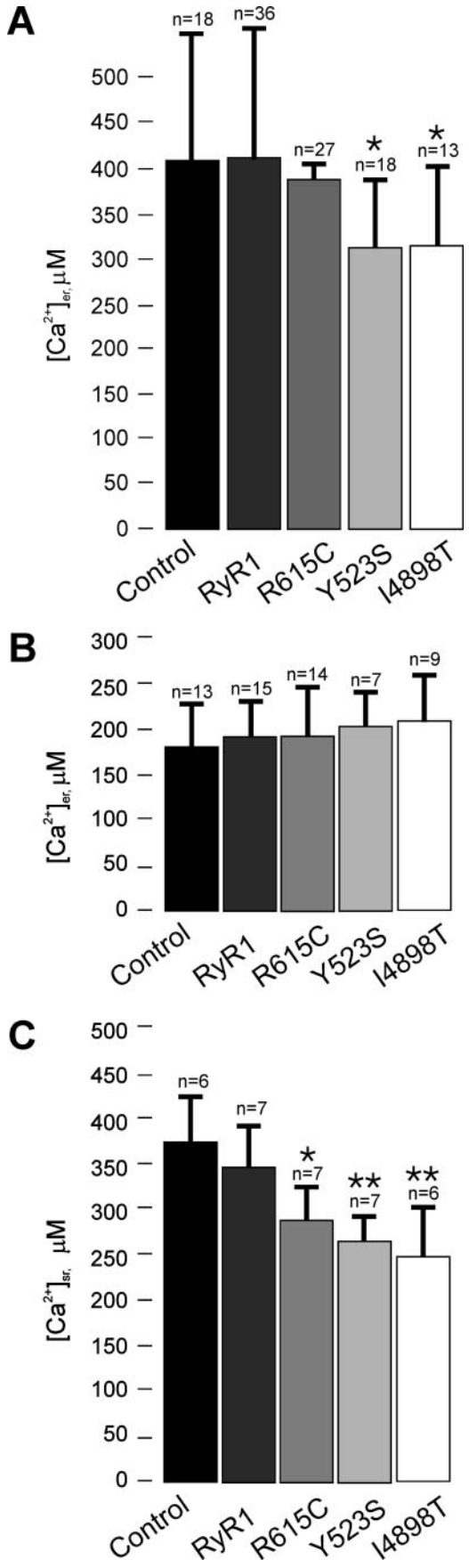

FIG. 2. **ER/SR Ca2+ concentration in HEK-293 cells (*panel A*) and in primary cultures of rat skeletal muscle myotubes (*panels B* and *C*).** Cells expressing the wt RyR1 receptor and the three mutants R615C, Y523S, and I4898T were monitored with recombinant erAEQ or srAEQ. *, $p < 0.05$; **, $p < 0.005$.

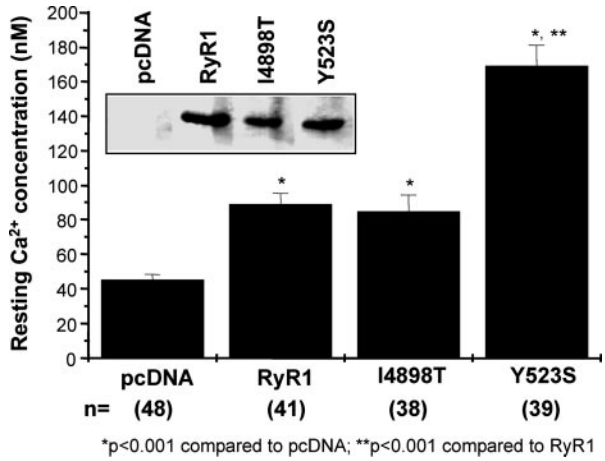

FIG. 3. **A comparison of resting Ca^{2+} in HEK-293 cells transfected with pcDNA, wild-type, and mutant RyR1 constructs.** HEK-293 cells were loaded with 4 μM Fura-2/AM 48 h after transfection, placed on the stage of an inverted microscope, and stimulated with 10 mM caffeine. Cells were then washed to allow them to return to resting Ca^{2+} levels before imaging measurements were carried out (see "Experimental Procedures"). The 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 free Ca^{2+} concentrations. Expression of RyR1 and mutants probed with 34C antibody to RyR1 (*inset*) shows that all three proteins were expressed in comparable amounts.

release channels within the ER induced by the overexpression. Resting $[Ca^{2+}]$ levels following expression of the I4898T CCD mutant were similar to those measured in cells transfected with wt RyR1, but resting $[Ca^{2+}]$ 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^{2+} values in HEK-293 cells, when compared with either non-transfected cells or cells transfected with wt RyR1 (14).

