Phenotyping malignant hyperthermia susceptibility by measuring halothane-induced changes in myoplasmic calcium concentration in cultured human skeletal muscle cells

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Background. Malignant hyperthermia (MH) is a potentially lethal disease triggered by volatile anaesthetics and succinylcholine in genetically predisposed individuals. Because of the heterogenetic nature of MH, a simple genetic-based diagnostic test is not feasible and diagnosis requires an invasive open muscle biopsy followed by the *in vitro* contracture test (IVCT). Our aim was to establish if measurements of halothane-induced increases in intracellular calcium ion concentration $[Ca^{2+}]_i$ in cultured human skeletal muscle cells can be used to phenotype MH susceptibility and if different mutations in the ryanodine receptor (RYR1) gene affect halothane-induced increases in $[Ca^{2+}]_i$.

Methods. Primary cultures of human skeletal muscle cells were established from 54 individuals diagnosed by the IVCT according to the protocol of the European MH Group as: MH susceptible (n=22), MH negative (n=18) or MH equivocal (n=14). All individuals were screened for the presence of the most common mutations in the RYR1 gene. $[Ca^{2+}]_i$ was measured by fluorescent digital microscopy using fura-2/AM in 10 cells from each patient at five different halothane concentrations.

Results. The halothane-induced increase in $[Ca^{2+}]_i$ differed significantly between the three diagnostic groups. Different mutations of the RYR1 gene did not have a specific impact on halothane-induced increases in $[Ca^{2+}]_i$.

Conclusions. Measurements of $[Ca^{2+}]_i$ in human skeletal muscle cells can be used to phenotype MH susceptibility; however, we did not observe a specific effect of any mutation in the RYR1 gene on the halothane-induced increase in $[Ca^{2+}]_i$.

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Malignant hyperthermia (MH) is a pharmacogenetic disease triggered by halogenated anaesthetics and succinylcholine in predisposed individuals.[1–3] The incidence of MH episodes is about 1 in 15 000 anaesthetics in children and 1 in 50 000 in adults, although these figures may underestimate the true prevalence, since many reactions occur in patients who have previously come into contact with trigger agents and had uneventful anaesthesia.[4 5] Symptoms characterizing an impending MH crisis include a rapid and sustained increase in body temperature, skeletal muscle rigidity, acidosis, rhabdomyolysis and tachycardia[6] or any combination of the above (see review by Gronert and colleagues[3]). If such a reaction is not recognized in its early phases and treatment quickly initiated, the patient is likely to die.

In their daily lives MH-predisposition does not pose a threat to MH-susceptible (MHS) individuals, so the major goal of MH diagnostics is to identify susceptible individuals before the administration of trigger agents.[7 8] To date the gold standard of MH diagnosis is the *in vitro* contracture test (IVCT). This invasive procedure involves an open muscle biopsy and *in vitro* challenge of muscle strips with halothane or caffeine. According to the guidelines of the

Table 1 Family of origin (a capital letter indicates members belonging to the same family, the absence of a letter indicates single members of a family), identification of malignant hyperthermia (MH)-linked ryanodine receptor (RYR1) mutation and *in vitro* contracture test (IVCT) of muscle biopsies. MHN=MH negative; MHS=MH susceptible; MHE=MH equivocal

				IVCT – halothane		IVCT – caffeine	
Number	**Status**	**Family**	**RYR1 mutation**	**Threshold (%)**	**Contracture (g) at 2%**	**Threshold (mM)**	**Contracture (g) at 2 mM**
1	MHN	A		>3	0.05	>4	0
2	MHN	A		>3	0.15	4	0
3	MHN	A		>3	0.05	>4	0
4	MHN	B		3	0.15	>4	0.1
5	MHN	C		>3	0.1	>4	0.1
6	MHN	C		3	0.15	4	0
7	MHN	D		>3	0.15	>4	0
8	MHN	D		3	0.1	4	0
9	MHN	D		>3	0.05	>4	0
10	MHN	D		>3	0.05	>4	0.05
11	MHN	D		>3	0.05	>4	0.05
12	MHN	D		>3	0.05	>4	0.05
13	MHN			>3	0.1	4	0
14	MHN			>3	0.05	>4	0
15	MHN			>3	0.1	>4	0.1
16	MHN			>3	0.1	>4	0
17	MHN			>3	0	4	0.1
18	MHN			>3	0.05	4	0.05
1	MHE	A	Arg614Cys	1	0.85	3	0
2	MHE	A		1	1.15	4	0.05
3	MHE	B		2	0.35	4	0.05
4	MHE	B		1	0.4	4	0.1
5	MHE	C		1	1.7	3	0
6	MHE	D		2	0.3	4	0
7	MHE	E		1	0.7	4	0
8	MHE	E		1	1	3	0.05
9	MHE			2	0.3	>4	0.1
10	MHE			0.5	1.3	3	0.1
11	MHE			2	0.3	>4	0.05
12	MHE			1	1.35	3	0.05
13	MHE			2	0.4	4	0.05
14	MHE			1	0.85	3	0.05
1	MHS	A	Arg614Cys	0.5	5.1	0.5	2.7
2	MHS	B	Gly2434Arg	0.5	1.3	2	0.25
3	MHS	C	Val2168Met	0.5	5.1	1.5	1.2
4	MHS	C	Val2168Met	0.5	4.6	1.5	2.7
5	MHS	C	Val2168Met	0.5	4.5	1	2.35
6	MHS	C	Val2168Met	0.5	2.1	1.5	1.35
7	MHS	D	Val2168Met	0.5	3.75	1	1.45
8	MHS	D	Val2168Met	0.5	2.65	1	1.65
9	MHS	E		0.5	3.9	1	1.9
10	MHS	F	Val2168Met	1	1.45	2	0.2
11	MHS	F	Val2168Met	0.5	3.55	1	2.25
12	MHS	G		0.5	3.5	1.5	0.4
13	MHS	G		0.5	3.05	1.5	0.65
14	MHS	G		1	1.6	0.5	1.6
15	MHS	G		0.5	2.6	1	1.3
16	MHS		Val2168Met	0.5	1.8	2	0.8
17	MHS		Arg2458Cys	0.5	1	1.5	0.7
18	MHS			1	2	1.5	0.4
19	MHS			0.5	1.95	1	1.3
20	MHS		Val2168Met	0.5	1.7	1.5	0.7
21	MHS		Val2168Met	1	1.05	1	1.3
22	MHS			1	0.9	2	0.25

