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Malignant hyperthermia causing Gly2435Arg mutation of the ryanodine receptor facilitates ryanodine-induced calcium release in myotubes

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We have investigated if cultivated muscle cells from malignant hyperthermia (MH) patients can be distinguished pharmacologically from controls. Muscle specimens from four individuals carrying the Gly2435Arg mutation of the skeletal muscle ryanodine receptor protein (RYR1) and from four controls were used to culture myotubes. Resting intracellular calcium concentration ($[Ca^{2+}]_i$) of MH myotubes was similar to controls. However, when ryanodine 0.5 μmol litre^{-1} was added, the kinetics of the increase in the calcium signals in MH and control cells were significantly different; the time for half maximum increase was mean 197 (SD 131) s for MH cells and 474 (61) s for controls (n=80 cells each). On average, the area under the MH response curves was twice the control value. These results give rise to hopes that the phenotype of MH can be characterized using cultured human muscle and that a culture-based test for MH susceptibility may eventually be developed.

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Malignant hyperthermia (MH) is a potentially fatal condition characterized by muscle rigidity and hypermetabolism triggered by certain inhalation anaesthetics and depolarizing skeletal neuromuscular blocking agents. An MH crisis starts with an unusual increase in body temperature.[1–3] It is believed to be caused by disturbed myoplasmic calcium homeostasis. More than half of the individuals susceptible to MH are likely to carry mutations in the gene encoding the skeletal muscle ryanodine receptor (RYR1).[4] The symptoms of muscle rigidity, hyperthermia and increased energy turnover are secondary effects.[5]

To date, 19 point mutations in the RYR1 gene have been associated with MH.[6 7] All mutations predict amino acid changes in the huge cytoplasmic domain of RYR1. Two of these (Arg614Cys and Gly2435Arg) bestow on the channel increased sensitivity to calcium, increased ryanodine binding affinity and less inhibitory effect of calmodulin. Thus the mutations render the release channel overactive.[8] Similar observations were made in pig muscle carrying a homologous mutation predicting Arg615Cys.[9] Genetic linkage studies indicate the existence of at least four other MH loci on chromosomes 3q,[10] 7q,[11] 1q[12] and 5p.[12] Recently, a point mutation in the CACLN1A3 gene (1q), coding for the α1 subunit of the skeletal muscle L-type Ca^{2+} channel was found to be associated with MH susceptibility.[13] Thus it is unlikely that a simple genetic test for MH susceptibility will be available in the near future.

At present, the only accepted test for MH is based on pharmacological criteria. The *in vitro* contracture test (IVCT)[14 15] requires bundles of muscle fibres obtained at open biopsy to be exposed to caffeine and halothane. Ryanodine[16 17] and/or 4-chloro-m-cresol[18 19] can also be used as trigger substances. Muscle with increased sensitivity to both halothane and caffeine is classified as MH susceptible (MHS). The IVCT has a high sensitivity and specificity but the procedure is invasive, complicated and has to be performed within a few hours of biopsy. A test based on cultured muscle would be less harmful and would allow study of the pathophysiology of the various MH phenotypes in detail, as cultures can be expanded, frozen and thawed.

Cultured muscle expresses the RYR1 gene, and porcine myotubes carrying the Arg615Cys mutation showed a lower threshold for contraction when stimulated with long-

Table 1 IVCT data of MHS and MHN individuals investigated in this study. IVCT=*In vitro* contracture test; MHS=malignant hyperthermia susceptible; MHN=malignant hyperthermia negative. *Muscle bundle broken during experiment. The time for response to ryanodine was calculated from predrug values to contracture (10 mN level)

Patient	Threshold for halothane (%)	Force at 2% halothane (mN)	Threshold for caffeine (mmol litre^{-1})	Force at caffeine 2 mmol litre^{-1} (mN)	Time for response to ryanodine (min)
MHS 1	2.0	11.8	1.5	4.6	17.0
MHS 2	0.5	20.2	1.5	2.4	– *
MHS 3	0.5	20.6	1.0	16.2	16.0
MHS 4	2.0	6.8	2.0	2.8	17.0
MHN 1	>4	0.0	3.0	0.0	>30.0
MHN 2	>4	0.0	4.0	0.0	>30.0
MHN 3	>4	1.0	4.0	0.0	26.5

lasting depolarizations.[20] Cultured muscle cells carrying another MH mutation of the RYR1 gene, predicting Arg163Cys, are more sensitive to halothane than controls.[21] This indicates that RYR1 gene mutations can cause functional changes at the cellular level. In preliminary studies with human myotubes, responses to halothane and caffeine were variable; only ryanodine caused reproducible increases in intracellular calcium concentration ($[Ca^{2+}]_i$).[22]

In this study, we have investigated more thoroughly calcium homeostasis in myotubes from MH patients with a distinct RYR1 mutation with special regard to the possibility of determining MH susceptibility using cultured muscle.