To measure cytosolic $[Ca^{2+}]$ 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 $CaCl_2$ and challenged after about 1 min with 20 mM caffeine. They were then washed for 2 min with KRB supplemented with 1 mM $CaCl_2$ and challenged with 100 μM 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^{2+} transients (for average values see Table I), did not differ significantly between wt RyR1 and the RyR1 mutants. The reduction in Ca^{2+} transients in cells transfected with RyR1 proteins were likely to be caused by the incomplete replenishment of the Ca^{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^{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

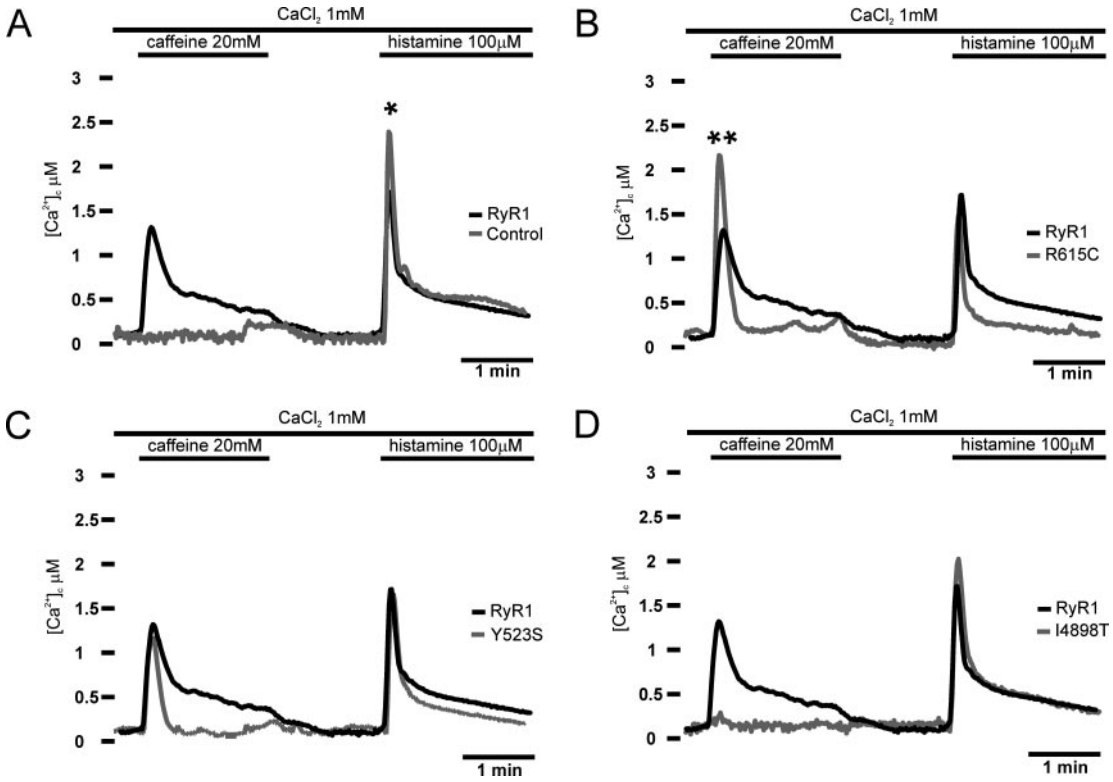

FIG. 4. **Monitoring of the cytosolic Ca^{2+} concentration, $[Ca^{2+}]_c$, with recombinant cytAEQ in HEK-293 cells expressing the wt RyR1 receptor (*panel A*), and the three mutants R615C (*panel B*), Y523S (*panel C*), and I4898T (*panel D*).** Aequorin reconstitution was carried out by incubating the cells with 5 μM coelenterazine, as described under "Experimental Procedures." Where indicated, the cells were challenged with 20 mM caffeine to induce Ca^{2+} release through the transfected RyR1, or with 100 μM histamine, an $InsP_3$ generating agonist. For details see "Experimental Procedures." *, $p < 0.05$; **, $p < 0.005$.

