Intracellular Calcium Homeostasis in Human Primary Muscle Cells from Malignant Hyperthermia-susceptible and Normal Individuals

Effect of Overexpression of Recombinant Wild-type and Arg163Cys Mutated Ryanodine Receptors

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Abstract

Malignant hyperthermia (MH) is a hypermetabolic disease triggered by volatile anesthetics and succinylcholine in genetically predisposed individuals. Nine point mutations in the skeletal muscle ryanodine receptor (RYR) gene have so far been identified and shown to correlate with the MH-susceptible phenotype, yet direct evidence linking abnormal Ca^{2+} homeostasis to mutations in the RYR1 cDNA has been obtained for few mutations. In this report, we show for the first time that cultured human skeletal muscle cells derived from MH-susceptible individuals exhibit a half-maximal halothane concentration causing an increase in intracellular Ca^{2+} concentration which is twofold lower than that of cells derived from MH-negative individuals. We also present evidence demonstrating that overexpression of wild-type RYR1 in cells obtained from MH-susceptible individuals does not restore the MH-negative phenotype, as far as Ca^{2+} transients elicited by halothane are concerned; on the other hand, overexpression of a mutated RYR1 Arg163Cys Ca^{2+} channel in muscle cells obtained from MH-negative individuals conveys hypersensitivity to halothane. Finally, our results show that the resting Ca^{2+} concentration of cultured skeletal muscle cells from MH-negative and MH-susceptible individuals is not significantly different. (*J. Clin. Invest.* 1998. 101:1233–1242.) Key words: Ca^{2+} channel • skeletal muscle • halothane • neuromuscular disorder • hypermetabolism

Introduction

Malignant hyperthermia (MH)[1] is an inherited pharmacogenetic muscle disorder triggered by halogenated anesthetic agents such as halothane, enflurane, isofluorane, desflurane, or sevoflurane and the depolarizing myorelaxant succinylcholine. An MH crisis is characterized by skeletal muscle rigidity, accelerated metabolism, and a rapid rise in body temperature; unless recognized immediately and treated, it is often fatal (1, 2). Initial studies in the molecular defect underlying this pathology indicated an abnormal regulation of the sarcoplasmic reticulum (SR) Ca^{2+} release mechanism (for a review, see reference 3). These results were substantiated by molecular genetic studies which suggested that in pigs and humans, MH mutations are located in the gene encoding the ryanodine receptor (RYR1) (4–7). The RYR1 is a large homotetrameric oligomer made up of four 565-kD subunits which forms a Ca^{2+} channel when incorporated into lipid bilayers (8). Indeed, to date, besides the Arg615Cys point mutation, at least eight other point mutations in the RYR1 gene have been reported to cosegregate with the MH phenotype in human pedigrees (9–11). In this context, it should be recalled that some MH phenotypes do not cosegregate with the RYR1 (12–16).

So far, all known mutations in the RYR1 gene induce a hypersensitivity of biopsied muscles to Ca^{2+}-releasing agents such as halothane, caffeine, 4-chloro-*m*-cresol, and ryanodine, and such a state is exploited in the in vitro muscle contracture test (IVCT). This test is carried out in patients with suspected or familial MH. The procedure is highly invasive and consists of dissection and in vitro treatment of muscle fibers with increasing doses of caffeine or halothane; the resulting contracture is recorded. Individuals are then classified according to their sensitivity to both compounds, as MH-susceptible (MHS), MH-negative (MHN), or MH-equivocal, the latter nevertheless considered MHS from a clinical point of view (17).

Direct biochemical and biophysical evidence linking point mutations in the RYR1 gene to an altered functional state of the Ca^{2+} release channel have been scant and to date have been published only for the Arg615Cys (18, 19) and the Gly2434Arg point mutations (20).

This study was undertaken with the aim of studying Ca^{2+} homeostasis in cultured human skeletal muscle cells obtained from fragments of biopsies of patients undergoing the IVCT. We show that the hypersensitive state to triggering agents which characterizes muscle fibers of MHS individuals is maintained at the single cell level, i.e., the intracellular Ca^{2+} transients elicited by halothane in cells from MHS and MHN individuals differ significantly. In addition, we present evidence demonstrating that MHN- and MHS-derived primary muscle cell cultures transfected with the wild-type or mutated RYR1 (Arg163Cys) cDNAs differ significantly in their response to halothane, as far as increases in the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, are concerned. Finally, the experimental approach used in this report may be exploited in the long run to offer a less invasive but equally sensitive alternative to the IVCT.

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; IVCT, in vitro contracture test; MH, malignant hyperthermia; MHN, MH-negative; MHS, MH-susceptible; RYR, ryanodine receptor; SR, sarcoplasmic reticulum.

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Methods

Materials

DME containing 4.5 mg/ml glucose, FCS, horse serum, penicillin G, streptomycin, and Lipofectin were all purchased from Life Technologies Ltd., Paisley, UK. Insulin was purchased from Eli Lilly and Company, Indianapolis, IN. Cell culture material was from Becton Dickinson GmbH, Heidelberg, Germany. Glutamine, Hepes, fura-2/AM, anti–α-actinin mAb, and anti–mouse–FITC conjugates were from Sigma Chemical Co., St. Louis, MO. Indo-1 and the calcium calibration kit were from Molecular Probes, Inc., Eugene, OR. Peroxidase-conjugated protein A was from Fluka AG, Buchs, Switzerland. Immunofluorescence mounting fluid was from Difco Laboratories Inc., Detroit, MI. The Western blot chemiluminescent kit was from DuPont-NEN, Brussels, Belgium. DNA-modifying enzymes were from Boehringer Mannheim, Mannheim, Germany. Halothane was from Halocarbon Labs, Inc., Hackensack, NJ. The Bluescript cloning vector was from Stratagene Inc., La Jolla, CA. The pEGFPC3 vector was from Clontech, Palo Alto, CA. The pRLDN expression vector was a kind gift of Dr. Schatzmann, SmithKline Beecham Pharmaceuticals, King of Prussia, PA. All other chemicals were reagent or highest available grade.

Patients

Primary human muscle cell cultures obtained from five patients with a positive family history for MH without any clinical signs for neuromuscular diseases, sent to the MH laboratory (Kantonsspital, University of Basel) for a diagnostic muscle biopsy, were involved in this study. Muscle biopsies were taken from the vastus medialis of the quadriceps muscles under regional anesthesia using 1% mepivacain. IVCTs were performed according to the protocol of the European MH Group as described previously (17, 21).

