

Patients with Malignant Hyperthermia Demonstrate an Altered Calcium Control Mechanism in B Lymphocytes

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Background: Altered Ca^{2+} homeostasis in skeletal muscle is a key molecular event triggering malignant hyperthermia (MH) in malignant hyperthermia-susceptible (MHS) individuals. Genetic studies have shown that mutations in the type 1 ryanodine receptor (*RYR1*) are associated with MH susceptibility. Because human B lymphocytes express the *RYR1*, it is hypothesized that Ca^{2+} homeostasis in B lymphocytes is altered in MHS individuals.

Methods: This study investigated the Ca^{2+} response of B cells to caffeine and 4-chloro-m-cresol in 13 MHS and 21 MH-negative (MHN) individuals who had been diagnosed by caffeine halothane contracture test (CHCT) and 18 healthy volunteers. Changes in $[Ca^{2+}]_i$ in B cells were measured directly in fluo-3 loaded cells using a dual-color flow cytometric technique. Further, B cell phenotype was correlated with CHCT results in a family with the Val2168Met (G6502A) mutation.

Results: Caffeine-induced (50 mM) increases in $[Ca^{2+}]_i$ in B cells were significantly greater in MHS than in MHN ($P = 0.0004$), control ($P = 0.0001$) or non-MHS (MHN and control) individuals ($P < 0.0001$). The 4-chloro-m-cresol-induced (400 μ M) increases in $[Ca^{2+}]_i$ were also significantly different between MHS and controls ($P = 0.003$) or between MHS and non-MHS (MHN and control) individuals ($P = 0.0078$). A study of a family with the Val2168Met mutation demonstrated expression of the *RYR1* mRNA mutant in B cells from the family members with MHS phenotype and a clear segregation of genotype with B-cell phenotype.

Conclusion: The Ca^{2+} responses to caffeine or 4-chloro-m-cresol in B lymphocytes showed significant differences between MHS and MHN (or control) individuals. Although the molecular mechanisms of these alterations are currently unde-

termined, the results suggest that the enhanced Ca^{2+} responses are associated with mutations in the *RYR1* gene in some MHS individuals.

MALIGNANT hyperthermia (MH) is a pharmacogenetic disorder of skeletal muscle. In MH-susceptible (MHS) individuals, halogenated anesthetic agents (halothane, enflurane, isoflurane, desflurane, and sevoflurane) and/or the depolarizing muscle relaxant, succinylcholine, trigger an abnormal acceleration of muscle metabolism that can cause a life-threatening reaction.¹⁻⁴ Initial pharmacologic and biochemical studies suggest that the underlying mechanisms in MH are conditional defects in mechanisms controlling myoplasmic concentrations of $[Ca^{2+}]_i$.⁵ Consistent with an involvement of Ca^{2+} , genetic linkage studies have shown linkage between MH and the type 1 ryanodine receptor (*RYR1*) gene of skeletal muscle on chromosome 19q13.1-13.2.^{6,7} To date, more than 22 mutations of *RYR1* have been reported to be associated with 25-50% of MHS individuals.⁷⁻¹⁰ Further support comes from gene expression studies, which demonstrated that cells expressing mutants of *RYR1* (for the 15 mutations) are more sensitive to halothane and caffeine, which initiate an increase in $[Ca^{2+}]_i$, than are the wild-type transfected cells.¹¹

Recently, we found that human B lymphocytes express not only the inositol 1,4,5-trisphosphate receptor, but also the ryanodine receptor.¹² Restriction fragment length polymorphism studies, cloning, and complementary DNA (cDNA) sequencing analysis indicated that the ryanodine receptor expressed in B cells is identical to skeletal muscle type, *RYR1*.¹² Immunoblotting and ryanodine binding studies showed that an intact 565 kd protein is indeed expressed in B cells. The RYR-stimulating agent 4-chloro-m-cresol (4CmC) induced Ca^{2+} release in a dose-dependent and ryanodine-sensitive fashion. Furthermore, depleting Ca^{2+} in the ryanodine-sensitive store by 4CmC decreased the magnitude of B-cell receptor (BCR)-mediated Ca^{2+} release, suggesting that *RYR1* is involved in regulating BCR-mediated Ca^{2+} signaling, perhaps in conjunction with the IP_3 receptor.¹² These results suggest that *RYR1* in B cells functions as a Ca^{2+} release channel during B-cell receptor-stimulated activation. We hypothesized that Ca^{2+} homeostasis in B cells was altered in MHS individuals. Therefore, this study compares Ca^{2+} response induced by two RYR-stimulating agents, caffeine or 4CmC, in B

This article is accompanied by an Editorial View. Please see: Allen PD, López JR: "Noninvasive" testing for malignant hyperthermia susceptibility. ANESTHESIOLOGY 2002 97:1045-6.

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Received from the Department of Anesthesiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland. Submitted for publication December 12, 2001. Accepted for publication May 30, 2002. Supported by grants from Uniformed Services University of the Health Sciences, Bethesda, Maryland (R08078), and Malignant Hyperthermia Association of the United States, Sherburne, New York (G28090). Presented at the 9th International Workshop on Malignant Hyperthermia, Copenhagen, Denmark, Aug 26, 1999, Annual Meeting American Society of Anesthesiologists, Dallas, Texas, Oct 9, 1999, and the 7th Malignant Hyperthermia Biopsy Center Meeting, Pittsburgh, Pennsylvania, March 23, 2001.

