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B-lymphocytes from Malignant Hyperthermia-susceptible Patients Have an Increased Sensitivity to Skeletal Muscle Ryanodine Receptor Activators*

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Malignant hyperthemia (MH) is a pharmacogenetic disease triggered by volatile anesthetics and succinylcholine in genetically predisposed individuals. The underlying feature of MH is a hypersensitivity of the calcium release machinery of the sarcoplasmic reticulum, and in many cases this is a result of point mutations in the skeletal muscle ryanodine receptor calcium release channel (RYR1). RYR1 is mainly expressed in skeletal muscle, but a recent report demonstrated the existence of this isoform in human B-lymphocytes. As B-cells can produce a number of cytokines, including endogenous pyrogens, we investigated whether some of the symptoms seen during MH could be related to the involvement of the immune system. Our results show that (i) Epstein-Barr virus-immortalized B-cells from MH-susceptible individuals carrying the V2168M RYR1 gene mutation were more sensitive to the RYR activator 4-chloro-*m*-cresol and (ii) their peripheral blood leukocytes produce more interleukin (IL)-1β after treatment with the RYR activators caffeine and 4-chloro-*m*-cresol, compared with cells from healthy controls. Our result demonstrate that RYR1-mediated calcium signaling is involved in release of IL-1β from B-lymphocytes and suggest that some of the symptoms seen during an MH episode may be due to IL-1β production.

Malignant hyperthermia (MH)[1] is a pharmacogenetic disease triggered by volatile anesthetics and the depolarizing muscle relaxant succinylcholine in predisposed individuals (1–4). The clinical signs of an impending MH reaction are highly variable and are caused by a hypermetabolic state with muscle rigidity, metabolic acidosis, rhabdomyolysis, tachycardia, and/or an increase in body temperature (5). In some individuals MH reactions appear to be triggered by physical exercise or emotional stress. The latter observation has led to the suggestion that MH, heat stroke, and exercise-induced rhabdomyolysis might have a common denominator (2, 6, 7). The underlying causes of MH are abnormalities in the skeletal muscle calcium metabolism (8, 9) and molecular genetic studies have mapped the primary locus of MH to chromosome 19q, the gene encoding the ryanodine receptor calcium release channel (RYR1) (2, 4, 10). Approximately 50% of MH families have mutations in the RYR1 gene, and mutations have been reported in other loci (for recent reviews, see Refs. 11 and 12).

The ryanodine receptors are large tetrameric oligomers that function as intracellular calcium release channels. Three different isoforms have been identified at the molecular level: type 1 (RYR1), which is preferentially expressed in skeletal muscle; type 2, which is in the heart and cerebellum; and type 3, which is in the central nervous system as well as in a variety of other tissues (13–16). RYR1 can be pharmacologically activated by a number of compounds, among which are caffeine, halothane, thymol, 4-chloro-*m*-cresol, E218, bastadin, polylysine, and calcium (17–21). Activation causes the channel to open and thus to a transient calcium flow from the sarcoplasmic reticulum, leading to an increase in the calcium concentration of the myoplasm.

In B-lymphocytes Ca^{2+} signaling has been implicated in various physiological responses such as cell proliferation, gene expression, and antibody secretion (22). In a recent report, Sei *et al.* (23) have presented evidence supporting the existence of RYR1 in B-lymphocytes. Thus, in this cell type, changes in the $[Ca^{2+}]_i$ may be under the control of both the inositol trisphosphate receptor and the RYR1. B-lymphocytes are capable of responding to and producing several cytokines among which IL-1, IL-6, and tumor necrosis factor (24), although what controls cytokine release is not fully understood. IL-1 is a mediator of the host inflammatory response in innate immunity and stimulates other cell types such as macrophages and endothelial cells to synthesize and secrete other cytokines. When released in large quantities, IL-1 causes fever, *i.e.* it is an endogenous pyrogen and can induce the synthesis of acute phase plasma proteins and initiate metabolic wasting (25). Because of the different clinical symptoms during an MH episode, we were interested in establishing whether there is a link between IL-1 release and malignant hyperthermia. The results of the pres-

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[1] The abbreviations used are: MH, malignant hyperthermia; RYR, ryanodine receptor calcium release channel; $[Ca^{2+}]_i$, intracellular free calcium concentration; BAPTA/AM, 1,2-bis (2-aminophenoxy)ethane-N, N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester); BAPTA, 1,2-bis (2-aminophenoxy)ethane-N, N,N′,N′-tetraacetic acid; EBV, Epstein-Barr virus; RT, reverse transcription; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; MHS, malignant hyperthermia susceptible.