Patients and methods

Patients

The *m. vastus lateralis* was biopsied in a patient who had undergone an MH crisis and in three of his relatives who wanted to be tested for MH susceptibility. Their ages were 16–63 yr. MH tests were performed according to the European protocol for the *in vitro* contracture test.[14] In addition to their responses to caffeine and halothane, the muscle bundles were also tested for their sensitivity to ryanodine.[16] All tests gave positive results (Table 1). PCR analysis revealed that the four MHS individuals were heterozygous for the Gly2435Arg mutation of the RYR1 gene.[8] For controls, we used muscle samples from three individuals who were classified as MH negative (MHN) according to the European IVCT protocol. A fourth control muscle sample was obtained from a patient with no muscle disease who had undergone orthopaedic surgery. The muscle specimens were transported to the laboratory in Krebs solution (Life Technologies, Eggenstein, Germany). A section of the sample from each patient was put aside for preparation of mononucleated cells. The study was conducted in accordance with the Helsinki convention and approved by the Ethics Commission of the University of Ulm. All donors gave informed consent to the experiments.

Muscle cultures

Muscle samples of 300–600 mg in weight were used for the enzymatic isolation of mononucleated cells or stored in Hank's solution (Biochrom, Berlin, Germany) for several hours at 4°C. Primary cultures were grown as described previously.[23] They were frozen before myotube formation began and stored for up to 1 yr at −196°C. On thawing, the first myotubes began to form after 6–8 days of culture. Recordings of $[Ca^{2+}]_i$ were performed between 5 and 10 days after the first myotube formation. The cultures had reached about 50% confluence at that time and the multinucleated myotubes could be distinguished easily form mononucleated fibroblast-like cells. To exclude artefacts, cultures were thawed and grown in pairs of MHS and control, and all recordings from these cultures at a corresponding stage were made on the same day in parallel.

Recordings of free cytosolic Ca^{2+}

$[Ca^{2+}]_i$ was determined using the calcium-sensitive dye fura-2. To load the cells with the dye, the cultures growing in a dish of 6 cm diameter were washed three times with Hank's solution and then incubated for 45 min at 37°C in Hank's solution containing fura2/AM· 1.2 μmol litre^{-1}. After loading, the cells were washed three times with standard external solution, and then 18 ml of external solution was added. The whole culture was heated to the desired temperature of 37°C and then kept for no longer than 1 h before experimentation.

An inverted microscope and equipment for UV excitation (alternating between 340 nm and 380 nm) and fast photometric detection (MPM 200, Zeiss, Oberkochen, Germany) was used to determine the ratio R of the fluorescent signals in cells or parts of single myotubes. A cell-free calibration of the recording system was performed using 15 different strongly Ca-buffered solutions of (mmol litre^{-1}) KCl 100; HEPES 10; EGTA/KOH 5; HEDTA/KOH 5; NTA/KOH 5; and fura-2 0.01 (pH 7.4). Appropriate amounts of Mg^{2+} and Ca^{2+} were added to this stock to obtain standards with a final free Mg^{2+} concentration of 1 mmol litre^{-1} and pCa values of 9.0, 8.3, 8.0, 7.7, 7.3, 7.0, 6.7, 6.3, 6.0, 5.7, 5.3, 5.0, 4.0, 3.0 or 2.0 (based on calculations and using the software described by Föhr, Warchol and Gratzl[24]). All standards were excited at set temperatures of 20–41°C (see Fig. 1) and the fluorescence intensities determined.

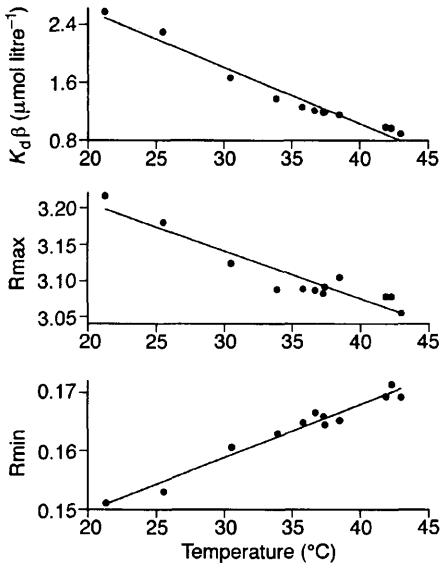

Fig 1 Temperature dependence of the conversion variables Rmin, Rmax and $K_d\beta$. *In vitro* calibrations with strongly calcium-buffered solutions containing defined free Ca^{2+} concentrations were performed at various temperatures (see Patients and methods). The resulting values for Rmin, Rmax and $K_d\beta$ were plotted against temperature and straight lines fitted to each set of data points. The calculated values for Rmin, Rmax and $K_d\beta$ were used to determine intracellular Ca^{2+} concentrations based on measured fluorescence emission intensities of fura-2 and equation (1).