TABLE I
Cytosolic $[Ca^{2+}]$ in HEK-293 cells transfected with the different forms of RyR

The peak values of the $[Ca^{2+}]_c$ transients produced by 20 mM caffeine and 100 μM histamine are expressed as averages and n is the number of experiments. p values were calculated by comparison with the wt RyR1.

	Caffeine $[Ca^{2+}]_c$	Histamine $[Ca^{2+}]_c$
	μM	μM
Control	-	2.16 ± 0.49 (n = 10)[a]
wt RyR1	1.37 ± 0.21 (n = 17)	1.78 ± 0.30 (n = 17)
R615C	2.22 ± 0.42 (n = 17)[b]	1.68 ± 0.26 (n = 17)
Y523S	1.32 ± 0.14 (n = 11)	1.74 ± 0.33 (n = 11)
I4898T	-	1.94 ± 0.21 (n = 13)

[a] Significant differences at $p < 0.05$.
[b] Significant differences at $p < 0.005$.

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^{2+} pumps, as observed by Tong *et al.* (14), or to the existence of regions of local depletion within the Ca^{2+} stores. Estimates of the area under the curves covering the caffeine-induced transients indicated that the total amount of released Ca^{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^{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^{2+} (Fig. 3). Estimates of the area under the curves covering the histamine-induced transients indicated that Ca^{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^{2+} releasing properties of the RyR1 mutants could be amplified by specific monitoring of $[Ca^{2+}]$ at the mouth of the Ca^{2+} release channels (transfected RyR1 or endogenous $InsP_3R$ channels). Previous work has shown that activated Ca^{2+} release from the endoplasmic reticulum generates microdomains of high $[Ca^{2+}]_c$, which are sensed and accumulated by vicinal mitochondria (30, 31). Thus, to monitor Ca^{2+} closer to the mouth of the release channels, mitochondrially 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 $[Ca^{2+}]_m$ in cells transfected with wt RyR1 (peak value about 3.5 μM, see Table II). As expected, HEK-293 cells transfected only with mtAEQ exhibited no increase in $[Ca^{2+}]_m$ when caffeine was applied. In cells expressing the R615C MH mutant, the height of the mitochondrial peak was about 5.6 μM, 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 μM, 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 $[Ca^{2+}]_m$. Results obtained with mitochondria thus matched those obtained with $[Ca^{2+}]_c$.

Fig. 5 also shows the mitochondrial Ca^{2+} uptake triggered by stimulation with histamine. After stimulation with caffeine,