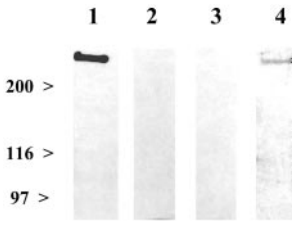

FIG. 1. **EBV-immortalized B-cells express type 1 ryanodine receptor.** Western blot analysis of rabbit skeletal muscle terminal cisternae (*lane 1*, 20 μg protein), rabbit heart total microsomes (*lane 2*, 30 μg protein), rabbit brain microsomes (*lane 3*, 30 μg protein) and human EBV-B-cell microsomes (*lane 4*, 30 μg protein). Proteins were separated on a 6% SDS-PAGE, blotted onto nitrocellulose and probed with a monoclonal anti-RYR antibody. Immunoreactivity was visualized with peroxidase conjugated anti-mouse IgG followed by chemiluminescence.

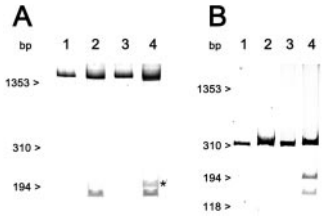

FIG. 2. **EBV-immortalized B-cells from MHS patients with the V2168M mutation carry and express a mutated ryanodine receptor.** *Panel A*- 6% polyacrylamide gel showing that PCR amplification of genomic DNA using a primer set spanning exons 39–40 of the RYR1 gene, gives rise to a DNA fragment of 1400 bp (*lanes 1* and 3). Digestion of this DNA fragment with the enzyme MslI gives rise to a band of ~192 bp (*lanes 2* and 4). The presence of the V2168M mutation creates an additional restriction site for the enzyme MslI, resulting in an additional band of ~196 bp (*) (*panel A*, *lane 4*). *Panel B*- 8% polyacrylamide gel showing that PCR amplification of cDNA from B-lymphocytes using a primer set spanning exons 39–40 of the RYR1 gene, gives rise to a DNA fragment of 338 bp (*lanes 1* and 3). The presence of the mutation V2168M creates a restriction site for the enzyme MslI, resulting in two extra bands of 192 and 146bp (*panel B*, *lane 4*), which are not present in the cDNA amplified from EBV-immortalized cells from healthy volunteers (*panel B*, *lane 2*). This experiment was repeated 4 times on different cDNA preparations.

ent study raise the possibility that some of the symptoms seen during an MH episode may be the result of IL-1 production.

EXPERIMENTAL PROCEDURES

Materials

fura-2/AM, 1,2-bis (2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA/AM), thapsigargin, and peroxidase-conjugated anti-mouse IgG were from Sigma; cDNA synthesis system kit, DNA isolation kit from mammalian blood, *Taq* polymerase, and BM chemiluminescence kit were from Roche Molecular Biochemicals. RNA isolation kit was from Biotecx Laboratories (Houston, TX). DNA-modifying enzymes were from New England Biolabs. Human IL-1β and IL-6 ELISA kits were from CLB (Amsterdam, The Netherlands). Cyclosporin A was from Novartis (Basel, Switzerland). Nitrocellulose and Ficoll-Hypaque were from Amersham Biosciences, Inc. Caffeine was from Merck (Darmstadt, Germany); 4-chloro-*m*-cresol was from Fluka Chemicals (Buchs, Switzerland). Tissue culture media and reagents were from Life Technologies, Inc. All other chemicals were reagent or of the highest available grade.

Methods

Mononuclear Cells and EBV-transformed Cell Lines—after informed consent whole blood was collected in EDTA-treated tubes from 4 healthy volunteers and from 4 patients who had undergone a diagnostic skeletal muscle biopsy to determine malignant hyperthermia susceptibility and were known to carry the MH-associated V2168M mutation. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. For infection with Epstein-Barr virus, mononuclear cells were exposed to supernatants of the B95.8 cell line in the presence of cyclosporin A and IL-6, according to standard procedures. Cells were cultured in RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 units of penicillin and streptomycin.