Human skeletal muscle cell cultures

Sterile muscle pieces not used for IVCT, weighing between 0.5 and 2.5 mg, were taken from the biopsies; each fragment was transferred to a sterile cell culture insert within a six-well plate containing DME plus 4.5 mg/ml glucose, 20% FCS, 5 ng/ml insulin, 200 mM glutamine, 600 ng/ml penicillin G and streptomycin, and 7 mM Hepes, pH 7.4. After ~ 8–10 d satellite cells could clearly be seen growing attached to the insert surrounding the biopsy fragment. The culture insert was then removed, and the cells were subsequently allowed to grow onto a new tissue culture dish; the medium was changed to a differentiation medium containing 10% horse serum, 5 ng/ml insulin, 200 mM glutamine, antibiotics, and 7 mM Hepes, pH 7.4. Cell cultures were obtained from each biopsy with a success rate of ~ 98%. The protocol was approved by the University of Basel Hospital Ethics Committee. For cryopreservation, ~ 10^6 cells were preserved in DME containing 40% FCS, 10% dimethyl sulfoxide and stored in liquid nitrogen. For intracellular Ca^{2+} measurements and immunofluorescence studies, confluent cells were transferred and grown on glass coverslips.

Genetic analysis of MHS patients

Total RNA/DNA was extracted from muscle cells growing in small tissue culture flasks according to Chomczynski and Sacchi (22) or the salting-out method. PCR amplification of RYR1 segments was carried out using published primer sequences and amplification conditions (23–26). The presence of the Arg163Cys mutation in MHS patient 1 was confirmed by restriction enzyme digestion and single-strand conformational polymorphism analysis as described by Quane et al. (23).

Construction of the expression vectors and DNA manipulations

The full-length rabbit skeletal muscle RYR1 cDNA was constructed as described previously (18). Replacement of C_{490} with T was performed using a cDNA cassette (EcoR1/vector-121/SalI 548) according to the method described by Kunkel et al. (27). DNA sequencing was performed by the dideoxy method of Sanger et al. (28). The wild-type and mutated (MHS Arg163Cys) RYR1 cDNAs were cloned into the pRLDN expression vector as described previously (18). Transfection of the wild-type and mutated RYR1 constructs was performed using lipofectin according to the manufacturer's instructions. To evaluate the efficiency of transfection, double transfection experiments (RYR1 cDNA constructs plus pEGFPC3 reporter vector) were performed using lipofectin.

Immunological methods

Immunofluorescence. 7 d after transfection, cells were used for intracellular Ca^{2+} measurements and subsequently fixed on the glass coverslips with an ice-cold solution of 50% methanol, 50% acetone at −20°C for 20 min. The cells were rinsed twice with PBS and preincubated for 60 min with 10% calf serum, 1% BSA in PBS. The cells were then incubated with an anti-RYR1 mAb (final concentration 20 μg/ml) raised in our laboratory, or an anti–α-actinin mAb for 90 min at 37°C in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), rinsed four times with TBST, and incubated with an FITC-conjugated anti–mouse IgG diluted 1:50 in TBST for 60 min at room temperature. The coverslips were rinsed four times with TBST, mounted and examined with an inverted fluorescent microscope (Diaphot 300; Nikon Inc., Melville, NY).

Western blot analysis. 7 d after transfection, skeletal muscle cells were rinsed twice in PBS, scraped from the petri dishes, and homogenized in 1 mM EDTA, 10 mM Hepes, pH 7.4, 0.25 M sucrose plus 1 μg/ml leupeptin, 1 mM PMSF, and 1 mM benzamidine in a glass/glass potter. Nuclei were sedimented at 3,000 rpm in an Eppendorf microfuge, and the supernatant was loaded on a 5% SDS PAGE. Protein concentration was determined according to the method of Bradford (29). Immunodetection was carried out using a mouse anti-RYR1 antibody, followed by peroxidase-conjugated protein A (1:5,000) as described previously (18). Rabbit terminal cisternae microsomes were prepared from skeletal muscle as described by Saito et al. (30).

$[Ca^{2+}]_i$ ratio measurements and calibration

The free cytosolic Ca^{2+} concentrations were determined in skeletal muscle cells using the fluorescent Ca^{2+} indicators fura-2 or indo-1. Briefly, cells grown on glass coverslips were loaded with either 5 μM fura-2/AM or 5 μM indo-1/AM at 37°C in Krebs-Ringer solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes, 1 mM $NaHPO_4$, 5.5 mM glucose, 1 mM $CaCl_2$, pH 7.4. After 60 min, cells were rinsed with Ringer's solution to remove unhydrolyzed indicator and stored at 20–22°C until used. On-line ratio measurements were recorded using a fluorescent microscope (Axiovert; Carl Zeiss GmbH, Jena, Germeny) attached to an infrared camera (Photonic Science Ltd., Robertsbridge, UK) and analyzed using the Zeiss imaging system. Coverslips were affixed to a chamber, and experiments were performed in a thermostatically controlled atmosphere at 37°C. 10 cells were identified in each field, and the average pixel (three frames averaged and ratioed every 3 s) value for each cell was measured at excitation wavelengths of 340 and 380 nm at various times (fura-2 measurements). Ca^{2+} calibration was performed using the EGTA/ionomycin/$MnCl_2$ quenching method (31), and the $[Ca^{2+}]$ was determined using a K_d of 225 nM (32). Alternatively, measurements were performed using a Diaphot 300 inverted fluorescent microscope (Nikon Inc.) attached to two photomultipliers (P100; Nikon Inc.). A single cell was identified, and the average fluorescence emitted at 410 and 480 nm was integrated and ratioed every 50 ms. Ca^{2+} calibration was performed using the cell free ratio measurement system with the Ca^{2+}-calibration kit and a K_d of 311 nM (33).

Statistical analysis

Statistical analysis was performed using the Student's *t* test for paired samples and the Origin and Prism computer programs (Microcal Software, Inc., Northampton, MA, and GraphPAD Software for Science, San Diego, CA).

Table I. IVCT of Muscle Biopsies from which Primary Cultures Were Derived

		Halothane		Caffeine	
Patient no.	Diagnosis	Threshold	Contracture*	Threshold	Contracture‡
		%	g	mM	g
1	MHS	0.5	3.0	2	0.2
2	MHS	0.5	2.25	1.5	0.9
3	MHN	3.0	0.1	0	0
4	MHN	3.0	0.1	0	0
5	MHN	> 3.0	0	0	0

Contracture at *2% halothane and at ‡2 mM caffeine.