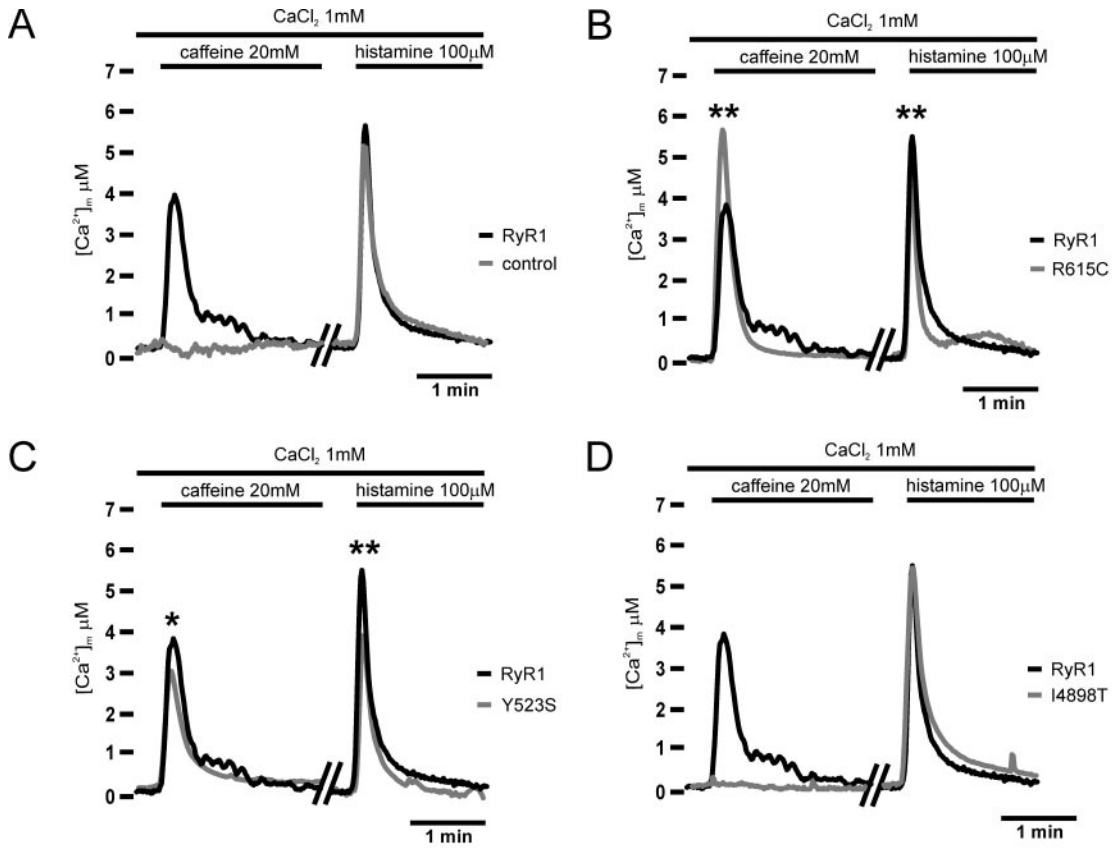

FIG. 5. **Monitoring of the mitochondrial Ca^{2+} concentration, $[Ca^{2+}]_m$, with wild-type recombinant mtAEQ in HEK293 cells expressing the RyR1 receptor (*panel A*), and the three mutants R615C (*panel B*), Y523S (*panel C*), and I4898T(*panel D*).** mtAEQ was reconstituted in the same way as cytAEQ (see legend to Fig. 4 and "Experimental Procedures"). Where indicated, the cells were challenged with 20 mM caffeine, or 100 μM histamine. *, $p < 0.05$; **, $p < 0.005$.

TABLE II
Mitochondrial $[Ca^{2+}]$ in HEK-293 cells transfected with the different forms of RyR

The peak values of $[Ca^{2+}]_m$ produced by 20 mM caffeine and 100 μM histamine. They are expressed as averages and the number of experiments. *p* values were calculated by comparison with the wt RyR.

	Caffeine $[Ca^{2+}]_m$	Histamine $[Ca^{2+}]_m$
	μM	μM
Control	-	5.22 ± 1.11 (n = 5)
wt RyR1	3.47 ± 0.87 (n = 14)	5.96 ± 1.62 (n = 14)
R615C	5.57 ± 0.76 (n = 19)[a]	3.91 ± 1.40 (n = 19)[a]
Y523S	2.76 ± 0.34 (n = 8)[b]	2.91 ± 1.50 (n = 8)[a]
I4898T	-	5.71 ± 0.44 (n = 6)

[a] Significant differences at $p < 0.005$.
[b] Significant differences at $p < 0.05$.

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

The peak values of the $[Ca^{2+}]_c$ transients produced by KCl, 20 mM caffeine and 500 μM carbachol in the presence of 10 μM atropine. They are expressed as averages and n is the number of experiments. p values were calculated by comparison with the wt RyR1.

	KCl $[Ca^{2+}]_c$	Caffeine $[Ca^{2+}]_c$	Carbachol $[Ca^{2+}]_c$
	μM	μM	μM
Control	1.62 ± 0.27 (n = 8)	0.81 ± 0.35 (n = 7)[a]	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)[a]	2.85 ± 0.28 (n = 6)
Y523S	1.67 ± 0.25 (n = 9)	0.88 ± 0.32 (n = 9)[a]	2.80 ± 0.69 (n = 6)
I4898T	1.66 ± 0.20 (n = 9)	0.93 ± 0.23 (n = 9)[a]	2.46 ± 0.38 (n = 6)

[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

The peak values of the $[Ca^{2+}]_c$ transients produced by KCl, 20 mM caffeine and 500 μM carbachol in the presence of 10 μM atropine are expressed as averages and n is the number of experiments. p values were calculated by comparison with the wt RyR1.