Equation (1) from Grynkiewicz, Poenie and Tsien[25] was

$$[Ca^{2+}]=K_d\beta\ (R-Rmin)/(Rmax-R) \qquad (1)$$

fitted to the data to determine Rmin (the ratio of fluorescence emission intensities at 340 and 380 nm excitation under Ca^{2+}-free conditions), Rmax (this same ratio with saturating Ca^{2+} concentrations) and β (the ratio of the 380-nm fluorescence under minimum and maximum $[Ca^{2+}]$ conditions, with K_d being the dissociation constant of Ca^{2+} and fura-2). The conversion variables Rmin, Rmax and $K_d\beta$ of equation (1) showed a more or less linear temperature dependence (Fig. 1). The regression variables and the ratio of the fluorescent signals from single cells were used for estimation of $[Ca^{2+}]_i$ in myotubes at various temperatures.

To record cytoplasmic Ca^{2+} concentrations in several myotubes of the same culture, the positions of 10 cells were stored and revisited using a computer-controlled microscope stage (Zeiss). The fluorescence emission intensities were recorded in rapid succession from the 10 cells, the recording time being 1 s per cell, and the procedure was repeated in 30-s intervals (loops, see Pressmar and colleagues[26]). Total recording time was 24 min. Ryanodine was normally applied after 3 min (i.e. after loop 6). In control experiments, no relevant artefacts were noted when water alone was applied.

Solutions and experimental conditions

The standard external solution used for recording Ca^{2+} signals contained (in mmol litre^{-1}): NaCl 150, KCl 3.5, $CaCl_2$ 1.0, $MgSO_4$ 1.0, glucose 10 and HEPES 5; pH was 7.4 and osmolarity was approximately 300 mosmol litre^{-1}. Ryanodine (Calbiochem, Bad Soden, Germany), highly purified and dissolved in water, was applied by adding the appropriate volume of the stock solution to the petri dish. Immediately after addition of the drug, the solution was stirred by gently pulling up and down the administering pipette. The final concentrations of ryanodine in the total volume of 18 ml in the dish were 0.5, 2 and 20 µmol litre^{-1}. To adjust the desired temperature during an experiment, we used a home-made heatable copper insert for the microscope stage. The insert, carrying the culture dish, was insulated against the microscope stage by a layer of Teflon. Peltier elements, attached to the copper plate, heated or cooled the dish to the desired temperature in the range 20–41°C with an accuracy of ±0.2°C. The temperature of the dish was recorded continuously and fed into the power control of the Peltier elements.

Statistical analysis

All data are given as mean (SD). To test for significant differences between the responses of MHN and MHS cells, the Wilcoxon rank test for paired samples was used as pairs of MHS and control cultures were tested on the same day.

Results

Temperature and concentration dependence of the ryanodine effect on $[Ca^{2+}]_i$ in control myotubes

Preliminary tests performed at room temperature showed that ryanodine concentrations >20 µmol litre^{-1} were needed to obtain any response. At 50 and 100 µmol litre^{-1}, the time course of calcium release was rather slow compared with other stimulating agents, as concentrations of Ca^{2+} took several minutes to reach a plateau about 1.5–3 times the resting level. No decline in $[Ca^{2+}]_i$ was noted during the recording time of 25 min.

Because of this low effectiveness, we performed most of our experiments at higher temperatures. At 25°C, a slight increase in $[Ca^{2+}]_i$ was observed with ryanodine 20 µmol litre^{-1}, and the increase was marked and remained more or less stable during the recording time when temperature was increased to 37°C (Fig. 2). A similar response was observed at 41°C. De-regulation of $[Ca^{2+}]_i$ (i.e. a rapid and irreversible increase in response to ryanodine in the micromolar range) was not observed during the whole series of experiments. All myotubes were morphologically intact after the recordings.