European MH Group, patients are then diagnosed as MHS or MH negative (MHN) on the basis of contractile threshold and sensitivity of the muscle bundles to halothane and caffeine. If contracture is achieved only with either caffeine or halothane, the patient is diagnosed as MH equivocal (MHE).[6 9]

The underlying cause of MH is an abnormality in skeletal muscle calcium metabolism.[10 11] Therefore, alterations in proteins involved in the regulation of the intracellular calcium concentration ($[Ca^{2+}]_i$), such as the calcium pump, the calcium release channel or other proteins implicated in excitation–contraction coupling, could potentially cause

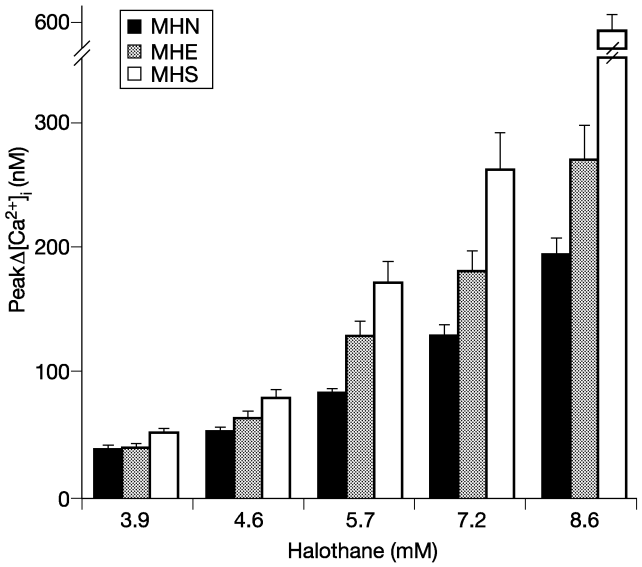


Fig 1 Average peak change (Δ) in $[Ca^{2+}]_i$ induced by different halothane concentrations in human skeletal muscle cells. Single-cell intracellular calcium measurements were performed with the fluorescent calcium indicator fura-2/AM, using a Zeiss imaging system attached to an Axiovert fluorescent microscope, as described in the Methods section. Results are mean (SEM); the number of cells measured ranged between 132 and 246 for each halothane concentration and diagnostic group. The halothane-induced increase in $[Ca^{2+}]_i$ was significantly different between the three diagnostic groups ($P<0.001$) and between the different halothane concentrations ($P<0.0001$; repeated measurements ANOVA). Fisher's PLSD post-hoc test revealed significant differences ($P<0.001$) between all halothane concentrations, with the exception of 3.9 *vs* 4.6 mM.

MH. Studies into the molecular mechanisms underlying this disease have demonstrated that the ryanodine receptor (RYR1) gene on human chromosome 19q is the primary locus of MH.[2 7 12]Mutation screening has identified more than 30 mutations in the RYR1 gene so far. Although approximately 50% of MH families have mutations in the RYR1 gene, linkage studies have revealed that this is a heterogenetic disease.[7 12–16]

The aim of the present study was to determine if halothane-induced increases in $[Ca^{2+}]_i$ in human skeletal muscle cells can be used to phenotype MH susceptibility and if different mutations in the RYR1 gene have a distinct effect on the halothane-induced increases in $[Ca^{2+}]_i$.