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Table 1. Caffeine Halothane Contracture Test Data and Clinical Profile of Susceptible Individuals with Malignant Hyperthermia in the Study

CHCT Data (mean tension, g)			
Halothane (3%)	Caffeine (2 mm)	Reason for Referral of Patient	<i>RYR1</i> Mutation
2.4	0.1	CE	Not detected
1.9	0.3	CE	Not detected
7.3	1.8	FH	Val2168Met*
4.8	0.6	FH	Asp2431Asn
1.1	0.0	CE	Not detected
8.0	1.2	CE	Not detected
5.4	1.1	FH	Gly2434Arg
3.0	0.0	FH	Gly2434Arg
8.7	2.0	FH	Not detected
1.0	0.0	CE	Not detected
3.7	1.3	CE	Arg2163His
0.7	0.2	FH	Not detected
6.5	1.4	CE	Not detected

Values indicate mean tension (g) of three strips. Diagnosis of malignant hyperthermia was made using three muscle strips for halothane and three for caffeine contracture.

*This patient is III-3 shown in figure 3A.

CHCT = caffeine halothane contracture test; CE = patient had either suspected or confirmed clinical episode; FH = patient had a family history of either suspected or confirmed clinical episode; Not detected = not detected after screening 21 known mutations.

cells from MHS individuals with that in B cells from MH-negative (MHN) individuals and healthy controls.

Materials and Methods

Patients

Using informed consent procedures approved by Uniformed Services University of the Health Sciences, we enrolled a total of 34 unrelated patients, who were referred to the Department for evaluation of MH status. We also enrolled five family members of a patient with the Val2168Met mutation. Blood samples were also obtained from 18 healthy volunteers. The 34 patients were referred for evaluation and diagnostic biopsy because of a personal or family history of MH or adverse metabolic response during surgery that was highly suspicious for MH. The patients were phenotyped as either MHS or MHN by the caffeine halothane contracture test (CHCT). The CHCT was performed according to a standardized protocol established by the North American MH group.¹³

The CHCT results and clinical profile of MHS patients are summarized in table 1. Mean age at the time of enrollment in the study was 27.9 yr (range, 19–49 yr), 23.8 yr (range, 6–53 yr), and 33.8 yr (range, 20–45 yr) for MHS, MHN, and control groups, respectively. Genetic screening of mutations in *RYR1* of these patients was previously performed.¹⁰ The mutations tested in the study were *Cys35Arg*, *Arg163Cys*, *Gly248Arg*, *Gly341Arg*, *Ile403Met*, *Arg552Trp*, *Arg614Cys*, *Arg614Leu*,

Arg2163Cys, *Arg2163His*, *Val2168Met*, *Val2214Ile*, *Ala2367Thr*, *Asp2431Asn*, *Gly2434Arg*, *Arg2435His*, *Arg2454Cys*, *Arg2454His*, *Arg2458Cys*, *Arg2458His*, and *Ile4898Thr*. Among 13 MHS patients, 5 patients were found to carry a mutation in *RYR1* (table 1). The remaining nine MHS patients were negative for the 21 *RYR1* mutations tested.

*Ca*²⁺ Mobilization Test Using B Cells

Relative changes in [*Ca*²⁺]_i in B cells were derived from changes in the fluorescence intensity of fluo-3-loaded cells.¹⁴ Peripheral mononuclear cells (PMCs) were isolated from 25-ml blood samples by Ficoll-Hypaque density gradient centrifugation. Cells (2 × 10⁶/ml) were loaded with 1 μM acetoxy-methyl ester of fluo-3 (Molecular Probes, Eugene, OR) by incubation in subdued light (30 min; 25°C). No difference in the viability of the cells was observed between MHS and MHN (or control) individuals. The resultant fluo-3-loaded cells were then stained with phycoerythrin (PE)-conjugated anti-CD19 mAb to selectively label B cells. Cells were then washed three times with Hanks balanced salt solution (HBSS), resuspended in 1 ml HBSS and analyzed by FACScan (Becton-Dickinson, San Jose, CA). Forward and right-angle scatter signals were displayed on a linear scale, with the forward scatter adjusted to gate cells from debris. For dual-color analysis of intracellular fluo-3 and surface-labeled PE, the fluo-3-fluorescence (excitation at 488 nm with emission at 525 nm) and PE (excitation at 488 nm, with emission at 585 nm) signals were detected after separation with 530 (FL-1) and 585 nm (FL-2) band-pass filters, respectively. FL-1 fluorescence and FL-2 fluorescence are recorded, amplified, and displayed on a logarithmic scale. Crossover of FL-1 fluorescence into the FL-2 detection window was compensated for by analog subtraction at the preamplifier stage. For each experiment, the fluo-3-loaded cells were analyzed to obtain an unstimulated baseline. Cells were then exposed to caffeine or 4CmC and analyzed every 15–30 s at rates of 400–1,000 cells/s. Transit time required for data acquisition was approximately 10–20 s. The measurement was done at room temperature. To analyze fluo-3 fluorescence shift of PE-labeled cells, FL-1 histograms of FL-2⁺ cells were obtained by gating FL-2-positive clusters in a FL-1 versus FL-2 dot plot display. The percentage of fluo-3⁺ cells relative to unstimulated baseline was then calculated and analyzed using Consort 30 program (Becton-Dickinson). Neither caffeine nor 4CmC affected fluo-3 fluorescence properties in this system. In some experiments, time-dependent continuous acquisition and analysis were performed using CellQuest software (Becton-Dickinson). For dose-response experiments, changes in [*Ca*²⁺]_i were examined after 25 and 50 mM caffeine and after 100, 200, and 400 μM 4CmC. The larger populations were then tested for 50 mM caffeine and 400 μM 4CmC. In a study of a family with the

Val2168Met mutation, 600 μM 4CmC was tested in addition to 200 and 400 μM 4CmC.