Next we investigated the concentration dependence of the ryanodine effect at 37°C, mainly to characterize the threshold concentration. Figure 3A shows typical responses of control myotubes to ryanodine applied at different concentrations. The lowest concentration showing an effect was 0.5 µmol litre^{-1}. Higher concentrations led to more pronounced and faster responses. Again, a plateau of $[Ca^{2+}]_i$

was reached after 5–10 min and there was no decline during the recording time of 25 min. Four independent experiments were carried out to determine the concentration dependence of the ryanodine effect. In each of these experiments, 10 cells of a single culture were tested at each ryanodine concentration. Figure 3B shows the results of one of the four experiments that confirmed the threshold concentration for an effect of ryanodine on the $[Ca^{2+}]_i$ of myotubes to be approximately 0.5 μmol litre^{-1}.

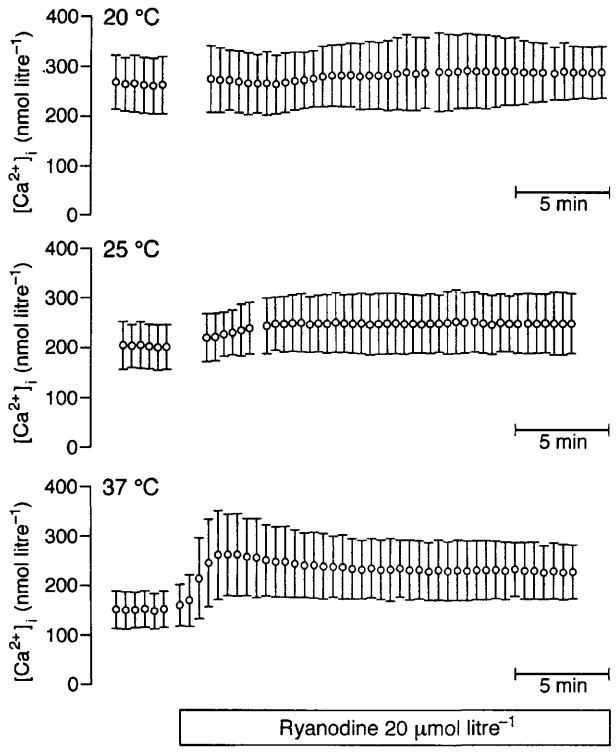

Fig 2 Effect of ryanodine on $[Ca^{2+}]_i$ of control myotubes, recorded at different temperatures. Each curve corresponds to averaged responses of 10 selected cells (mean (SD)) after application of ryanodine 20 μmol litre^{-1}. All data originate from 10 $[Ca^{2+}]_i$ recordings lasting 1 s each. Ryanodine was added after loop 6 (see bar). After the solution containing the drug had been applied and stirred, the culture was inspected to ensure that all selected cells remained in the correct position.

MHS myotubes: resting Ca^{2+} concentrations and effects of ryanodine at 37°C

Mean resting Ca^{2+} concentrations of MHS myotubes (156 (48) nmol litre^{-1}; $n=140$ from four probands) were not significantly different from controls (140 (55) nmol litre^{-1}, $n=140$). All cultures showed increases in $[Ca^{2+}]_i$ on application of ryanodine 20, 2 and 0.5 μmol litre^{-1}. At the two highest concentrations, the responses of MHS and control myotubes were not remarkably different, but at 0.5 μmol litre^{-1}, the responses of the MHS cultures were faster (Fig. 4A) and/or reached higher levels than controls (Fig. 4B). To illustrate these differences in the kinetics of the ryanodine effect, we plotted the times required for reaching half-maximum response. At ryanodine 20 and 2 μmol litre^{-1}, MHS and control cells were not different but at 0.5 μmol litre^{-1}, the rate of rise of $[Ca^{2+}]_i$ in MHS myotubes was twice that of controls (Fig. 5A).

A similar difference between MHS and controls was observed when the areas under the response curves obtained with ryanodine 0.5 μmol litre^{-1} were plotted. For MHS myotubes these integrated $[Ca^{2+}]_i$ values were, on average, more than twice those of controls (Fig. 5B) and this difference was even more prominent when data of tested pairs of MHS and control myotubes were compared individually. In seven of eight tests, the MHS/control ratios were: 1.9, 2.1, 3.3, 3.8, 4.3, 6.6 and 6.8. In only one experiment (represented by one of the open squares in Fig. 5), was the ratio reversed (0.7).