Methods

Study protocol

In order to determine if halothane-induced increases in $[Ca^{2+}]_i$ in human skeletal muscle cells can be used to phenotype MH susceptibility, we compared the results obtained by the IVCT with results obtained by measuring the halothane-induced increase in $[Ca^{2+}]_i$ in cultured skeletal muscle cells. Patients included in the study were tested by the IVCT according to the protocol of the European MH Group.[9] On the same day, primary skeletal muscle cell cultures were established from surplus fragments of the muscle biopsies. For each patient, increases in $[Ca^{2+}]_i$ were measured in 10 muscle cells after exposure to 3.9, 4.6, 5.7, 7.2 or 8.6 mM halothane. These concentrations were chosen because in a previous study[17] we found that the biggest differences in $[Ca^{2+}]_i$ between cells from MHS and MHN individuals were obtained in this range of halothane concentrations.

In order to determine if different mutations have a different effect on halothane-induced increases in $[Ca^{2+}]_i$, all patients were screened for nine of the most common mutations in the RYR1 gene (described below) and the increases in $[Ca^{2+}]_i$ in subjects carrying different mutations, as well as in subjects carrying the same mutation in the RYR1 gene, were analysed and compared.

Patient selection

We selected 54 patients from 26 families with a positive history of MH without clinical signs of neuromuscular disease. The age range of the patients was 8–59 yr. Twenty-two patients were diagnosed as MHS, 18 as MHN and 14 as MHE by IVCT. Patients were classified as MHS if a contracture force $\geqslant$0.2 g was elicited by at least 2% halothane and 2 mM caffeine, as MHE if a contracture force $\geqslant$0.2 g was elicited only by either caffeine or halothane, and MHN if contractures $\geqslant$0.2 g were not reached with either trigger agent. The characteristics of the individuals involved in this study, including contractures obtained from the muscle strips during the IVCT, family of origin and mutation found are given in Table 1.

The protocol for the study was approved by the University of Basel Hospital Ethics Committee and written informed consent was obtained from patients.

Materials

Dulbecco modified Eagle (DME) medium containing 4.5 mg ml^{-1} glucose, fetal calf serum (FCS), horse serum, penicillin G and streptomycin was purchased from Life Technologies Ltd, Paisley, UK. Insulin was purchased from Eli Lilly and Co., Indianapolis, IN, USA. Cell culture material was from Becton Dickinson GmbH, Heidelberg, Germany. Halothane was from Halocarbon Labs, Inc., Hackensack, NJ, USA. The mammalian blood DNA isolation kit and Taq polymerase and DNA restriction enzymes were from Roche Molecular Biochemicals (Basel, Switzerland). The kit for DNA isolation from tissue was from Machery-Nagel GmbH, Düren, Germany. The polymerase chain reaction (PCR) purification kit was from Qiagen GmbH, Hilden, Germany. Fura-2/AM and ionomycin were from Sigma Chemical Co., St Louis, MO, USA. Primers were from Microsynth GmbH, Balgach,

Table 2 Halothane-induced changes (Δ) in $[Ca^{2+}]_i$, 340/380 nm ratio and integral calcium in primary cultures derived from MHN individuals: definition of cut-off values

		Percentile				
Halothane concentration (mM)	***n***	**50th**	**75th**	**90th**	**95th**	**97th**
Δ $[Ca^{2+}]_i$						
3.9	198	31	50.6	85.1	110.3	117.2
4.6	198	47	74	101	127	137.9
5.7	205	72.7	105.5	167	203.5	230.6
7.2	178	106	158.6	239.2	310.4	507.2
8.6	173	139.5	265.6	391.4	513.8	570.5
Δ 340/380 nm ratio						
3.9	198	0.07	0.09	0.13	0.15	0.176
4.6	198	0.09	0.13	0.17	0.201	0.233
5.7	205	0.13	0.187	0.27	0.347	0.409
7.2	178	0.2	0.28	0.35	0.376	0.43
8.6	173	0.25	0.422	0.57	0.677	0.733
Integral calcium						
3.9	220	2.5	4.8	7.5	9.1	9.9
4.6	209	3.8	7.6	10.4	11.7	12.8
5.7	206	4.3	9.6	12	15.3	18.3
7.2	216	8.7	13.5	22	28.7	31.1
8.6	206	13.1	23.3	34.7	40.9	45.6

Table 3 Diagnosis of MH susceptibility by single-cell calcium measurements (changes in (Δ)$[Ca^{2+}]_i$, 340/380 nm ratio and integral calcium) compared with *in vitro* contracture test (IVCT)-based diagnosis. MHN=MH negative; MHS=MH susceptible; MHE=MH equivocal

	Diagnosis by IVCT		
	MHN (*n*=18)	**MHE (*n*=14)**	**MHS (*n*=22)**
Δ $[Ca^{2+}]_i$ >95th percentile	1 (6%)	4 (29%)	15 (68%)
Δ $[Ca^{2+}]_i$ 97th percentile	0	2 (14%)	11 (50%)
Δ ratio >95th percentile	1 (6%)	5 (36%)	15 (68%)
Δ ratio >97th percentile	1 (6%)	4 (29%)	13 (59%)
Integral calcium >95th percentile	2 (11%)	7 (50%)	12 (55%)
Integral calcium >97th percentile	1 (6%)	5 (36%)	11 (50%)

Switzerland. All other chemicals were reagent grade or of highest available grade.