	KCl $[Ca^{2+}]_m$	Caffeine $[Ca^{2+}]_m$	Carbachol $[Ca^{2+}]_m$
	μM	μM	μM
Control	7.56 ± 1.82 (n = 9)	3.35 ± 0.92 (n = 9)[a]	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)[a]	11.33 ± 0.92 (n = 6)
Y523S	8.95 ± 2.52 (n = 11)	3.38 ± 0.65 (n = 11)[a]	10.09 ± 1.96 (n = 9)
I4898T	7.11 ± 1.64 (n = 12)	4.04 ± 1.24 (n = 10)[a]	9.00 ± 2.44 (n = 12)

[a] Significant differences at $p < 0.005$.

creased, 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 mutants 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 mutations (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 $[Ca^{2+}]$ 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^{2+} release, suggesting that it was no longer activated by caffeine. Moreover, the homotetrameric channel failed to bind [^{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^{2+} release were reduced. Caffeine sensitivity was not altered, but resting Ca^{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^{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^{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^{2+} levels remains an open question, since no direct measurements of resting cytosolic Ca^{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^{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^{2+} content of HEK-293 cells, and the SR Ca^{2+} content of skeletal myotubes.

These results suggest that the mutant channels have abnormal permeability to Ca^{2+} under resting conditions, but this reduction was not reflected in the increase of resting cytosolic Ca^{2+}, as I4898T RyR1 channels produced cytosolic Ca^{2+} values which were similar to those observed for wt RyR1 channels (Fig. 3). However, when the I4898T RyR1 mutant was expressed in the hetrotetrameric form together with wt RyR1 and SERCA1a (to compensate for the possible reduction in the ER Ca^{2+} store), it produced an enhanced resting Ca^{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^{2+}-induced Ca^{2+} release mechanism, but with very low sensitivity to Ca^{2+} (35). If localized Ca^{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^{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^{2+} stores could have deleterious consequences on the contraction mechanism of muscle cells. In addition, the inefficient voltage gating of Ca^{2+} release observed in myotubes co-expressing wt RyR1 and the I4898T RyR1 mutant (21) would impair Ca^{2+} release and could be responsible for the reduction of muscle strength.

The direction of the derangement of cytosolic and mitochondrial Ca^{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^{2+} transients following cell stimulation. By contrast, the mitochondrial Ca^{2+} transients were reduced in cells expressing the Y523S CCD mutation, reflecting a pronounced reduction in the Ca^{2+} content of intracellular stores. The increase in the amplitude of the $[Ca^{2+}]_m$ transient in the R615C mutant would have the effect of increasing the mitochondrial metabolic rate, including ATP production, while the increase in the $[Ca^{2+}]_c$ transient would be account for the contracture typical of MH patients. Similarly, the reduction in the $[Ca^{2+}]_m$ 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^{2+} homeostasis (protracted store depletion) would cause CCD, whereas acute defects (enhanced Ca^{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 $[Ca^{2+}]$ 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 $[Ca^{2+}]$ 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^{2+} release induced by channel activators. The fact that the RyR1 mutations had no effect on the size of the ER/SR Ca^{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^{2+} depletion in terminal cisternae could evoke only localized Ca^{2+} handling alterations which may have gone underdetected, but still be sufficient to generate the pathological phenotype.

REFERENCES

1. Berchtold, M. W., Brinkmeier, H., and Muntener, M. (2000) *Physiol. Rev.* **80,** 1215–1265
2. Loke, J., and MacLennan, D. H. (1998) *Am. J. Med.* **104,** 470–486
3. MacLennan, D. H., and Phillips, M. S. (1992) *Science* **256,** 789–794
4. MacLennan, D. H., Loke, J., and Odermatt, A. (1999) in *Calcium as a Cellular Regulator* (Carafoli, E., and Klee, C., eds), pp. 610–630, Oxford University Press, Oxford-New York
5. MacLennan, D. H. (2000) *Eur. J. Biochem.* **267,** 5291–5297
6. MacLennan, D. H., and Kranias, E. G. (2003) *Nat. Rev. Mol. Cell. Biol.* **4,** 566–577
7. McCarthy, T. V., Quane, K. A., and Lynch, P. J. (2000) *Hum. Mutat.* **15,** 410–417
8. Dirksen, R. T., and Avila, G. (2002) *Trends Cardiovasc. Med.* **12,** 189–197
9. Balshaw, D., Gao, L., and Meissner, G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3345–3347
10. Gao, L., Balshaw, D., Xu, L., Tripathy, A., Xin, C., and Meissner, G. (2000) *Biophys. J.* **79,** 828–840
11. Zhao, M., Li, P., Li, X., Zhang, L., Winkfein, R. J., and Chen, S. R. (1999) *J. Biol. Chem.* **274,** 25971–25974
12. Du, G. G., Guo, X., Khanna, V. K., and MacLennan, D. H. (2001) *J. Biol. Chem.* **276,** 31760–31771
13. MacLennan, D. H., Philips, M. S., and Zhang, Y. (1996) in *Molecular Biology of Membrane Transport Disorders* (Schultz, S. G., ed), pp. 181–200, Plenum Press, New York
14. Tong, J., McCarthy, T. V., and MacLennan, D. H. (1999) *J. Biol. Chem.* **274,** 693–702
15. Lynch, P. J., Tong, J., Lehane, M., Mallet, A., Giblin, L., Heffron, J. J., Vaughan, P., Zafra, G., MacLennan, D. H., and McCarthy, T. V. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4164–4169