Selective Reverse Transcription-Polymerase Chain Reaction Followed by Restriction Fragment Length Polymorphism Analysis

Expression of the *RYR1* messenger RNA (mRNA) in B cells was examined as follows. Because only small number of cells were available for RNA isolation after the Ca^{2+} assay, B cells were not isolated from PMCs since we have shown that *RYR1* mRNA from PMCs are mainly from B cells.¹⁵ Total RNA was extracted using SV Total RNA Isolation System (Promega, Madison, WI); reverse transcription was performed to the first strand of cDNA using a cDNA synthesis kit (Promega). Synthesized cDNA was then amplified by reverse transcription (RT)-polymerase chain reaction (PCR). The PCR amplifications were carried out using Expand Long PCR system (Boehringer Mannheim, Indianapolis, IN), using a primer set that amplifies the exon 39 portion of the *RYR1*, forward primer; 5'-dCGTGGGAAGACACCATGAGCCTGCT-3' and reverse primer; 5'-ACCCGATGCTCTGGATCATGA-3'. PCR was performed in a 50- μl reaction mixture containing 100 ng cDNA, 15 pmol of each primer, 0.5 mM dNTP, 2.5 U Expand Long polymerase mixture and Expand Long PCR buffer 3 (Boehringer Mannheim). The PCR amplification conditions were 95°C for 2 min, followed by 35 cycles of 95°C for 1 min, 58°C for 2 min, and 68°C for 3 min, followed by a 7-min extension at 68°C. The RT-PCR products were then digested using a restriction enzyme, BspI, to identify G6502A substitution. Based on the known sequences of human RYR isoforms, Bsp I cuts the amplified 113 base pairs (bp) *RYR1* product into 86- and 27-bp fragments. The restriction fragments were then resolved by electrophoresis on a 2% agarose gel and visualized on a UV transilluminator. As a control of mRNA input, β -actin mRNA concentrations were determined for each sample in separate RT-PCR reactions. For β -actin amplification, PCR was performed with 25 cycles to ensure that the amplification was completed within the linear range. The sequences of primers for β -actin were 5'-dAAGAGAGGCATCCTCACCCCT-3' (sense) and 5'-dTGCTGATCCACATCTGCTGGA-3' (antisense). The signal ratio of *RYR1* to β -actin was determined on the basis of the ratio of the intensity of the PCR product compared with the corresponding β -actin band. The PCR products were imaged and the relative optical density of each band was measured and analyzed using NIH Image software (National Institutes of Health, Bethesda, MD).

Nested Polymerase Chain Reaction Followed by Restriction Fragment Length Polymorphism

The *RYR1* mRNA expressed in B cells from members of a family with the Val2168Met mutation was also tested for the mutation following nested PCR. Nested PCR is a

two-step PCR that amplifies an internal target by the second PCR from longer PCR products obtained by the initial PCR. This technique eliminates the possibility of amplification from contaminated undigested genomic DNA in the cDNA samples. Thus, the 1.2-kilobase pair (kbp) sequence consisted of multiple exons (exons 39–47) was first amplified so that the cDNA-derived amplicons (1.2 kbp) could be selectively obtained by separating genomic-derived amplicons (> 6 kbp) on gel electrophoresis. The *RYR1* was amplified using primers (upstream primer; 5'-dTGGGCCCAAGAGGACTTCGT-3', downstream primer; 5'-dAGCACCATGGACGCCTTGTG-3'). The PCR amplification conditions were 95°C for 2 min, followed by 30 cycles of 95°C for 1 min, 58°C for 2 min and 68°C for 3 min, followed by a 7-min extension at 68°C. The RT-PCR products were resolved by electrophoresis on a 1% agarose gel and visualized on a UV transilluminator. A PCR product of approximately 1,200 bp was purified from a 1% agarose gel with QIAquick gel extraction kit (QIAGEN, Valencia, CA). The 1.2-kbp amplicon was then tested for Val2168Met (G6502A) by PCR-based restriction fragment length polymorphism as described previously (Selective Reverse Transcription-Polymerase Chain Reaction Followed by Restriction Fragment Length Polymorphism Analysis).

Statistical Analysis

Kruskal-Wallis and Mann-Whitney tests were used for nonparametric evaluations, where applicable. Dose-response data for 4CmC were analyzed using repeated measures (RM) analysis of variance (ANOVA).