Discussion

Tissue cultures have been extremely useful for investigation of neuromuscular disorders. The major problems encoun-

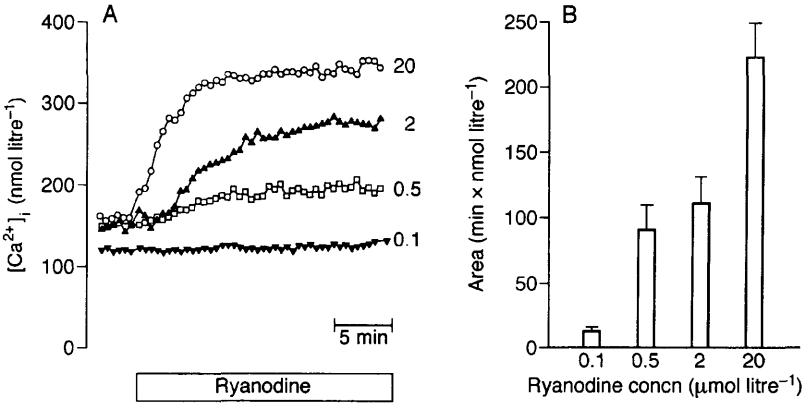

Fig 3 Concentration dependence of the effect of ryanodine on myotubes. (A) Cytoplasmic calcium concentration of four selected control myotubes before and during application of ryanodine 0.1, 0.5, 2 and 20 μmol litre^{-1}; drug was applied after loop 6 (bar). The four traces are typical responses of control myotubes to ryanodine. Recordings of $[Ca^{2+}]_i$ were made every 30 s; the recording time per data point was 1 s. (B) Averaged responses of 10 myotubes (each column) to ryanodine (mean (SD)). The data originate from experiments, as shown in A. The area included by the response curves was calculated for the various ryanodine concentrations. All recordings were made at 37°C using control myotubes.

tered with cultured muscle are: (i) in comparison with adult muscle, maturation is incomplete and (ii) there are embryonic isoforms for various muscle proteins. Expression[27 28] and upregulation[29] of RYR1 has been demonstrated in cells at the myotube stage. In addition to the RYR1 gene, myotubes express RYR3 to a smaller extent,[30] but the formation of the triad system and the functional existence of depolarization-induced Ca^{2+} release indicate that the RYR1 function is predominant.[31] Therefore, with respect to the expression of an MHS phenotype in culture, it was tempting to study muscle cell cultures from patients with mutations in the RYR1 gene.

Our finding that myotubes carrying the Gly2435Arg mutation in RYR1 have an increased sensitivity to ryanodine is compatible with another result obtained with the same mutant RYR1 receptor. When studied in SR vesicle preparations, the receptor showed enhanced sensitivity to activating calcium concentrations and, among other altered pharmacological properties, increased affinity to ryanodine.[8] Thus the faster kinetics of the $[Ca^{2+}]_i$ increase in the myotubes prepared from our probands may be explained by two mechanisms: (i) the higher affinity of the mutant receptors for ryanodine and (ii) an increase in calcium-induced calcium release as a consequence of local $[Ca^{2+}]$ changes in the microenvironment of the receptors.

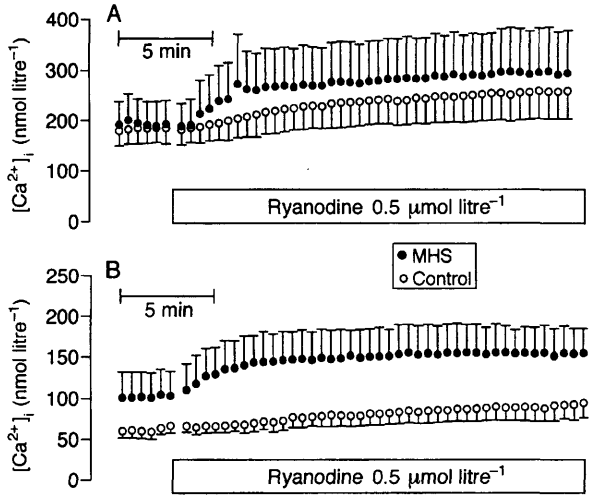

Fig 4 Effect of ryanodine 0.5 μmol litre^{-1} on $[Ca^{2+}]_i$ of MHS and control myotubes. Application of ryanodine is indicated by the bar. Data from each of the 10 MHS and 10 control myotubes are given. Mean (SD) $[Ca^{2+}]_i$ values obtained in experiments performed as shown in Figure 3. Results from two typical experiments are shown in A and B. Myotubes with the MHS genotype carry the Gly2435Arg mutation of RYR1. In some experiments, $[Ca^{2+}]_i$ of control cells was almost unaffected by ryanodine (B), indicating that 0.5 μmol litre^{-1} is the threshold concentration

In Figure 5, some overlap of the responses of MHS and control myotubes is evident. However, comparison of the responses of pairs of cultures tested on the same day clearly showed that under the most discriminating conditions (ryanodine 0.5 μmol litre^{-1} and 37°C) MHS myotubes responded considerably stronger than controls. Probably factors other than genotype (e.g. cell density or stage of maturation of the culture) have a modulating influence on the response to ryanodine. This would explain why in some tests responses of either cell type (MHS or control) was stronger than in others. In our view, high standardization and maturation of cultures is recommended, and a control culture should be tested in parallel with each MHS case.