Results

One of the aims of this report was to investigate whether cultured human muscle cells express differences characteristic of MHS muscles, even after several passages in culture. Table I shows the halothane- and caffeine-induced muscle contractures obtained from biopsies of the patients involved in this study and classified as MHN or MHS. Genotypic analysis indicated that MHS patient 1 carries the Arg163Cys mutation (Fig. 1), whereas in MHS patient 2, we could not detect four of the most frequent MH mutations.

Muscle satellite cells are small mononucleated cells present in skeletal muscle fibers which are normally quiescent but can be activated during postnatal growth, after muscle injury, or in response to intensive physical activity, after which they can proliferate and fuse (Fig. 2). To confirm that the human primary muscle cells we used in this report were indeed of skeletal muscle lineage, we performed immunofluorescence studies on glass coverslip–grown cells. The expression of skeletal muscle cytoskeletal proteins was studied using an anti–α sarcomeric actinin mAb. Fig. 3 shows the immunofluorescent staining of the cultured human muscle cells; regardless of whether they were obtained from biopsies of MHN (*A*) or MHS (*B*) patients, the pattern of fluorescence remained the same. COS-7 cells, which are not expected to express sarcomeric proteins, were negative when assayed with the anti–α-actinin antibody (*C*). We also studied whether the human muscle–derived cells express native SR proteins. Fig. 4 shows indirect immunofluorescence of muscle cells derived from MHN (*A*) and MHS (*B*) individuals using an anti-RYR1 mAb. As shown, the fluorescent pattern appears as discreet punctuated fluorescence uniformly distributed throughout the cell. COS-7 cells, which do not express endogenous RYR1, were negative (*C*).

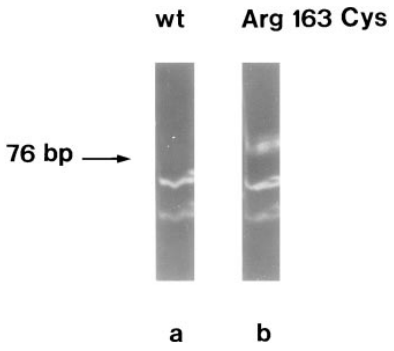


Figure 1. Detection of the RYR1 Arg163Cys mutation in DNA from MHS patient 1. PCR amplification of a 76-bp product was achieved using the primer pairs 457F, 532R. 20% acrylamide gel of (*a*) digestion of the 76-bp product from a normal control (MHN) and (*b*) from MHS patient 1 with the restriction endonuclease BstU1. The presence of the mutation Arg163Cys abolishes a BstU1 site. *wt*, wild-type.

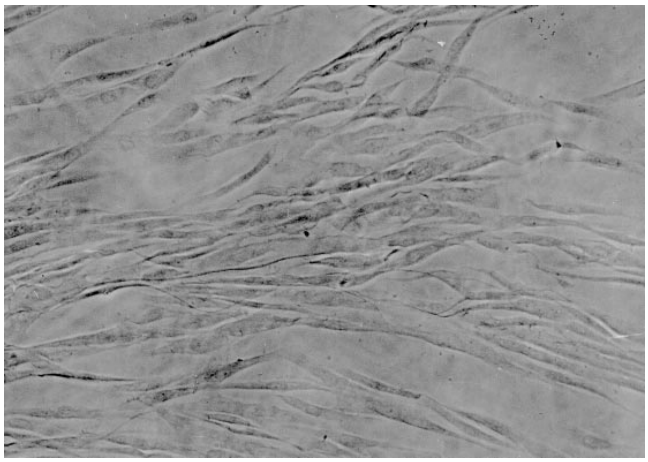


Figure 2. Phase–contrast micrograph of primary cultures derived from human skeletal muscle satellite cells (×250). The average length (n = 20) of individual muscle cells was 461±2.2 μm.

Since an MH crisis is the result of an abnormal increase in the myoplasmic Ca^{2+} concentration in response to "trigger agents," we investigated intracellular Ca^{2+} homeostasis of muscle cells from MHN and MHS individuals. Human skeletal muscle cells were grown on glass coverslips, and single cell calcium measurements using fluorescence microscopy were performed on fura-2–loaded cells. Fig. 5 shows the single cell intracellular Ca^{2+} measurements of cultured muscle cells obtained from one MHN (*top*) and one MHS (*bottom*) individual. For each experiment, a total of 72 digital images were recorded at 340 and 380 nm and ratioed. The first image represents the resting $[Ca^{2+}]_i$; the next two images were taken 20 and 30 s after perfusion with 5.7 mM halothane; and the last image shows the fluorescence ratio after reperfusion with Krebs buffer (t = 140 s). As shown in Fig. 5, halothane increases substantially the myoplasmic $[Ca^{2+}]$ of muscle cells derived from the MHS individual but affects only slightly that of cells derived from the MHN individual. Fig. 6 shows the actual fura-2 fluorescence ratio measurements (340/380 nm) obtained from four randomly chosen cells imaged in Fig. 5; as indicated, the addition of halothane causes an immediate increase in the fluorescence ratio of MHS-derived (*solid line*) but not MHN-derived (*dotted line*) muscle cells. All experiments were performed at least three separate times on four individual MHS and MHN patients, and gave qualitatively identical results.

To evaluate in greater detail the differences between MHN- and MHS-derived muscle cells, we also performed a dose–response curve to halothane. Fig. 7 shows that the half-maximal halothane concentration causing an increase in the fura-2 ratio was approximately two-fold lower in MHS- than in MHN-derived cells (5.8 vs. 9.5 mM, respectively).