16. Otsu, K., Nishida, K., Kimura, Y., Kuzuya, T., Hori, M., Kamada, T., and Tada, M. (1994) *J. Biol. Chem.* **269,** 9413–9415
17. Treves, S., Larini, F., Menegazzi, P., Steinberg, T. H., Koval, M., Vilsen, B., Andersen, J. P., and Zorzato, F. (1994) *Biochem. J.* **301,** 661–665
18. Tong, J., Oyamada, H., Demaurex, N., Grinstein, S., McCarthy, T. V., and MacLennan, D. H. (1997) *J. Biol. Chem.* **272,** 26332–26339
19. Censier, K., Urwyler, A., Zorzato, F., and Treves, S. (1998) *J. Clin. Investig.* **101,** 1233–1242
20. Avila, G., and Dirksen, R. T. (2001) *J. Gen. Physiol.* **118,** 277–290
21. Avila, G., O'Brien, J. J., and Dirksen, R. T. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4215–4220
22. Tong, J., Du, G. G., Chen, S. R., and MacLennan, D. H. (1999) *Biochem. J.* **343 Pt 1,** 39–44
23. Montero, M., Brini, M., Marsault, R., Alvarez, J., Sitia, R., Pozzan, T., and Rizzuto, R. (1995) *EMBO J.* **14,** 5467–5475
24. Brini, M., De Giorgi, F., Murgia, M., Marsault, R., Massimino, M. L., Cantini, M., Rizzuto, R., and Pozzan, T. (1997) *Mol. Biol. Cell* **8,** 129–143
25. Rizzuto, R., Brini, M., Bastianutto, C., Marsault, R., and Pozzan, T. (1995) *Methods Enzymol.* **260,** 417–428
26. Cantini, M., Massimino, M. L., Catani, C., Rizzuto, R., Brini, M., and Carraro, U. (1994) *In Vitro Cell Dev. Biol. Anim.* **30,** 131–133
27. Brini, M., Marsault, R., Bastianutto, C., Alvarez, J., Pozzan, T., and Rizzuto, R. (1995) *J. Biol. Chem.* **270,** 9896–9903
28. Barrero, M. J., Montero, M., and Alvarez, J. (1997) *J. Biol. Chem.* **272,** 27694–27699
29. Robert, V., De Giorgi, F., Massimino, M. L., Cantini, M., and Pozzan, T. (1998) *J. Biol. Chem.* **273,** 30372–30378
30. Rizzuto, R., Brini, M., Murgia, M., and Pozzan, T. (1993) *Science* **262,** 744–747
31. Rizzuto, R., Pinton, P., Carrigton, W., Fay, F. S., Fogarty, Ke., Lifshitz, L. M., Tuft, R. A., Pozzan, T. (1998) *Science* **280,** 1763–1766
32. Rizzuto, R., Simpson, A. W. M., Brini, M., and Pozzan, T. (1992) *Nature* **358,** 325–328
33. Avila, G., O'Connell, K. M., Groom, L. A., and Dirksen, R. T. (2001) *J. Biol. Chem.* **276,** 17732–17738
34. Dirksen, R. T. (2002) *Front Biosci.* **7,** d659–670
35. Du, G. G., Khanna, V. K., Guo, X., and MacLennan, D. H. (2004) *Biochem. J.* **382,** 557–564

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

Marisa Brini, Sabrina Manni, Nicola Pierobon, Guo Guang Du, Parveen Sharma, David H. MacLennan and Ernesto Carafoli

J. Biol. Chem. 2005, 280:15380-15389.
doi: 10.1074/jbc.M410421200 originally published online February 2, 2005