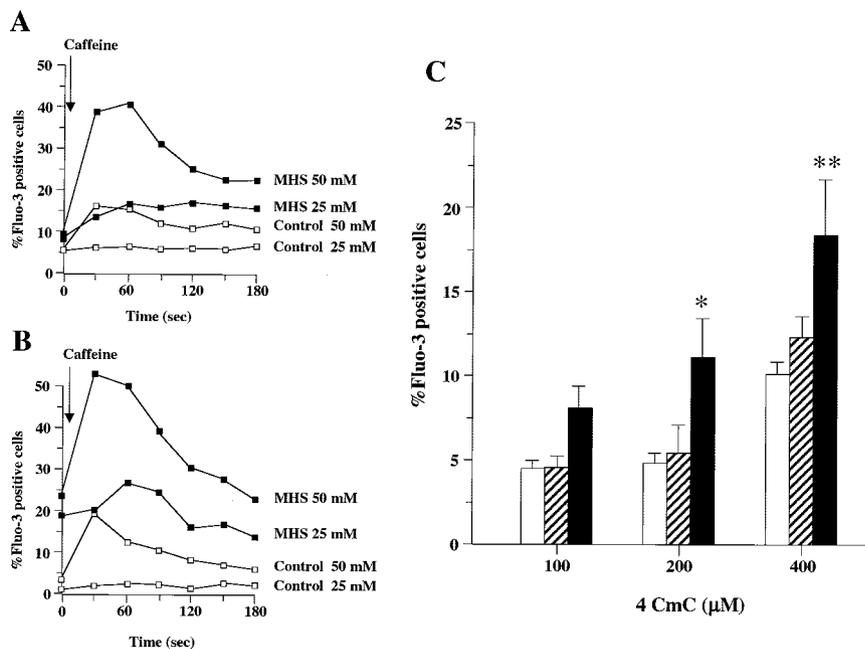
Results

Ca²⁺ Responses of B Lymphocytes to Caffeine and 4CmC

The difference in the Ca^{2+} response in B cells between MHS ($n = 6$) individuals and controls ($n = 6$) was statistically significant following addition of 50 mM caffeine (33.0 ± 3.6 vs. 15.5 ± 3.1 , $P < 0.005$) but not following 25 mM caffeine (8.5 ± 1.7 vs. 3.4 ± 2.0 , $P > 0.05$). The representative results from two pairs of MHS and control individuals for Ca^{2+} response to caffeine were shown in figures 1A and B. Differences in 4CmC-induced changes in $[\text{Ca}^{2+}]_i$ between the three groups were greater at 400 μM than at 200 or 100 μM (fig. 1C). The RM ANOVA test at 400 μM indicated a significance of $P = 0.010$. The difference at 200 μM indicated a $P = 0.042$, while there was no difference at 100 μM . Based on the results obtained from the dose-response experiments, concentrations of 50 and 400 μM were chosen to test for the effects of caffeine and 4CmC on B cells.

The Ca^{2+} response of B cells to caffeine (50 mM) and 4CmC (400 μM) in 13 MHS individuals (11 MHS individuals for 4CmC), 21 MHN individuals and 18 controls are

Fig. 1. (A,B) Caffeine (25 and 50 mM)-induced Ca^{2+} responses in B cells from malignant hyperthermia-susceptible (MHS) (closed squares) and control individuals (open squares). Two sets of experiments (A,B) from four unrelated individuals are shown. Changes in $[Ca^{2+}]_i$ in B cells were analyzed as percent increase of fluo-3⁺ cells relative to the base line established by standard calibration (% fluo-3⁺). The percent increase of fluo-3⁺ cells in CD19⁺ B cells was monitored using flow cytometry as described in *Materials and Methods*. Plotted are mean % fluo-3⁺ cells of 500 to 1,000 CD19⁺ B cells at each time point. (C) The 4-chloro-m-cresol (4CmC) (100, 200, and 400 μ M)-induced Ca^{2+} responses in CD19⁺ B cells. Data were expressed as the mean \pm SEM of Delta % fluo-3⁺ cells from MHS (filled bars, $n = 6$ at 100 μ M, 8 at 200 μ M and 12 at 400 μ M), malignant hyperthermia-negative (MHN) (hatched bars, $n = 8$ at 100 μ M, 13 at 200 μ M and 21 at 400 μ M) and control individuals (open bars, $n = 8$ at 100 μ M, 9 at 200 μ M and 18 at 400 μ M). Delta % fluo-3⁺ cells were calculated as peak % fluo-3⁺ cells-basal % positive cells. Repeated measures analysis of variance (RM ANOVA) revealed significant differences between the groups at 200 μ M (* $P = 0.042$) and 400 μ M (** $P = 0.010$).



shown in figure 2. Caffeine-induced Ca^{2+} response significantly differed between the MHS, MHN, and control groups ($P = 0.0001$, Kruskal-Wallis test). Caffeine-induced (50 mM) increases in $[Ca^{2+}]_i$ in B cells were significantly greater in MHS than in MHN ($P = 0.0004$), control ($P = 0.0001$), and non-MHS individuals (MHN plus control, $P < 0.0001$) by Mann-Whitney test. Similarly, 4CmC-induced (400 μ M) Ca^{2+} responses significantly differ among the three groups ($P = 0.028$). Mann-Whitney test indicated that 4CmC-induced (400 μ M) increases in $[Ca^{2+}]_i$ were significantly greater in MHS individuals than in controls ($P = 0.003$) or non-MHS individuals (MHN individuals plus controls, $P = 0.0078$). The nonparametric Spearman rank correlation test indicated a significant correlation between Ca^{2+} response to caffeine (50 mM) and 4CmC (400 μ M) (fig. 2C) ($r = 0.608$, $P < 0.0001$).

Expression of the RYR1 Mutant and Ca^{2+} Response in B Cells in the Family with the Val2168Met Mutation

The CHCT and genotype results of a family with the Val2168Met mutation were reported previously.¹⁰ Correlation between muscle phenotype and genotype was seen in this family (fig. 3A). The referred patient was found to be positive for MHS by CHCT. That individual's parent and sibling also had positive CHCT results in 1981 and 1988, respectively. A maternal sibling died during anesthesia under circumstances consistent with MH. All three CHCT-positive individuals in this family had Val2168Met (G6502A), while the CHCT-negative relatives had no mutation in *RYR1*.

The 4CmC caused a dose-dependent increase in $[Ca^{2+}]_i$ in B cells from this family (fig. 3B). ANOVA revealed a significant MH status by 4CmC concentration interaction ($P = 0.004$), indicating that 4CmC-induced increases in $[Ca^{2+}]_i$ over concentration depend on the MHS status. The 4CmC-induced Ca^{2+} increases were significantly greater at 600 μ M in the three MHS members than in the other members (% fluo-3⁺ cells; 39.3 ± 5.5 and $17.6 \pm 3.3\%$, $P = 0.028$). It was also noted that all three MHS individuals showed increases in $[Ca^{2+}]_i$ at 200 μ M from unstimulated basal concentrations, while the mutation-negative members exhibited no increase, indicating a lower threshold to 4CmC in the MHS B cells in the family. Caffeine-induced (50 mM) increases in $[Ca^{2+}]_i$ in mutation-positive members were not different from those in mutation-negative members (% fluo-3⁺ cells; 16.4 ± 3.2 and $19.8 \pm 5.0\%$, $P > 0.05$).