Human skeletal muscle cell cultures

Primary human muscle cell cultures were established from surplus fragments taken from biopsies of patients undergoing diagnostic IVCT as described previously.[17] Cells were grown in DME, 10% horse serum, insulin 5 ng ml^{-1}, 2 mM glutamine, antibiotics and 7 mM HEPES, pH 7.4 (proliferative medium) under standard cell culture conditions. For cryopreservation, about 10^6 cells were resuspended in DME containing 40% FCS and 10% DMSO and were stored in liquid nitrogen.

$[Ca^{2+}]_i$ measurements

For measurements of $[Ca^{2+}]_i$, cells were trypsinized and transferred from tissue culture flasks to glass coverslips and allowed to grow in proliferative medium until groups of cells were visible. We have previously demonstrated that under these culture conditions the cells acquire skeletal-muscle-specific proteins such as sarcomeric α-actinin and type-1 RYR,[17] although they do not fully differentiate into multinuclear myotubes. Such primary cultures exhibit a degree of variability in the maturity of individual cells.

Cells to be tested were loaded with the fluorescent calcium indicator fura-2/AM. Single-cell calcium measurements using fluorescence microscopy were performed on 10 cells before and after the addition of halothane. A new coverslip containing fura-2/AM-loaded cells was used for each halothane concentration. For each experiment a total of 72 digital images were recorded at excitations of 340 and 380 nm and the ratio calculated, as per our previous study.[17] The values obtained during the first 10 cells (i.e. before the application of halothane) were used to calculate the resting $[Ca^{2+}]_i$. Values were then taken during halothane perfusion as well as after addition of EGTA/ionomycin and calcium ions to obtain R_{min} (the value obtained in the presence of EGTA) and R_{max} (the value obtained in the presence of high

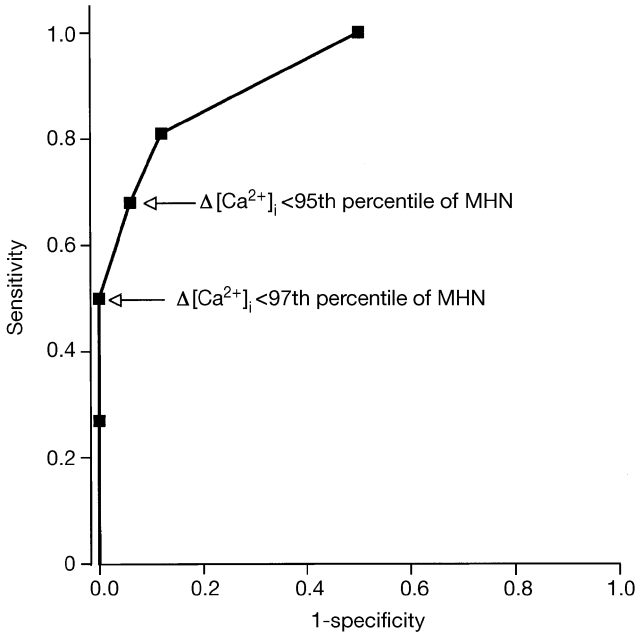


Fig 2 Receiver operating characteristic (ROC) curve comparing the diagnostic results of $[Ca^{2+}]_i$ measurements at different cut-off values (50–99th percentile) with IVCT diagnosis. The curve shows sensitivity and specificity of the $[Ca^{2+}]_i$ measurements for the diagnosis of MH susceptibility.

calcium after the addition of ionomycin). Each run lasted approximately 6 min, including the time taken to run the calibration at the end of the experiment. Halothane in DMSO was administered at the indicated concentrations from a gas-tight vial by means of a roller pump. Its actual concentration was verified by gas chromatography in several test runs using fixed flow rates, temperature and tubing. Calibration was performed using the EGTA/ionomycin/manganese chloride method.[18] The changes in fluorescence were converted into $[Ca^{2+}]_i$ using the formula $[Ca^{2+}]_i=[k_D\times(R-R_{min})/(R_{max}-R)]\times Sf2/Sb2$, where k_D (dissociation constant) of fura-2/AM was assumed to be 225 nM and Sf2 and Sb2 are the fluorescent values for Ca^{2+} free (f) and bound (b) of the indicator. These values are a constant.

The ratios were analysed and converted into $[Ca^{2+}]_i$ values (nM) using a programmable database application (Omnis 7/3 from Blyth Holding Inc., Suffolk, UK). The increase in $[Ca^{2+}]_i$ was determined by the difference between resting $[Ca^{2+}]_i$ and peak $[Ca^{2+}]_i$ after the addition of halothane. The whole transient elicited by a given halothane concentration was used to calculate the integral calcium, which reflects the total amount of calcium released.

Mutation screening

Screening for the presence of nine of the most frequent MH-linked mutations (Arg163Cys, Gly341Arg, Arg614Cys, Arg614Leu, Arg2163Cys, Val2168Met, Gly2434Arg, Arg2458Cys, Arg2458His) was performed by genomic DNA PCR amplification followed by restriction enzyme digestion and polyacrylamide gel electrophoresis. Total genomic DNA was isolated from either peripheral blood or muscle fragments not used for IVCT. PCR conditions and primer sequences were as described previously.[19]

Statistical analysis

The $[Ca^{2+}]_i$ measurements from the three diagnostic groups were compared using repeated measurements ANOVA. Within each halothane concentration, the results from the three diagnostic groups were compared using Fishers's protected least significant difference (PLSD) post-hoc test.