Significantly different results with MHS and control myotubes were obtained only when ryanodine was applied at the threshold concentration of 0.5 μmol litre^{-1}, and not with 2 or 20 μmol litre^{-1}. This is similar to the IVCT in which the best distinction between MHS and control muscle bundles is achieved when caffeine and halothane are used at low concentrations. Interestingly, the concentration required for a significant increase in $[Ca^{2+}]_i$ in myotubes is close to the concentration used for the ryanodine contracture test (myotubes 0.5 μmol litre^{-1}; muscle bundles 1 μmol litre^{-1}).[16] In an earlier study, a concentration of ryanodine of at least 20 μmol litre^{-1} was necessary to elicit

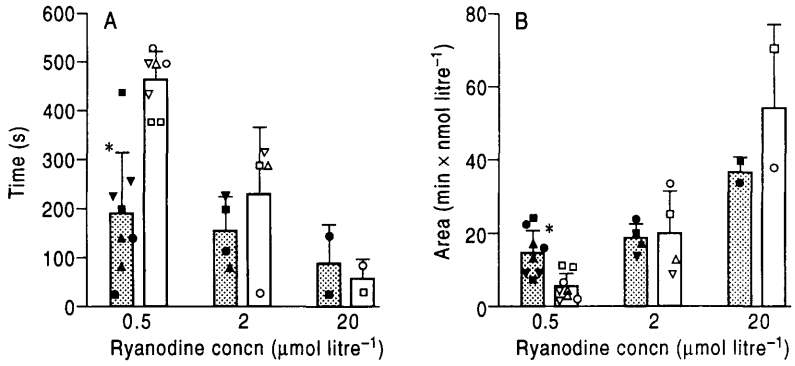

Fig 5 Summarized results from experiments, as shown in Figure 3. (A) Data from the four patients with the Gly2435Arg mutation (filled symbols) and four controls (open symbols). The time required for the half maximum response to ryanodine is plotted. Each symbol represents the mean (SD) results obtained with one culture (10 selected cells). Identical symbols refer to the same patient. Mean results of the two, four or eight experiments per genotype and ryanodine concentration are given as columns. *Significantly different from control ($P<0.02$, Wilcoxon rank test for paired samples). (B) Data evaluated from the same experiments as in (A). Here, the area included by the response curve is plotted for the different genotypes and applied ryanodine concentrations. Mean (SD) results of the two, four or eight experiments per genotype and ryanodine concentration are given as columns. *Significant difference ($P<0.005$).

an increase in $[Ca^{2+}]_i$ in myotubes.[22] The discrepancy in our data is explained by the fact that the earlier data were obtained at room temperature. We now know that the effect of ryanodine is temperature dependent (Fig. 2).

Other possible explanations for our results would be that: (i) in the myotubes of MH patients, expression of the RYR1 gene is generally increased or (ii) RYR3 gene expression is increased. We consider these two possibilities unlikely because over-expression of the normal RYR1 in control myotubes alone does not confer the MHS phenotype to control myotubes.[21] Further, RYR3 is present only at low levels in myotubes and its expression is regulated by neurotrophic factors and electrical stimulation, factors which have little influence or should at least be constant in our culture system. Thus RYR3 probably contributes little to the observed ryanodine effects. In summary, the best explanation for the observed data is that in muscle cultured from patients, the threshold for activation of the mutant RYRs is lowered.

It is still not clear if muscle fibres from MHS probands have a higher resting cytoplasmic Ca^{2+} concentration than controls. In some studies with porcine MH, it was claimed that resting cytoplasmic Ca^{2+} concentrations were not increased[32] whereas in others a four-fold increase was reported.[33] A similar increase in resting $[Ca^{2+}]_i$ was reported for MHS individuals.[34] Our results with myotubes do not support increased resting concentrations but possibly other mutations lead to different results. For the RYR1 mutation, Arg163Cys, the calcium resting concentration of cultured myotubes was similar to controls.[21] Pigs homozygous for the Arg615Cys mutation have a tendency to develop muscle hypertrophy without exposure to any trigger substance. An increase in Ca^{2+} release at rest or during normal muscle activity could be the reason for increased muscle growth.