Expression of the *RYR1* mRNA in B cells was examined using semiquantitative RT-PCR (fig. 3C). The B cells from all individuals expressed *RYR1* mRNA (fig. 3C). Relative levels of the *RYR1* expression were determined by comparing with levels of a housekeeping gene β -actin (fig. 3C) and expressed as the signal ratio of *RYR1* to β -actin (fig. 3C). There was a tendency for the high 4CmC responders (MHS) to have higher expression levels of *RYR1*, but overall results showed no clear relation between the 4CmC Ca^{2+} response and *RYR1* expression in the family members (*RYR1*/ β -actin ratio, MHS vs. MHN; 0.85 ± 0.15 vs. $0.52 \pm 0.20\%$, $P > 0.05$; fig. 3C).

Finally, we examined whether the Val2168Met (G6502A) mutant was expressed in B cells. DNase-treated RNA isolated from B cells was tested for the

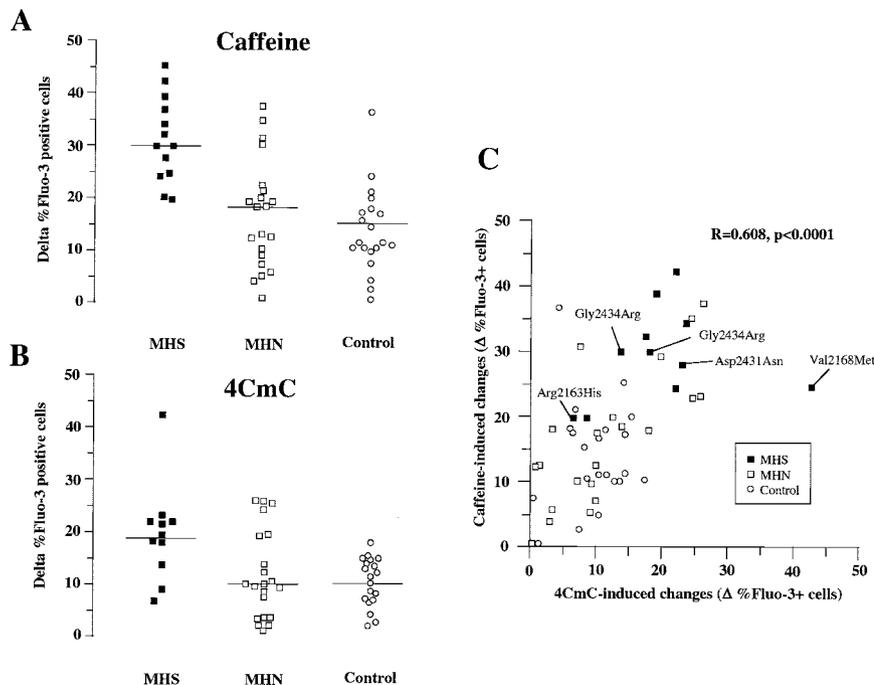


Fig. 2. (A,B) Caffeine (50 mM)-induced and 4-chloro-m-cresol (4CmC) (400 μ M)-induced Ca^{2+} responses in CD19+ B cells in malignant hyperthermia-susceptible (MHS) (n = 11–13), malignant hyperthermia-negative (MHN) (n = 21), and control individuals (n = 18). Delta % fluo-3+ cells are calculated as peak % fluo-3+ cells-basal % positive cells. Caffeine-induced increases in $[\text{Ca}^{2+}]_i$ in B cells were significantly greater in MHS (n = 13) than in MHN ($P = 0.0004$) or control ($P = 0.0001$). The 4CmC-induced increases in $[\text{Ca}^{2+}]_i$ were significantly greater in MHS (n = 11) than in controls ($P = 0.003$). There was no significant difference in 4CmC-induced changes in $[\text{Ca}^{2+}]_i$ between the MHS versus MHN ($P = 0.108$). Bars = median. (C) The correlation between the Ca^{2+} response of B cells to caffeine (50 mM) and 4CmC (400 μ M) in a total of 51 individuals, 11 MHS (closed square), 21 MHN (open square) and 18 control (open circle). There was a significant correlation between Ca^{2+} response to caffeine and 4CmC ($P < 0.0001$). Patients who had mutations, Gly2434Arg, Arg2163His, Asp2431Asn, or Val2168Met are indicated. The patient with Val2168Met was

III-3 in figure 3A. The test values from this patient in this figure were obtained from his first visit and slightly different from the values presented in figure 3B.

mutation. Consistent with the results obtained from genomic DNA, the mutation-positive members (II-1, III-1, and III-3) expressed mutant RNA in B cells, while I-2, II-1, and III-2 expressed normal RNA (fig. 3D).