Mutation Screening—The presence of the MH-linked V2168M RYR1 gene mutation in selected patients from four unrelated families was determined by DNA PCR amplification, followed by restriction enzyme digestion. Genomic DNA was isolated using a DNA isolation kit for mammalian blood. Total RNA was isolated using an RNA isolation kit; poly(A^+) RNA was converted into cDNA using a cDNA synthesis system kit following the instructions provided by the manufacturer (Roche Molecular Biochemicals, catalog no. 1117831). Approximately 100 ng of DNA were used for each PCR amplification using a GeneAmp2400 thermocycler (PerkinElmer Life Sciences). The following primers were used to amplify genomic DNA and cDNA: forward, 5′-GGG CCC AAG AGG ACT TCG TGC; reverse, 5′-GCC CCC GAG GAC GTT GAC CAT. Amplification conditions were: 5 min, 95 °C followed by 35 cycles for genomic DNA or 40 cycles for cDNA: 30-s annealing at 60 °C, 45-s extension at 72 °C, 30-s denaturation at 92 °C, and extension for 4 min at 72 °C. The presence of the mutation was detected by restriction enzyme digestion using *Msl*I.

Subcellular Membrane Fractionation and Western Blotting—Total microsomes were isolated from rabbit brain, heart, and EBV-transformed B-lymphocytes as described previously (20) and stored in liquid nitrogen. Terminal cisternae were isolated from the white muscle of New Zealand White rabbits as described by Saito *et al.* (26). Proteins were separated on a 6% SDS-PAGE transferred onto nitrocellulose and probed with a monoclonal antibody specific for type 1 RYR (27). Peroxidase-conjugated anti-mouse IgG was used to detect the primary antibody followed by chemiluminescence.

Intracellular Ca^{2+} Measurements—changes in the intracellular calcium concentration of the EBV-transformed B-lymphocyte cell lines were monitored with the fluorescent calcium indicator fura-2/AM (final concentration, 5 μM) as described (20, 28). Fluorescent changes (ratio 340/380 nm) were measured in a PerkinElmer Life Sciences spectrofluorometer equipped with a magnetic stirrer and thermostated at 37 °C. All measurements were made in Ca^{2+}-free Krebs-Ringer containing 0.5 mM EGTA.

Release of Cytokines—Peripheral blood mononuclear leukocytes were placed in the wells of a microtiter plate in Krebs-Ringer solution and incubated 37 °C for 60 min under the specified conditions. For experiments in which the $[Ca^{2+}]_i$ was to be buffered, cells were pre-incubated with 50 μM of the calcium chelator BAPTA/AM for 30 min in Ca^{2+}-free Krebs-Ringer. Cells were then washed with Ca^{2+}-free Krebs-Ringer medium and treated as indicated. After 60 min, cells were centrifuged and the amount of IL-1β (or IL-6) released into the supernatant was determined by using the CLB PeliKine Compact indirect ELISA kit following the manufacturer's instructions. All tests were performed in triplicate.

Data Analysis—For the two group comparisons, Student's *t* test was used; three or more groups were compared by one-way ANOVA. Dose-response measurements were compared by repeated measurement ANOVA. Where ANOVA revealed a significant difference, the Fisher protect least significant difference *post hoc* test was performed. The overall statistical significance level was set to 5%. StatView™ from SAS Institute Inc. was used for statistical analysis.

RESULTS

The EBV-transformed B-cell lines were assessed for the presence of type 1 RYR using a monoclonal antibody we previously developed and characterized (27). Fig. 1 shows that an immunopositive high molecular weight band is present in the microsomal fraction obtained from the EBV-immortalized B-lymphocytes (*lane 4*). The antibody failed to react with type 2 RYR, which is present in heart microsomes (*lane 2*), or with any high molecular weight protein present in the microsomal fraction of rabbit brain (*lane 3*). The antibody reacted strongly with the RYR present in rabbit skeletal muscle terminal cisternae, a fraction that is highly enriched in this protein (*lane 1*).