Even at higher concentrations of ryanodine (20 or 50 μmol litre^{-1}) we did not observe de-regulation of $[Ca^{2+}]_i$ (i.e. an uncontrolled increase in cytoplasmic Ca^{2+} into the micromolar range). Such a de-regulation is assumed to occur during an MH crisis. Presumably, expression of Ca^{2+} control proteins, including the SR Ca^{2+} pumps, is balanced in cultured muscle in such a way that increased Ca^{2+} release from RYRs cannot produce an 'MH crisis' in culture.

We conclude that muscle cultures are a promising tool for the study of MHS muscle. It seems possible to recognize the MHS phenotype in myotubes at the cellular level and to investigate if different RYR mutations lead to different responses. Thus it may be feasible to develop an MHS test based on muscle cultures.

Acknowledgements

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References

1 Denborough MA, Lovell RRH. Anaesthetic deaths in a family. *Lancet* 1960; **2**: 45

2 Gronert GA. Malignant hyperthermia. In: Engel AG, Franzini-Armstrong C, eds. *Myology. Basic and Clinical*, 2nd Edn. New York: McGraw-Hill, 1994; 1661–78

3 Denborough M. Malignant hyperthermia. *Lancet* 1998; **352**: 1131–6

4 Phillips MS, Fujii J, Khanna VK, *et al.* The structural organization of the human skeletal muscle ryanodine receptor (RYR1) gene. *Genomics* 1996; **34**: 24–41

5 Mickelson JR, Louis CF. Malignant hyperthermia: excitation–contraction coupling, Ca^{2+} release channel, and cell Ca^{2+} regulation defects. *Physiol Rev* 1996; **76**: 537–92

6 Manning BM, Quane KA, Ording H, *et al.* Identification of novel mutations in the ryanodine-receptor gene (RYR1) in malignant hyperthermia: genotype–phenotype correlation. *Am J Hum Genet* 1998; **62**: 599–609

7 Barone V, Massa O, Intravaia E, *et al.* Mutation screening of the RYR1 gene and identification of two novel mutations in Italian malignant hyperthermia families. *J Med Genet* 1999; **36**: 115–18

8 Richter M, Schleithoff L, Deufel T, Lehmann-Horn F, Herrmann-Frank A. Functional characterization of a distinct ryanodine receptor mutation in human malignant hyperthermia-susceptible muscle. *J Biol Chem* 1997; **272**: 5256–60

9 Mickelson JR, Litterer LA, Jacobson BA, Louis CF. Stimulation and inhibition of [^{3}H]ryanodine binding to sarcoplasmic reticulum from malignant hyperthermia susceptible pigs. *Arch Biochem Biophys* 1990; **278**: 251–7

10 Sudbrak R, Procaccio V, Klausnitzer M, *et al.* Mapping of a further malignant hyperthermia susceptibility locus to chromosome 3q13.1. *Am J Hum Genet* 1995; **56**: 684–91

11 Iles DE, Lehmann Horn F, Scherer SW, *et al.* Localization of the gene encoding the alpha 2/delta-subunits of the L-type voltage-dependent calcium channel to chromosome 7q and analysis of the segregation of flanking markers in malignant hyperthermia susceptible families. *Hum Mol Genet* 1994; **3**: 969–75

12 Robinson RL, Monnier N, Wolz W, *et al.* A genome wide search for susceptibility loci in three European malignant hyperthermia pedigrees. *Hum Mol Genet* 1997; **6**: 953–61

13 Monnier N, Procaccio V, Stieglitz P, Lunardi J. Malignant-hyperthermia susceptibility is associated with a mutation of the alpha 1-subunit of the human dihydropyridine-sensitive L-type voltage-dependent calcium-channel receptor in skeletal muscle. *Am J Hum Genet* 1997; **60**: 1316–25

14 European MH group. A protocol for the investigation of malignant hyperpyrexia (MH) susceptibility. *Br J Anaesth* 1984; **56**: 1267–9

15 Larach MG. Standardization of the caffeine halothane muscle contracture test. North American Malignant Hyperthermia Group. *Anesth Analg* 1989; **69**: 511–15