Discussion

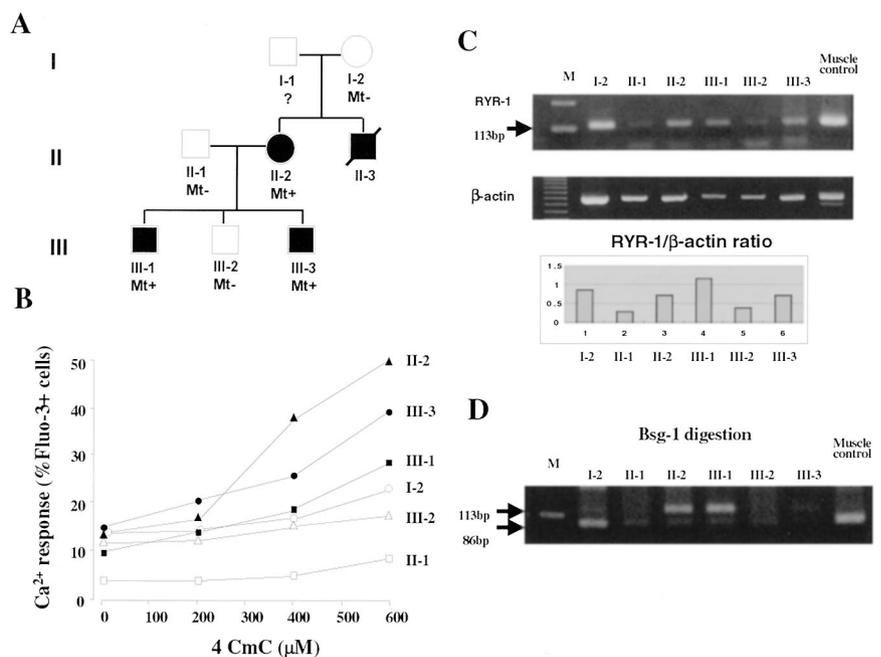
Previously, we reported that *RYR1* was expressed in human B lymphocytes and functioned as a Ca^{2+} -release channel during B-cell receptor-stimulated Ca^{2+} signaling process.¹² In the present study, we tested 52 unrelated individuals (13 MHS individuals, 21 MHN individuals, and 18 controls) and 6 members of a single family for the Ca^{2+} response of B cells to RYR-stimulating agents caffeine or 4CmC using flow cytometric dual-color analysis. The study showed that Ca^{2+} responses induced by caffeine and 4CmC in B cells from MHS patients are larger than the responses from MHN and control individuals. We also show that B cells express mutant mRNA of *RYR1* in patients with the Val2168Met mutation (G6502A) and that the B-cell Ca^{2+} phenotype segregates with genotype and CHCT results in a family with this mutation. These results suggest that the B-cell Ca^{2+} phenotype may be altered in MHS individuals and that the alterations are associated with mutations in the *RYR1* gene in some MHS individuals. This type of study may help in studying the genotype-phenotype association in patients with various MH mutations.

Pharmacologic characterization of effects of caffeine and 4CmC on Ca^{2+} response in B cells suggests that a

key protein in the heightened Ca^{2+} response in MHS individuals is *RYR1* or possibly proteins associated with the *RYR1*. 4CmC has been shown to induce Ca^{2+} release from a ruthenium red/caffeine-sensitive Ca^{2+} release channel in skeletal muscle, from heparin-treated cerebellar microsomes and from IP_3 -insensitive pools in PC12 cells.¹⁶ These findings suggest that 4CmC selectively activates RYR-mediated Ca^{2+} release in different types of cells. Similarly, in B cells, 4CmC activates *RYR1*-mediated Ca^{2+} release, as shown by its ability to release Ca^{2+} after depletion of IP_3 -sensitive pools by cross-linking membrane immunoglobulin M.¹² The dose-responses for 4CmC suggested a lower threshold for 4CmC-induced Ca^{2+} release in MHS individuals than in MHN or control individuals. The result was consistent with the 4CmC dose-response results from the skeletal muscle.¹⁷

In contrast to 4CmC, which appears to activate mainly Ca^{2+} release in B cells, we found that caffeine induced Ca^{2+} influx from the extracellular compartment.¹⁸ Thus, the caffeine-induced increase in $[\text{Ca}^{2+}]_i$ was abolished in EGTA-treated or Ca^{2+} -free media.¹⁸ At concentrations of 1–50 mM, caffeine causes an increase in $[\text{Ca}^{2+}]_i$ in a concentration-dependent fashion.¹⁸ The increase in $[\text{Ca}^{2+}]_i$ becomes apparent at the high concentration of 25 mM.¹⁸ Requirement of more than 20 mM caffeine for activating Ca^{2+} response is not unusual. In nonmuscle cells, such as neurons and chromaffin cells, concentrations as high as 40–50 mM are often required to obtain maximal Ca^{2+} response.^{19,20} Although the nature of the Ca^{2+} channel and the mechanism whereby caffeine elic-

Fig. 3. (A) Segregation of Val2168Met (G6502A) mutation and caffeine halothane contracture test results in a malignant hyperthermia family. This family was published previously.¹⁰ Patients III-1, II-2 and III-3 had positive caffeine halothane contracture test (CHCT) results (filled symbols). The II-2 and II-3 had clinical episodes of malignant hyperthermia. BsgI restriction endonuclease analysis revealed that all three CHCT-positive II-2, III-1, and III-3 had *Val2168Met* mutations (Mt+) while II-1 and III-2 (CHCT-negative) had no mutation (Mt-). Symbols: bold lined individuals received CHCT; filled, CHCT-positive; open, CHCT-negative. **(B)** The 4-chloro-m-cresol (4CmC) (200, 400, and 600 μM)-induced Ca^{2+} responses in CD19⁺ B cells. Plotted are mean peak %fluo-3⁺ cells of 500 to 1,000 CD19⁺ B cells at each concentration. **(C)** Agarose gel electrophoresis of PCR products following selective RT-PCR for type 1 ryanodine receptor (*RYR1*) and β -actin. The complementary DNAs obtained from peripheral mononuclear cells of each family member (I-2, II-1, II-2, III-1, III-2, and III-3) were amplified using primer sets that specifically amplified *RYR1* and β -actin. A bar graph indicates relative expression of *RYR1* messenger RNA (mRNA) obtained by densitometric quantification of the *RYR* mRNA transcript over the β -actin mRNA transcript. The B cells from all individuals expressed *RYR1* mRNA, but no clear relation between malignant hyperthermia phenotype and expression levels of *RYR1* was found. **(D)** Expression of the *RYR1* mRNA mutant (G6502A) in B cells from II-2, III-1, and III-3. The BsgI digestion of the 113 bp RT-PCR fragment yielded fragments of 86 and 27 bp for normal RT-PCR product and a single undigested fragment of 113 bp plus digested fragments of 86 and 27 bp for RT-PCR product containing the heterozygous mutation. A small fragment of 27 bp is not clearly visible in this figure. The lanes for II-2, III-1, and III-3 show more undigested fragments of 113 bp than digested fragments, indicating the presence of the heterozygous mutation in these individuals.