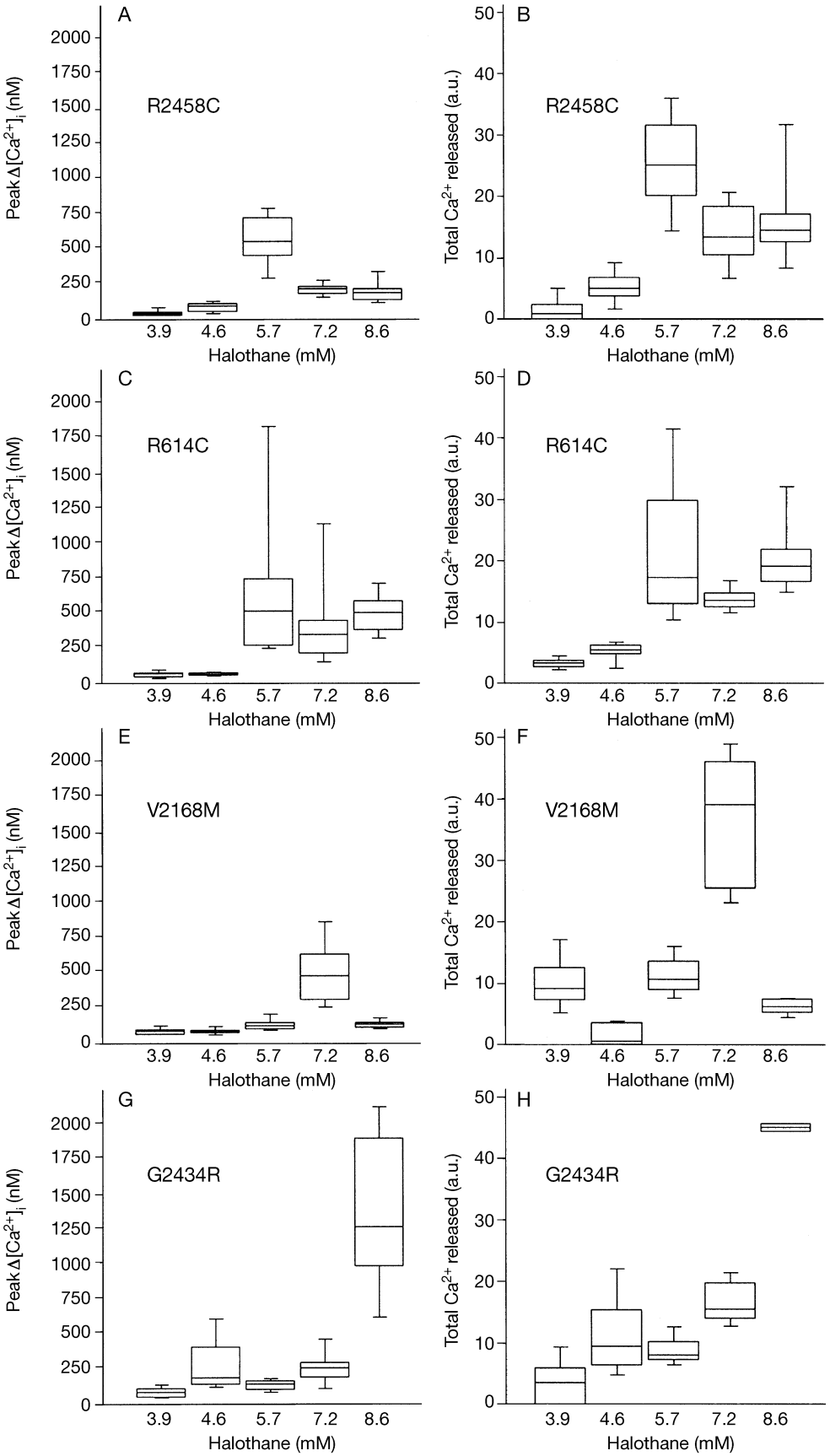
To determine normal $[Ca^{2+}]_i$ values, all measurements of the MHN cells were pooled and median values, as well as 75th, 90th, 95th, 97th and 99th percentiles, were calculated for each halothane concentration. These percentile values were used to define different cut-off values. Median values were calculated for each individual at the five halothane concentrations and compared with the cut-off values defined by the MHN population. If the cut-off value was exceeded at any concentration of halothane, the patient was classified as MHS.

StatView (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis.