We also studied the resting myoplasmic $[Ca^{2+}]$ of skeletal muscle cells derived from MHN and MHS individuals using phototubes/photometers and the Ca^{2+} indicator indo-1. Under our experimental conditions, we did not find any significant

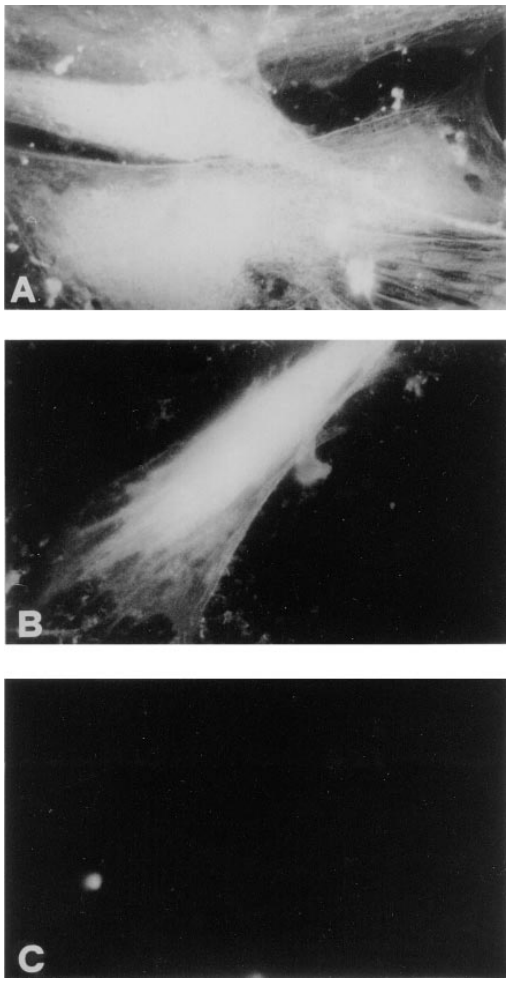


Figure 3. Indirect immunofluorescent staining for sarcomeric α-actinin. Human skeletal muscle cells were from an MHN (*A*) and an MHS (*B*) individual. COS-7 cells, which do not express this marker, were negative (*C*) (×1,500).

differences between the mean resting $[Ca^{2+}]_i$ of MHN or MHS cells (the mean $[Ca^{2+}]_i$ was 37.2±1.7 nM, $n = 12$, and 40.6±2.0 nM, $n = 18$, respectively).

We thought that the primary human skeletal muscle cells would offer the ideal system (*a*) to study the effects of various point mutations found to associate with MH, and (*b*) to investigate whether the MH phenotype in cells from MHS individuals was dominant or could be reversed by the addition of wild-type RYR1 cDNA. The full-length wild-type and mutated RYR1 (RYR Arg163Cys) cDNAs were constructed and inserted into the mammalian expression vector under the control of Rous sarcoma virus long terminal repeat. Because the mutation at nucleotide 490 abolishes a BstU1 site, we used loss of this site as an analytical tool to detect the mutation (Fig. 8). Substitution of T for C was also confirmed by direct sequencing of the DNAs (not shown).

Expression of the recombinant RYR was monitored using a combination of immunocytochemical and biochemical techniques. Indirect immunofluorescence staining for the RYR in skeletal muscle cells derived from biopsies of MHN and MHS individuals revealed a uniformly distributed fluorescent pattern (Fig. 9, *A* and *B*, respectively), while the fluorescent pattern of α-actinin remained identical to that of untransfected cells (compare Fig. 10, *A* and *B,* with fluorescent staining of cells in Fig. 3). Western blot analysis of the 3,000 *g* supernatant obtained from cells transfected either with the cDNA encoding the wild-type (Fig. 11, lane *3*) or the mutated RYR1 (Fig. 11, lane *5*) showed an immunopositive band having an apparent molecular weight similar to the rabbit skeletal muscle RYR (Fig 11, lane *1*). An immunopositive band is also present in mock-transfected MHS- (lane *2*) and MHN (lane *4*)–derived cells, but appears to be more abundant in the transfected cells. The efficiency of transfection was also assessed in double transfectant muscle cells; using the reporter molecule green fluorescent protein for monitoring the degree of transfection, we found that under our conditions, ~ 80% of the cells were

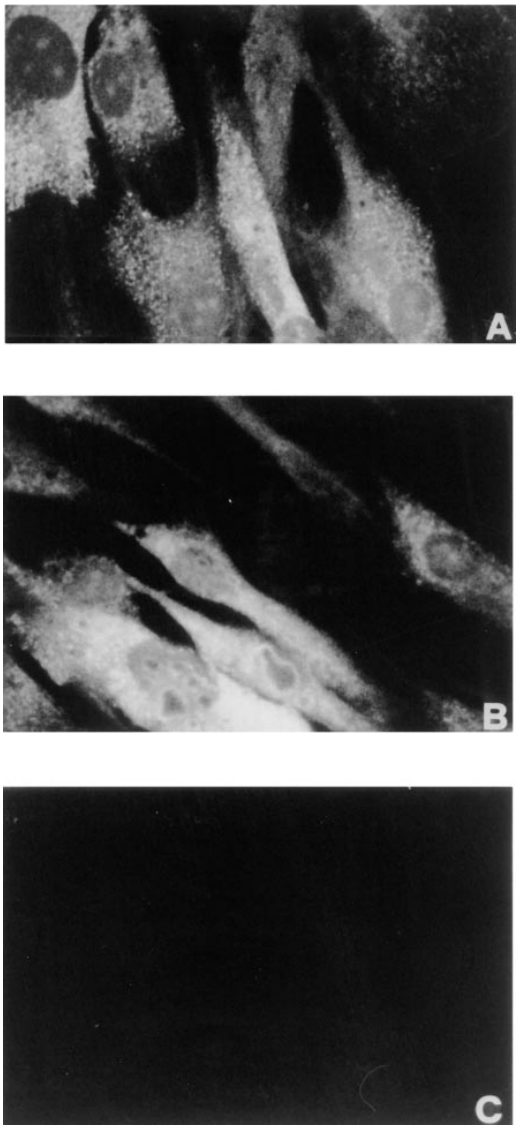


Figure 4. Indirect immunofluorescence staining for skeletal muscle RYR. Human skeletal muscle cells from MHN (*A*) and MHS (*B*) individuals show distinct punctuated fluorescence, while COS-7 cells, which do not express the skeletal muscle RYR, are negative (*C*) (×1,500).