its opening are currently unknown, our previous studies suggest that caffeine activates nonselective cation channels in B cells.¹⁸ Thus, the Ca^{2+} channel activated by caffeine is Mn^{2+} permeable, and is insensitive to a store-operated Ca^{2+} channel (SOC) blocker SKF-96365.¹⁸ We hypothesize that caffeine induces Ca^{2+} influx *via* a nonselective cation channel and then Ca^{2+} activates a *RYR1*-mediated opening of SOC, as has been demonstrated in *RYR1*-transfected HEK 293 cells.²¹ In this hypothesis, the caffeine-induced Ca^{2+} response involves *RYR1* and can be substantially enhanced in MHS individuals who have mutations in the *RYR1*. Alternatively, there is a common mechanism in caffeine and 4CmC-induced Ca^{2+} response that is likely mediated by *RYR1*, as suggested by a positive correlation between the two measures (fig. 2C). However, the caffeine-induced Ca^{2+} response likely involves other cellular factors and thus could be more sensitive but less specific to *RYR1*-related mechanism than is the 4CmC-induced Ca^{2+} response. This may account for greater differences between the groups in the caffeine-induced Ca^{2+} response than in 4CmC-induced responses. It should be stressed that other components that modulate the caffeine-induced Ca^{2+} phenomenon may be relevant to other causal genes for MH.

There was a segregation of Val2168Met (G6502A) with a CHCT phenotype and B-cell Ca^{2+} response in a family

of three generations. In this family, the B cells from MHS patients express the *RYR1* mutants and abnormal Ca^{2+} response and thus verified our rationale of testing B cells for the *RYR*-stimulating agents. Moreover, expression of *RYR1* was confirmed for all the members tested for the Ca^{2+} response. Therefore, the Ca^{2+} results were valid for evaluating the status of *RYR1* for every family member. Alternatively, if *RYR1* was not expressed, low caffeine- or 4CmC-induced Ca^{2+} responses could not be explained by altered function of the *RYR1*. Consistent with dose-response data (fig. 1C), the dose-response results from this family indicated that all mutation-positive members showed increases in Ca^{2+} at 200 μM from the basal concentrations, whereas mutation-negative members did not. This is consistent with observations that the MHS skeletal muscle showed a lower threshold to 4CmC than did MHN muscle.¹⁷ In this family study, we have tested 600 μM 4CmC for Ca^{2+} response. The dose-response experiments indicated a clearer separation of MHS individuals from MHN individuals at 600 μM 4CmC than was obtained at 400 μM . Although the concentration 400 μM was initially applied to compare the larger populations for the Ca^{2+} response to 4CmC (fig. 2B), the results from this family study suggest that 600 μM 4CmC would give better separation of MHS from MHN than 400 μM . There was no difference in caffeine-induced Ca^{2+} response between mutation-positive and

-negative members in this family. The main reason was that caffeine failed to enhance Ca^{2+} response in mutation-positive members. Thus, Ca^{2+} response to caffeine should be further examined to study a mechanism of lack in enhancement in these mutation-positive individuals.

Phenotyping of patients as either MHS or MHN has been performed by a procedure involving muscle biopsy of the vastus lateralis, followed by *in vitro* CHCT, in North America, and *in vitro* contracture test, in Europe.¹³ The mechanism of these contracture tests is thought to depend on the abnormal Ca^{2+} regulation mediated by RYR-1 or associated molecules in muscle from MHS compared with MHN individuals. Altered Ca^{2+} homeostasis in B cells in MHS individuals, similar to the alteration demonstrated for muscle cells, increases the possibility of a minimally invasive blood test. A scattergram showing Δ % responding cells (fig. 2A and B) reveals significant differences, but with imperfect discrimination between MHS, MHN, and control groups for the Ca^{2+} response. It is apparent that heterogeneity of MH genotype would at least complicate discrimination between MHS and MHN by B-cell Ca^{2+} phenotype. Further studies will determine whether Ca^{2+} signaling in B cells can be used for reliable prediction of MHS.

The enhanced Ca^{2+} response to these RYR-stimulating agents, which mimics that of skeletal muscle from MHS patients, suggests that B cells and skeletal muscles share a common mechanism of controlling $[\text{Ca}^{2+}]_i$, which is conditionally hyperresponsive to RYR-stimulating agents. Thus, although the validity of the test was not proven in this study, B-cell Ca^{2+} phenotyping may be useful in the future as a complementary test to CHCT. It should be also stressed that *RYR1* mRNA expressed in B cells can be used for effective screening of mutations in the *RYR1* gene and also for gene transfection studies. Moreover, studying the mechanism of Ca^{2+} homeostasis in B cells may provide a unique opportunity to investigate molecular mechanisms of MH and possibly to find other candidate genes causal for MH.