Results

MH phenotyping by $[Ca^{2+}]_i$ measurements

We investigated intracellular calcium homeostasis in primary skeletal muscle cells obtained from 22 individuals diagnosed as MHS, 14 individuals diagnosed as MHE and 18 individuals diagnosed as MHN by IVCT, according to the European MH Group protocol. Single-cell $[Ca^{2+}]_i$ measurements were performed at the five halothane concentrations in 10 fura-2/AM-loaded cells; the fluorescent changes induced by halothane were converted into $[Ca^{2+}]_i$ values by performing signal calibration at the end of each experiment. Some cells contracted when high intracellular calcium concentrations were reached, so the fluorescence signal could not be calibrated. These cells were therefore excluded from Figure 1, in which we show the peak $[Ca^{2+}]_i$ induced by halothane. The results depicted in Figure 1 show that halothane-induced increases in $[Ca^{2+}]_i$ in primary skeletal muscle cells differed significantly between MHS, MHE and MHN individuals. The increases in $[Ca^{2+}]_i$ were dose dependent ($P<0.0001$; repeated measurements ANOVA; Figure 1). In addition, the increases in $[Ca^{2+}]_i$ in muscle cells from MHS and MHE individuals were shifted to lower halothane concentrations when compared with cells from MHN individuals ($P<0.0001$; repeated measurements ANOVA, Figure 1). Fisher's PLSD post-hoc test revealed significant differences ($P<0.001$) between all


A
R2458C
Peak Δ[Ca2+]i (nM)
2000
1750
1500
1250
1000
750
500
250
0
3.9
4.6
5.7
7.2
8.6
Halothane (mM)
B
R2458C
Total Ca2+ released (a.u.)
50
40
30
20
10
0
3.9
4.6
5.7
7.2
8.6
Halothane (mM)
C
R614C
Peak Δ[Ca2+]i (nM)
2000
1750
1500
1250
1000
750
500
250
0
3.9
4.6
5.7
7.2
8.6
Halothane (mM)
D
R614C
Total Ca2+ released (a.u.)
50
40
30
20
10
0
3.9
4.6
5.7
7.2
8.6
Halothane (mM)
E
V2168M
Peak Δ[Ca2+]i (nM)
2000
1750
1500
1250
1000
750
500
250
0
3.9
4.6
5.7
7.2
8.6
Halothane (mM)
F
V2168M
Total Ca2+ released (a.u.)
50
40
30
20
10
0
3.9
4.6
5.7
7.2
8.6
Halothane (mM)
G
G2434R
Peak Δ[Ca2+]i (nM)
2000
1750
1500
1250
1000
750
500
250
0
3.9
4.6
5.7
7.2
8.6
Halothane (mM)
H
G2434R
Total Ca2+ released (a.u.)
50
40
30
20
10
0
3.9
4.6
5.7
7.2
8.6
Halothane (mM)

halothane concentrations, with the exception of 3.9 *vs* 4.6 mM.

Cut-off values for the determination of MH susceptibility by $[Ca^{2+}]_i$ measurements were calculated on the basis of results from the individuals diagnosed as MHN by the IVCT and are presented in Table 2. Using the 95th percentile as a cut-off value, MH susceptibility was diagnosed by measurements of $[Ca^{2+}]_i$ in 15 of 22 MHS patients (68%), 4 of 14 MHE patients (29%) and 1 of 18 MHN patients (6%) (Table 3). We calculated sensitivity and specificity by comparing results obtained by the $[Ca^{2+}]_i$ measurements with results obtained by the IVCT. Using the 95th percentile as a cut-off value, the specificity was 0.94 (95% confidence intervals [CI] 0.73 to 1.0, Table 3, Fig. 2) and the sensitivity was 0.68 (95% CI 0.45 to 0.86). Taking into account that the specificity of the IVCT using the European MH Group protocol is 0.93,[20] the actual number of MHS could be 20 instead of 22. This would increase the sensitivity of $[Ca^{2+}]_i$ measurements to 0.75 (95% CI 0.51 to 0.91).

Effect of RYR1 gene mutations on halothane-induced increases in $[Ca^{2+}]_i$

We next investigated whether the presence of different RYR1 gene mutations has an impact on halothane-induced $[Ca^{2+}]_i$ transients and whether cells from different patients carrying the same mutation respond in a similar way. Figure 3 shows a box plot of the halothane-induced $[Ca^{2+}]_i$ transients (peak $[Ca^{2+}]_i$ and total amount of $[Ca^{2+}]_i$ released) from individuals carrying four different MH-linked mutations. The increase in $[Ca^{2+}]_i$ is particularly evident at halothane concentrations $\geqslant$5.7 mM and was irrespective of the mutation.

The halothane 'trigger concentration' appears to vary between individuals with different mutations.[19 21] In order to study if this is specific for a given mutation or a more general phenomenon, we analysed the halothane-induced peak $[Ca^{2+}]_i$ in cells obtained from unrelated individuals carrying the same Val2168Met mutation. Halothane trigger concentrations and peak $[Ca^{2+}]_i$ were different in these unrelated individuals (Fig. 4), suggesting that the genetic background influences the halothane-induced increase in $[Ca^{2+}]_i$.