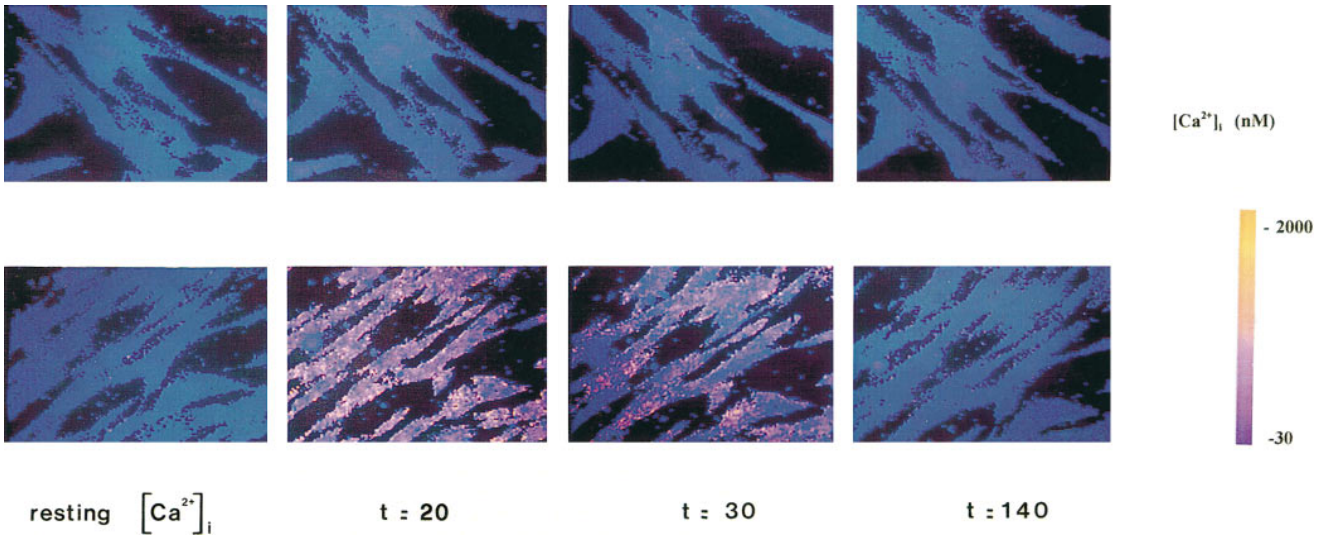


Figure 5. Single cell intracellular Ca^{2+} measurements of cultured human skeletal muscle cells stimulated with 5.7 mM halothane. Cells from an MHN (*top*) or an MHS individual (*bottom*) were grown on glass coverslips for 1 wk and loaded with the fluorescent Ca^{2+} indicator fura-2; single cell intracellular Ca^{2+} measurements were performed using the Zeiss imaging system attached to an Axiovert fluorescent microscope as described in Methods. Images were taken at the indicated times after halothane addition.

transfected as demonstrated by bright green cytoplasmic fluorescence (not shown).

Thus, we studied the effect of overexpression of both wild-type and Arg163Cys mutated recombinant RYR1 cDNAs in skeletal muscle cells derived from MHS and MHN individuals. The effect of overexpression of RYR1 on intracellular Ca^{2+} homeostasis was evaluated by plotting the change in fluorescence ratio (340/380 nm) as a function of time after halothane addition (Fig. 12). Each trace represents the change in fluorescence ratio occurring in a single cell, and for clarity, the traces of four distinct cells are shown. Results are representative of experiments carried out at least three times in skeletal muscle cells derived from the biopsies of three MHN and two MHS individuals. Halothane addition to MHN cells transfected with wild-type RYR evoked a calcium transient which was similar if not identical to that of mock-transfected MHN cells (Fig. 12 *A*). On the contrary, when the same experiment was performed on MHS-derived cells overexpressing the wild-type RYR, halothane was still capable of causing an increase in the $[Ca^{2+}]$, yet to a lower extent compared with mock-transfected MHS cells (Fig. 12 *B*). As expected, transfection of MHN-derived cells with the RYR1 cDNA carrying the Arg163Cys mutation affected dramatically the kinetics of the calcium transients. Fig. 12 *C* shows that halothane induces a very rapid and large increase in the myoplasmic $[Ca^{2+}]$; in addition, it can be appreciated that the elicited calcium transient is very similar to

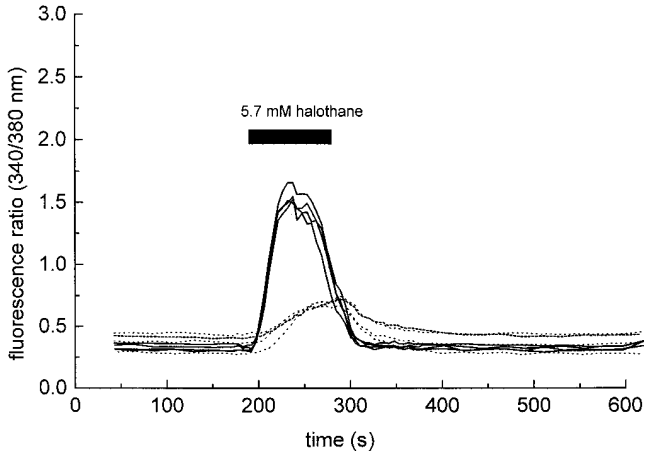


Figure 6. Halothane-induced Ca^{2+} responses of cultured human skeletal muscle cells. Cells were from an MHN (*dotted lines*) and an MHS individual (*solid lines*). Traces represent the changes in fluorescence ratio (340/380 nm) of four randomly chosen cells from Fig. 5.

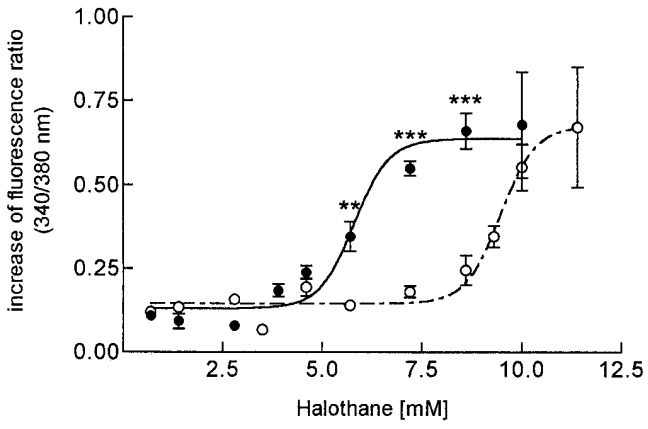


Figure 7. Dose–response curve to halothane. $[Ca^{2+}]_i$ measurements were carried out as described in Fig. 5. Values represent the mean±SEM ($n = 10$) increase in fura-2 fluorescence ratio (340/380 nm). $^{**}P < 0.01$; $^{***}P < 0.001$.