The authors thank Drs. Anthony S. Basile, Ph.D. (Senior Investigator, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland), Rulph Bunger, Ph.D. (Professor, Uniformed Services University of the Health Sciences, Maryland), Tommy McCarthy Ph.D. (Professor, University College, Cork, Ireland), Leslie McKinney, Ph.D. (Assistant Professor, Uniformed Services University of the Health Sciences, Maryland), Paul Mongan, M.D. (Associate Professor, Uniformed Services University of the Health Sciences, Maryland), Thomas E. Nelson, Ph.D. (Professor, Wake Forest University School of Medicine), Henry Rosenberg, M.D. (Professor, Thomas

Jefferson University-Jefferson Medical College, Philadelphia, Pennsylvania), Shizuko Sei, M.D. (Laboratory Chief, NCI at Frederick, Maryland) and Frank Wappler, M.D. (Professor, University Hospital Eppendorf, Hamburg, Germany) for helpful discussions and critical comments.

References

- Nelson TE, Flewelling EH: Current concepts: The malignant hyperthermia syndrome. *N Engl J Med* 1983; 309:416-8
- Heffron JJA: Malignant hyperthermia: Biochemical aspects of the acute episode. *Br J Anesth* 1988; 60:274-8
- Rosenberg H: Clinical presentation of malignant hyperthermia. *Br J* 1988; 60:268-73
- Allen GC, Larach MG, Kunselman AR: The sensitivity and specificity of the caffeine-halothane contracture test. The North American Malignant Hyperthermia Registry of MHAUS. *ANESTHESIOLOGY* 1998; 88:579-88
- 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
- MacLennan DH, Phillips MS: Malignant hyperthermia. *Science* 1992; 256:789-94
- McCarthy TV, Quane KA, Lynch PJ: Ryanodine receptor mutations in malignant hyperthermia and central core disease. *Hum Mutat* 2000; 15:410-7
- Manning BM, Quane KA, Lynch PJ, Urwyler A, Tegazzin V, Krivosic-Horber R, Censier K, Comi G, Adnet P, Wolz W, Lunardi J, Muller CR, McCarthy TV: Novel mutations at a CpG dinucleotide in the ryanodine receptor in malignant hyperthermia. *Hum Mutat* 1998; 11:45-50
- Barone V, Masa O, Intravia E, Bracco A, DiMartino A, Tegazzin V, Cozzolino S, Lorrentino V: Mutation screening of the RYR1 gene and identification of two novel mutations in Italian malignant hyperthermia families. *J Med Genet* 1999; 36:115-8
- Sambuughin N, Sei Y, Gallagher KL, Wyre HW, Madsen D, Nelson TE, Fletcher JE, Rosenberg H, Muldoon SM: North American Malignant Hyperthermia population. Screening of the ryanodine receptor gene and identification of novel mutations. *ANESTHESIOLOGY* 2001; 95:594-9
- Tong J, Oyamada H, Demareux N, Grinstein S, McCarthy TV, MacLennan DH: Caffeine and halothane sensitivity of intracellular Ca^{2+} release is altered by 15 calcium release channel (ryanodine receptor) mutations associated with malignant hyperthermia and/or central core disease. *J Biol Chem* 1997; 272:26332-9
- Sei Y, Gallagher KL, Basile AS: Skeletal muscle type ryanodine receptor is involved in calcium signaling in human B lymphocytes. *J Biol Chem* 1999; 274:5995-6002
- Larach MG: Standardization of the caffeine halothane muscle contracture test. *Anesth Analg* 1989; 69:511-5
- Sei Y, Arora P: Quantitative analysis of calcium mobilization after stimulation with mitogen and anti-CD3 antibodies: Simultaneous fluo-3 and immunofluorescence flow cytometry. *J Immunol Method* 1991; 137:237-44
- Hosoi E, Nishizaki C, Gallagher KL, Wyre HW, Matsuo Y, Sei Y: Expression of the ryanodine receptor isoforms in immune cells. *J Immunol* 2001; 167:4887-94
- Zorzato F, Scutari E, Tegazzin V, Clementi E, Treves S: Chlorocresol: An activator of ryanodine receptor-mediated Ca^{2+} release. *Mol Pharmacol* 1993; 44:1192-201
- Baur CP, Bellon L, Felleiter P, Fiege M, Fricker R, Glahn K, Heffron JJ, Hermann-Frank A, Jurkat-Rott K, Klingler W, Lehane M, Ordning H, Tegazzin V, Wappler F, Georgieff M, Lehmann-Horn F: A multicenter study of 4-chloro-m-cresol for diagnosing malignant hyperthermia susceptibility. *Anesth Analg* 2000; 90:200-5
- Sei Y, Gallagher KL, Daly JW: Multiple effects of caffeine on Ca^{2+} release and influx in human B lymphocytes. *Cell Calcium* 2001; 29:149-60
- Bennet DL, Bootman MD, Berridge MJ, Check TR: Ca^{2+} entry into PC12 cells initiated by ryanodine receptors or inositol 1,4,5-triphosphate receptors. *Biochem J* 1998; 329:349-57
- Liu PS, Lin YJ, Kao LS: Caffeine-sensitive calcium stores in bovine adrenal chromaffin cells. *J Neurochem* 1991; 56:172-7
- Kiselyov K, Shin DM, Wang Y, Pessah IN, Allen PD, Muallem S: Gating of store-operated channels by conformational coupling to ryanodine receptors. *Mol Cell* 2000; 6:421-31