Discussion

In the present report we show that halothane-induced increases in $[Ca^{2+}]_i$ in human skeletal muscle cells can be used to phenotype MH susceptibility. However, our results show that different mutations in the RYR1 gene have no specific effect on halothane-induced increases in $[Ca^{2+}]_i$. Measurements of halothane-induced increases in $[Ca^{2+}]_i$ in human skeletal muscle cells confirmed the IVCT diagnosis of MH susceptibility in 15 of 22 MHS individuals (68%) when the 95th percentile of the values obtained from the MHN population was used as a cut-off value. Taking into account that the specificity of the IVCT following the protocol of the European MH Group is 0.93, this could reduce the number of MHS individuals to 20, leading to an increase in the number of coinciding MHS diagnoses (15 of 20, 75%). Only one MHN individual exceeded the 95th percentile cut-off value. This discordance could be due to either a wrong IVCT diagnosis or oversensitivity of diagnosis based on measurements of halothane-induced increases in $[Ca^{2+}]_i$.

In seven of 22 individuals the IVCT diagnosis of MHS could not be confirmed by halothane-induced increases in $[Ca^{2+}]_i$. This could be due to either a reduced specificity of the IVCT (not every MHS diagnosis can be confirmed by molecular genetic analysis), or to an overestimation of the halothane-induced increases in $[Ca^{2+}]_i$ of the true 'normal' population—in the present study 'normal' $[Ca^{2+}]_i$ values were obtained from individuals from MHS families, not from non-MH-linked individuals.

In this study we confirm and extend our previous results. In fact, our earlier work was based on observations carried out on two MHS subjects and three MHN subjects while in this study we also included 14 MHE individuals, and the number of patients involved was 10 times larger. In the study by Censier and colleagues,[17] we observed that the maximum difference between MHS and MHN, as far as halothane-induced increases in $[Ca^{2+}]_i$ are concerned, occurred at halothane concentrations of 5.7–9.5 mM. In addition, we confirm that the mean increase in $[Ca^{2+}]_i$ of cells from MHN individuals occurring at 8.6 mM halothane is similar to that of cells from MHS individuals at 5.7 mM halothane (203 nM *vs* 167 nM, respectively; P=0.269, student's t-test for unpaired samples).

The response of cells presented in our previous report[17] seem to be different from those found in this study. In the present study we examined a larger sample than previously and we believe that the values obtained are more likely to represent the MHN population. We would like to point out that it is very important to perform $[Ca^{2+}]_i$ measurements at several halothane concentrations and we suggest concentrations ranging around 5.7 mM since this is the concentration where maximal differences can be observed.

Fig 3 Halothane-induced $[Ca^{2+}]_i$ response of cultured human skeletal muscle cells for four individuals. Single-cell $[Ca^{2+}]_i$ measurements were performed as described in Figure 1. The box plot shows median peak Δ $[Ca^{2+}]_i$ and median total calcium release induced at each halothane concentration, together with 10th, 25th, 75th and 90th percentiles. Panels A, C, E and G show the peak $[Ca^{2+}]_i$; panels B, D, F and H show total amount of calcium released (arbitrary units, a.u.) by halothane in individuals carrying the indicated RYR1 mutation. Mutation screening was performed as described in the Methods section.