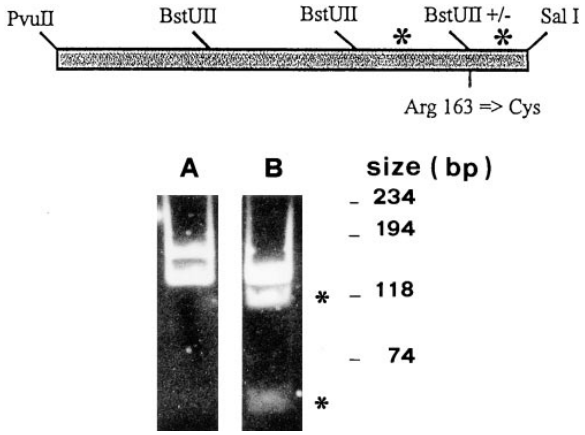


Figure 8. BstU1 restriction endonuclease digestion of the RYR1 PvuII 96–Sal1 546-bp cDNA fragment. Lane *1*, RYR1 cDNA carrying the Arg163Cys point mutation; lane *2*, wild-type RYR1. *The bands of 120 and 54 bp which are replaced by a band of 174 bp in the mutated RYR cDNA. The other two fragments of 144 and 146 bp migrated as a single band (*top*) in this gel.

that exhibited by mock-transfected MHS cells. Fig. 13 compares the average (mean±SEM) peak in the $[Ca^{2+}]_i$ induced in RYR1 wild-type/Arg163Cys mutated cDNA–transfected or mock-transfected skeletal muscle cells from MHN and MHS individuals. Overexpression of the wild-type RYR1 cDNA in MHS-derived cells caused a significant decrease (~ 50%) in the peak $[Ca^{2+}]_i$ induced by halothane; however, overexpression of a channel carrying the Arg163Cys point mutation in muscle cells derived from an MHN individual caused a fourfold increase in the peak $[Ca^{2+}]_i$ induced by halothane. Over-

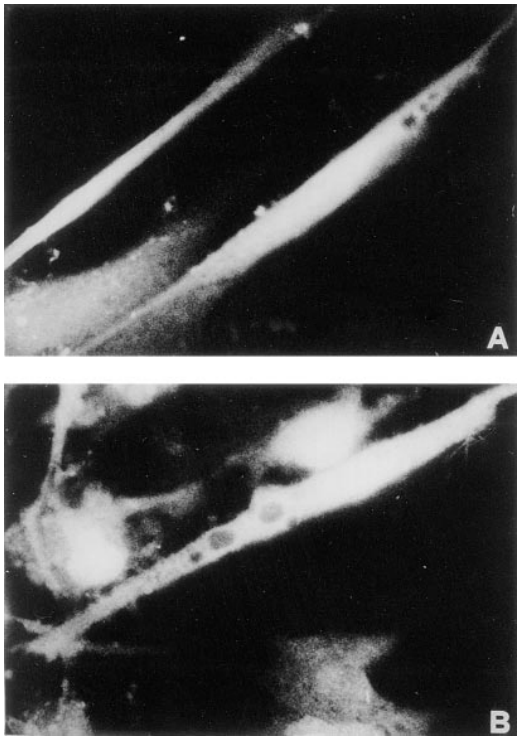


Figure 9. Indirect immunofluorescent staining for skeletal muscle RYR in human skeletal muscle cells overexpressing RYR1. Cells from MHN (*A*) and MHS (*B*) individuals were transfected with the RYR1 cDNA carrying the Arg163Cys point mutation (*A*) or the wild-type RYR cDNA (*B*), as detailed in Methods (×1,500).

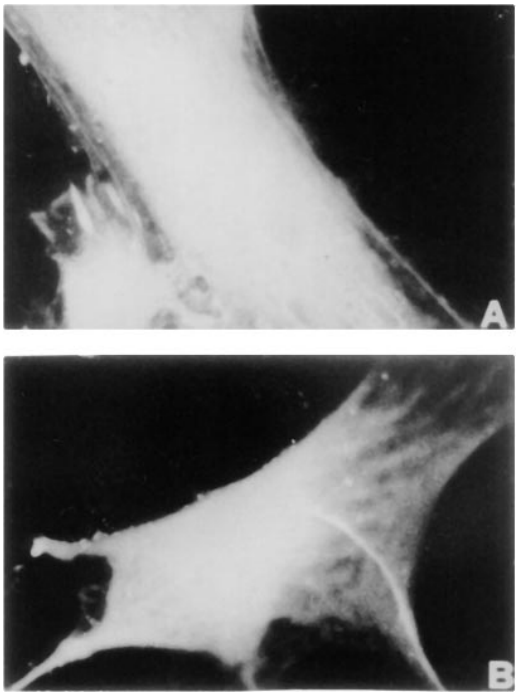


Figure 10. Indirect immunofluorescent staining for sarcomeric α-actinin in human skeletal muscle cells overexpressing RYR1. Cells from an MHN individual were transfected with the RYR1 cDNA carrying the Arg163Cys point mutation (*A*); cells from an MHS individual were transfected with the wild-type RYR1 cDNA (*B*). Overexpression of the RYR does not affect the fluorescent pattern for sarcomeric α-actinin (×1,500).

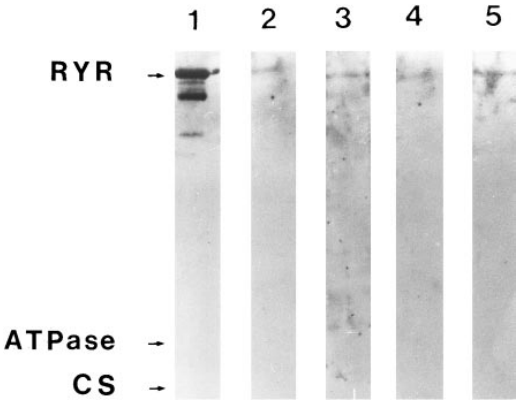
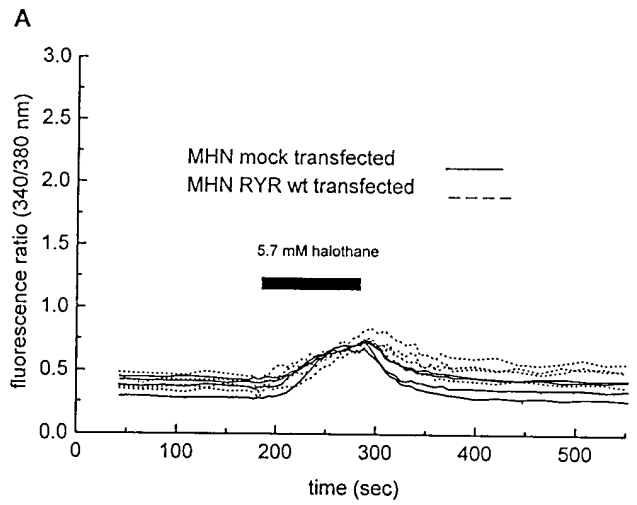
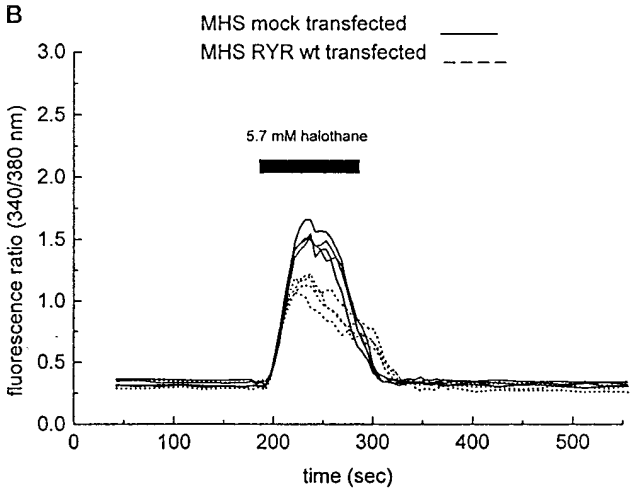