16 Hartung E, Koob M, Anetseder M, *et al.* Malignant hyperthermia (MH) diagnostics: a comparison between the halothane–caffeine- and the ryanodine-contracture-test results in MH susceptible, normal and control muscle. *Acta Anaesthesiol Scand* 1996; **40**: 437–44

17 Hopkins PM, Ellis FR, Halsall PJ, Stewart AD. An analysis of the predictive probability of the in vitro contracture test for determining susceptibility to malignant hyperthermia. *Anesth Analg* 1997; **84**: 648–56

18 Ording H, Glahn K, Gardi T, Fagerlund T, Bendixen D. 4-Chloro-m-cresol test–a possible supplementary test for diagnosis of malignant hyperthermia susceptibility. *Acta Anaesthesiol Scand* 1997; **41**: 967–72

19 Herrmann-Frank A, Richter M, Sarkozi S, Mohr U, Lehmann-Horn F. 4-Chloro-m-cresol, a potent and specific activator of the skeletal muscle ryanodine receptor. *Biochim Biophys Acta* 1996; **1289**: 31–40

20 Gallant EM, Jordan RC. Porcine malignant hyperthermia: genotype and contractile threshold of immature muscles. *Muscle Nerve* 1996; **19**: 68–73

21 Censier K, Urwyler A, Zorzato F, Treves S. 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. *J Clin Invest* 1998; **101**: 1233–42

22 Uetz U, Pressmar J, Brinkmeier H, Iaizzo PA, Rüdel R, Lehmann-Horn F. Bestimmung der intrazellulären Kalziumkonzentration an menschlichen Skelettmuskelfasern und Myotuben von Patienten mit Anlage zur malignen Hyperthermie. *Akt Neurol* 1995; **22**: S23

23 Brinkmeier H, Mutz JV, Seewald MJ, Melzner I, Rüdel R. Specific modifications of the membrane fatty acid composition of human myotubes and their effects on the muscular sodium channels. *Biochim Biophys Acta* 1993; **1145**: 8–14

24 Föhr KJ, Warchol W, Gratzl M. Calculation and control of free divalent cations in solutions used for membrane fusion studies. *Methods Enzymol* 1993; **221**: 149–57

25 Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985; **260**: 3440–50

26 Pressmar J, Brinkmeier H, Seewald MJ, Naumann T, Rüdel R. Intracellular Ca^{2+} concentrations are not elevated in resting cultured muscle from Duchenne (DMD) patients and in MDX mouse muscle fibres. *Pflügers Arch* 1994; **426**: 499–505

27 Kyselovic J, Leddy JJ, Ray A, Wigle J, Tuana BS. Temporal differences in the induction of dihydropyridine receptor subunits and ryanodine receptors during skeletal muscle development. *J Biol Chem* 1994; **269**: 21770–7

28 Ray A, Kyselovic J, Leddy JJ, Wigle JT, Jasmin BJ, Tuana BS. Regulation of dihydropyridine and ryanodine receptor gene expression in skeletal muscle. Role of nerve, protein kinase C, and cAMP pathways. *J Biol Chem* 1995; **270**: 25837–44

29 Schmoelzl S, Leeb T, Brinkmeier H, Brem G, Brenig B. Regulation of tissue-specific expression of the skeletal muscle ryanodine receptor gene. *J Biol Chem* 1996; **271**: 4763–9

30 Tarroni P, Rossi D, Conti A, Sorrentino V. Expression of the ryanodine receptor type 3 calcium release channel during development and differentiation of mammalian skeletal muscle cells. *J Biol Chem* 1997; **272**: 19808–13

31 Flucher BE, Andrews SB, Fleischer S, Marks AR, Caswell A, Powell JA. Triad formation: organization and function of the sarcoplasmic reticulum calcium release channel and triadin in normal and dysgenic muscle in vitro. *J Cell Biol* 1993; **123**: 1161–74

32 Iaizzo PA, Klein W, Lehmann Horn F. Fura-2 detected myoplasmic calcium and its correlation with contracture force in skeletal muscle from normal and malignant hyperthermia susceptible pigs. *Pflügers Arch* 1988; **411**: 648–53

33 Allen PD, Lopez JR, Sanchez V, Ryan JF, Sreter FA. EU 4093 decreases intracellular $[Ca^{2+}]$ in skeletal muscle fibers from control and malignant hyperthermia-susceptible swine. *Anesthesiology* 1992; **76**: 132–8

34 Lopez JR, Gerardi A, Lopez MJ, Allen PD. Effects of dantrolene on myoplasmic free $[Ca^{2+}]$ measured in vivo in patients susceptible to malignant hyperthermia. *Anesthesiology* 1992; **76**: 711–19