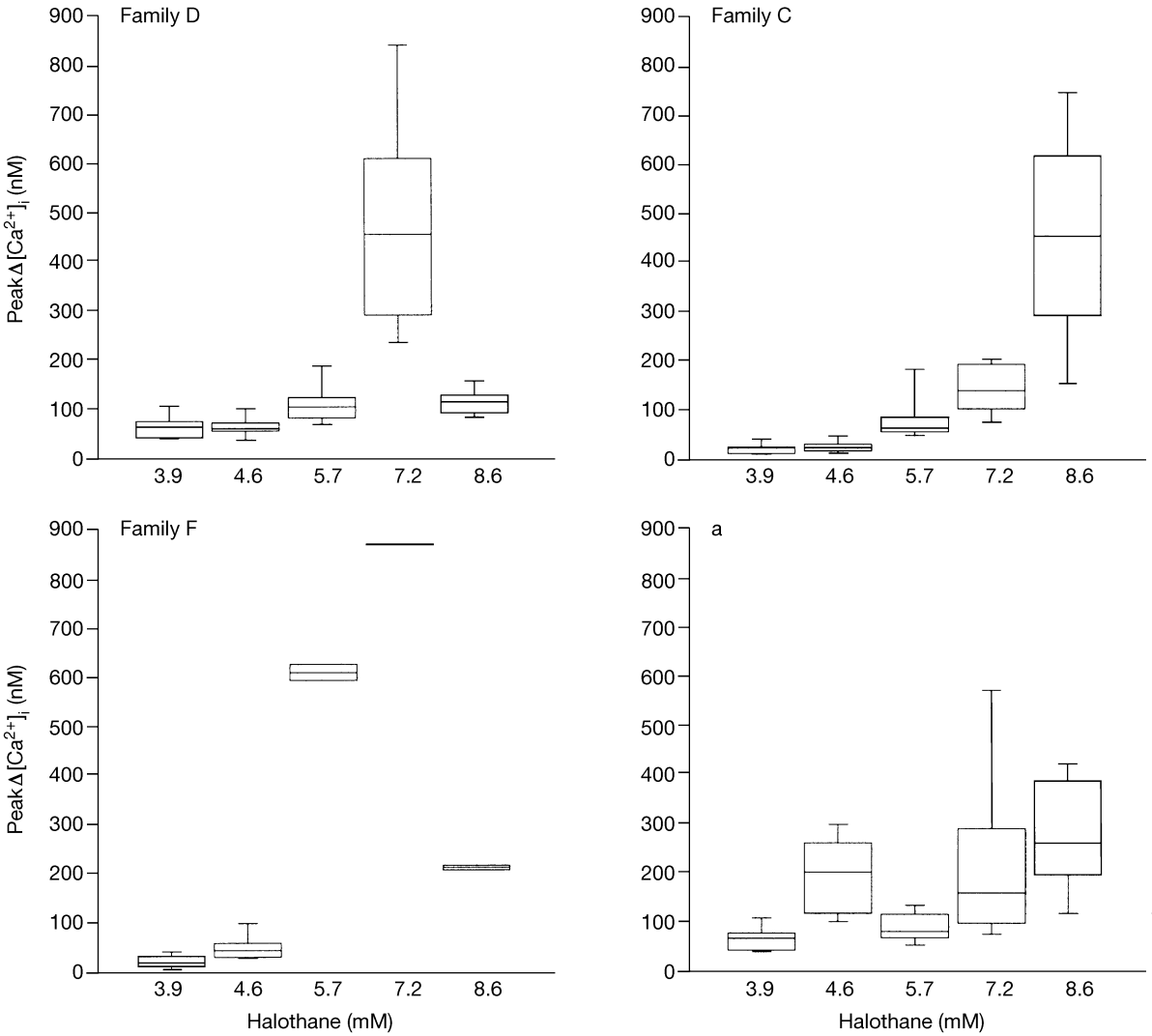


Fig 4 Halothane-induced $[Ca^{2+}]_i$ response of cultured human skeletal muscle cells from single members of four families carrying the same Val2168Met mutation (families D, C and F – as given in Table 1; the individual shown in panel a was the only member of this family investigated). Median peak Δ $[Ca^{2+}]_i$ induced by each halothane concentration, together with 10th, 25th, 50th, 75th and 90th percentile are shown in the box plot. Conditions are described in the legend to Figure 1.

In this study we used small fragments of tissue (2–4 mm^3) left over from muscle biopsies. We have already assessed the feasibility of obtaining primary cultures from needle biopsies (unpublished observations). This approach offers obvious physical advantages for the patients, as well as being cheaper. Obviously there are some limitations in the use of this novel approach: (i) the need for cell culture and fluorescent calcium imaging facilities, (ii) the fact that cells cultured from the biopsies give rise to heterogeneous cell populations that do not respond to halothane in a uniform way, (iii) not all individuals diagnosed as MHS by IVCT had an halothane-induced increase in $[Ca^{2+}]_i$ beyond that of the MHN population.

As to the future of MH diagnostics, one can envisage that individuals from families with defined mutations could first undergo genetic testing.[22] If no mutation is found, they could undergo a skeletal muscle needle biopsy followed by measurements of halothane-induced increases in $[Ca^{2+}]_i$. If measurements of $[Ca^{2+}]_i$ do not reveal MH susceptibility, then an IVCT could be performed. With this less invasive and stepwise approach, the high sensitivity of the IVCT and thus the high safety level of MH diagnostics is maintained. In fact if these criteria were used, only three of the 22 MHS individuals and seven of the 14 MHE individuals would still require the invasive IVCT. For the time being, however, all the MHN individuals would still require IVCT testing.

The common final pathway for MH is the loss of the fine regulation of calcium homeostasis in muscle cells. The present investigation is not only potentially important from a clinical point of view, but also from a biological one. In fact, it indicates that although a variety of factors such as metabolic processes, muscle training, protein composition

and enzyme activation may influence the IVCT (at least as far as contracture force is concerned), there is an intrinsic 'defect' in the machinery involved in $[Ca^{2+}]_i$ homeostasis in MHS individuals. Each individual may compensate for the molecular defect in a variety of ways, which may be influenced by the specific mutation present as well as other genetic and 'environmental' factors. This hypothesis is supported by the fact that the halothane-induced changes in $[Ca^{2+}]_i$ in cells from MHS individuals bearing the same point mutation but with a different genetic background do not coincide, and because MHS individuals with RYR1 mutations do not undergo MHS reactions at every contact with trigger agents.[4 5] Our results also show that the penetrance of a given mutation varies between individuals. This observation would be expected in view of the fact that the RYR must assemble as a tetramer in order to function as a calcium channel. The results of this study, as well as data from IVCT, tend to favour the hypothesis that mutated channels are probably randomly distributed, giving rise to 'heterogeneous' responses, but the presence of a single mutated channel is sufficient to confer the MHS phenotype. This hypothesis is supported by our previous work,[17] in which the normal phenotype was not reconstituted by transfecting MHS cells with wild-type channels.

In conclusion, measurements of $[Ca^{2+}]_i$ may be useful in increasing the accuracy of MH phenotyping and may therefore be used as a complementary method for the diagnosis of MH susceptibility.

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