Figure 11. Western blot analysis of human skeletal muscle cells transfected with the cDNA encoding RYR1. Lane *1*, 15 μg rabbit skeletal muscle terminal cisternae; lanes *2* and *4*, 3,000 rpm supernatant of mock-transfected MHS and MHN skeletal muscle cells; lane *3*, 3,000 rpm supernatant of MHS cells transfected with the wild-type RYR1 cDNA; lane *5*, 3,000 rpm supernatant of MHN cells transfected with the RYR1 cDNA carrying the Arg163Cys point mutation (80 μg protein loaded per lane). *Arrows*, The RYR (565 kD), the Ca^{2+} ATPase (110 kD), and calsequestrin (65 kD).





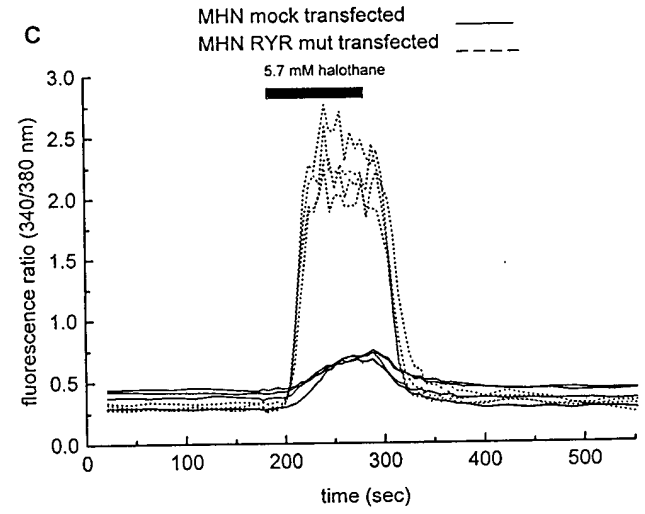


Figure 12. Halothane-induced Ca^{2+} responses of cultured human skeletal muscle cells. Mock-transfected cells (*solid lines*) and RYR1 cDNA–transfected cells (*dotted lines*). (*A*) Cells from an MHN patient transfected with wild-type RYR1 cDNA; (*B*) cells from an MHS patient transfected with wild-type RYR1 cDNA; (*C*) cells from an MHN individual transfected with RYR1 cDNA containing the Arg163Cys point mutation. Traces represent the changes in fluorescence ratio (340/380 nm) of four randomly chosen cells which had been either mock-transfected or transfected with the appropriate RYR1 cDNA construct. Conditions as described in Fig. 5.

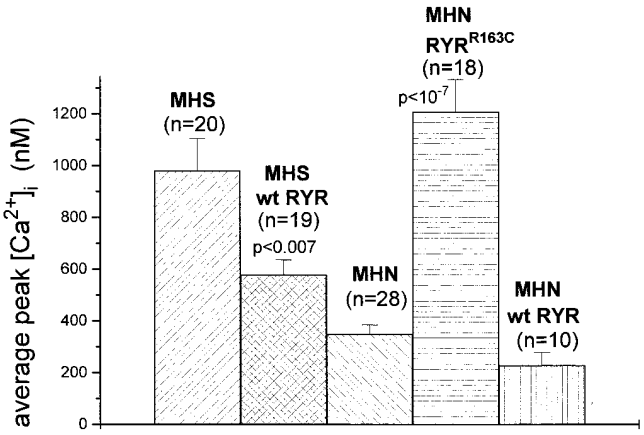


Figure 13. Average peak $[Ca^{2+}]_i$ induced by 5.7 mM halothane in mock-transfected or RYR1 cDNA–transfected human skeletal muscle cells. Results are the mean±SEM of *n* cells. *wt*, wild-type.

expression of the wild-type RYR1 cDNA in MHN-derived cells did not change significantly the peak $[Ca^{2+}]_i$ induced by halothane.

The halothane dose–response curves in Fig. 14 show that the transfection of MHN cells with Arg163Cys RYR1 cDNA decreases approximately twofold the half-maximal halothane concentration necessary to cause an increase in the fluorescence ratio of fura-2 (4.9 vs. 9.5 mM for Arg163Cys RYR1 cDNA– and mock-transfected cells, respectively). On the other hand, the half-maximal halothane concentration necessary to increase the fura-2 ratio of MHS cells transfected with the wild-type RYR1 cDNA was higher than that of MHS mock-transfected cells (6.9 vs. 5.8 mM, respectively), but lower than that of MHN mock-transfected cells. The latter result indicates that the presence of altered Ca^{2+} release channels is sufficient to convey halothane hypersensitivity to skeletal muscle cells.

Discussion

Satellite cells give rise to a heterogeneous population of cells which can undergo differentiation into myoblasts and myotubes under appropriate culture conditions. In the past few years, several reports have appeared concerning the biochemical characterization of satellite cell–derived skeletal muscle cell cultures (34, 35): as far as the expression of cytoskeletal proteins and proliferative capacity are concerned, numerous species-specific differences exist (36). Cells obtained from human biopsies appear to have undergone some sort of differentiation step towards the skeletal muscle phenotype, and express skeletal muscle–specific cytoskeletal proteins (37).

One of the major objectives of this report was to evaluate the feasibility of substituting the IVCT with Ca^{2+}-imaging

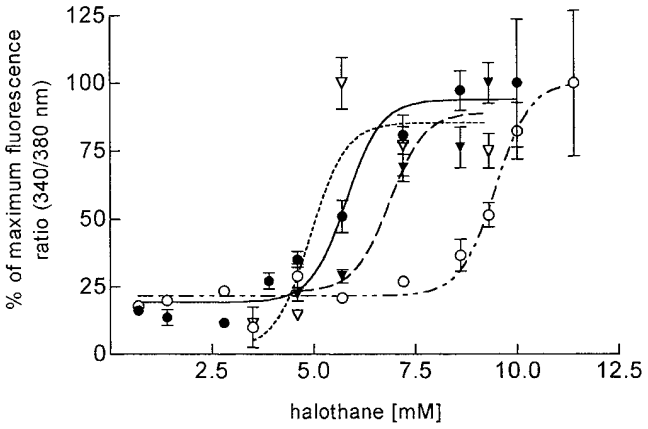


Figure 14. Halothane dose–response curve in MHN and MHS cells transfected with RYR1 cDNA constructs. Conditions as described in Fig. 7. Results are expressed as mean±SEM ($n = 10$) percent fluorescence increase. 100% was the maximal fluorescence ratio increase induced by the highest halothane concentration. *Filled circle*, *solid line*, MHS. *Open circle*, *uneven lines*, MHN. *Open triangle*, *dotted line*, MHN RYR mutated. *Filled triangle*, *dashed line*, MHS RYR wild-type.

studies on primary cultures of human skeletal muscle–derived cells. So far, we have obtained primary cultures from the tested biopsies with a very high efficiency, and positive IVCT contracture tests have correlated well (correlation coefficient = 0.88, $P = 0.0192$) with an increased halothane sensitivity of cultured skeletal muscle cells (38). In these experiments, we used millimolar halothane concentrations. We are aware that clinically used halothane concentrations are < 1 mM, and that the threshold concentration for abnormal muscle contractures in the IVCT is 0.44 mM halothane. Nevertheless, there are reasons that may account for using higher concentrations of halothane in our experiments: (*a*) a cell culture model has general differences compared with the in vivo situation; (*b*) it is interesting that Otsu et al. (19) have used a 5.7 mM halothane concentration to demonstrate differences in the effects of halothane on $[Ca^{2+}]_i$ between wild-type and Arg615Cys-transfected C_2C_{12} cells; (*c*) when we performed experiments using low doses of halothane (< 2 mM), we did not observe significant increases in the $[Ca^{2+}]_i$ in cells derived from either MHS or MHN individuals; (*d*) a halothane dose–response curve shows that the effect of halothane on the $[Ca^{2+}]_i$ of MHN- and MHS-derived cells is saturable; and (*e*) we are able to clearly demonstrate a significant difference in the EC_{50} to halothane between MHS and MHN human muscle cells. Moreover, it is possible that these high doses of halothane may also inhibit the SR CaATPase (39), and thus unmask the release of Ca^{2+} from intracellular stores which is higher in MHS than MHN cells.

The RYR mediates Ca^{2+} release from the SR. The primary structure of the skeletal muscle RYR has been deduced from the cDNA of several species: the carboxy-terminal segment contains the hydrophobic pore-forming region, while the remaining 70–80% of the molecule forms a large hydrophilic region which has been assigned to the cytoplasmic side of the SR (40, 41). Though in humans, MH is a heterogeneous genetic disorder (12–16), all known mutations in the RYR1 cDNA have been mapped to the large hydrophilic amino-terminal portion of the molecule, and it has been assumed that they cause functional alterations in the Ca^{2+} release properties of the RYR. In this study, we show for the first time that the transfection of human skeletal muscle cells with the cDNA encoding the mutated RYR1 Arg163Cys and subsequent challenge with halothane cause a twofold decrease in the EC_{50} for halothane, thus supporting the hypothesis that at least some mutations associated with MH lead to an increase in the rate of Ca^{2+} release from the SR (42).

As to the overexpression of wild-type RYR1 in skeletal muscle cells from MHS individuals, the situation is more complex; under our experimental conditions, the hypersensitivity to halothane which characterizes MHS was reverted only partially by transfection of muscle cells with the wild-type RYR1 cDNA, and halothane was still capable of causing an increase in $[Ca^{2+}]_i$, though to a significantly lower extent. Thus, it seems that the defective (MH mutated) channels maintain their hypersensitive state to MH-triggering agents regardless of the overexpression of wild-type channels. These results support and extend previous studies (43) which indicated that a single defective subunit in the tetrameric channel is sufficient to confer the MH phenotype.

One of the most frequent yet unresolved questions concerning MH is whether the resting myoplasmic $[Ca^{2+}]$ of the muscle cells from MHS individuals is significantly different from that of MHN individuals (44, 45). Indeed, evidence in favor of this hypothesis has been gathered mainly using Ca^{2+} electrodes. Though sensitive, the main drawback of this approach is that it damages the muscle fiber, causing leakage of ions into the myoplasm. In fact, other investigators (46) have reported that the resting Ca^{2+} levels in muscle fibers from MHN and MHS swine are not significantly different, a result supported by these findings. In terms of free Ca^{2+}, we found that the cytoplasmic concentration was much lower than that reported by Benders et al. (47) for human cultured skeletal muscle cells (35–40 nM in this report vs. 125 nM in reference 47), but similar to that reported for resting mouse cultured skeletal muscle cells (48). However we would like to point out that (*a*) we obtained identical results using two different Ca^{2+} indicators and two distinct methodologies; (*b*) it is a well-established fact that the use of fluorescent Ca^{2+} indicators does not give exact quantitative values; (*c*) the K_d of Ca^{2+} indicators is obtained in solution and may vary somewhat within the cytoplasm (49); and (*d*) though the values may be altered in quantitative terms, they are not affected qualitatively; thus, though the mean resting $[Ca^{2+}]$ may be underestimated, this occurs for both MHS and MHN cells. At any given time, the mean resting myoplasmic Ca^{2+} concentration is the net between efflux from the SR and activity of the Ca^{2+}-extrusion mechanisms. If MH is characterized by an increased rate of Ca^{2+} release, i.e., an increase in Ca^{2+} leakage from the SR via the RYR1, one might expect the resting myoplasmic $[Ca^{2+}]$ to be higher in MHS individuals; however, the Ca^{2+}-extrusions systems are capable of compensating for the increased leakage by increasing their activity so that the result is an unaltered resting free Ca^{2+} concentration.

The use of skeletal muscle primary cultures seems to be the ideal tool to study the physiology and biochemistry of cells isolated from MH-equivocal individuals and the effects on intracellular Ca^{2+} homeostasis of other point mutations genotypi-

cally linked to MH, as well as the effects of newly developed drugs.

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