Having determined that the B-cell lines do indeed express RYR 1, we performed RT-PCR analysis to confirm the presence of the mutated cDNA in cells derived from patients carrying the V2168M RYR1 mutation. Fig. 2 shows a polyacrylamide gel of the PCR-amplified genomic DNA (*panel A*) and cDNA (*panel B*) from EBV-immortalized B-lymphocytes. The primers used span exons 39–40, and the presence of the mutation V2168M creates a restriction site for the enzyme *Msl*I. Amplification of genomic DNA and cDNA yields fragments of ~1400 bp (*panel*

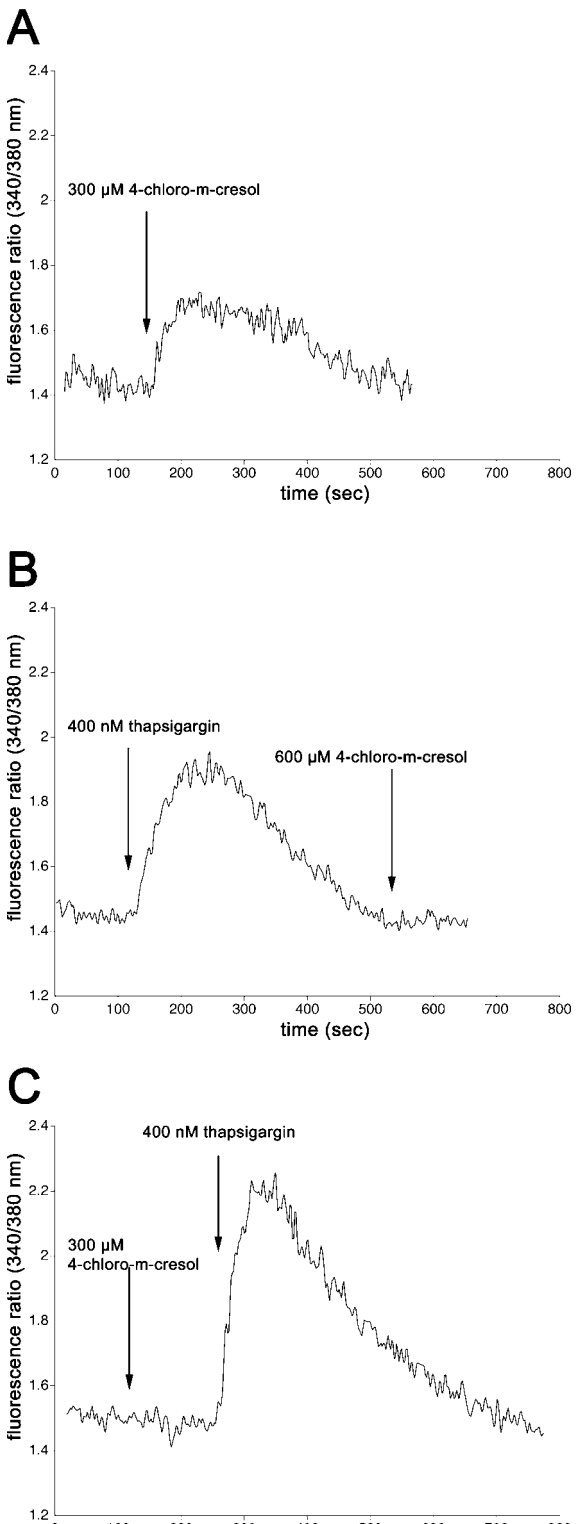

FIG. 3. **Effect of 4-chloro-*m*-cresol on the $[Ca^{2+}]_i$ of EBV-immortalized B-cells and HL-60 cells.** In each experiment 0.5×10^6 cells/ml loaded with 5 μM fura-2/AM were added to a thermostated, magnetically stirred cuvette in nominally calcium-free Krebs-Ringer and the fluorescence ratio recorded. Once a steady state $[Ca^{2+}]_i$ was reached, the effect of various compounds was determined. Where indicated 300 μM 4-chloro-*m*-cresol (A) or 400 nM thapsigargin followed by 600 μM 4-chloro-*m*-cresol (B) were added to EBV-immortalized B-lymphocytes. (C) Addition of 4-chloro-*m*-cresol to fura-2-loaded HL-60 cells a human myelomonocytoid cell line, did not induce an increase in the $[Ca^{2+}]_i$, though the stores could be depleted by treatment with 400 nM thapsigargin.

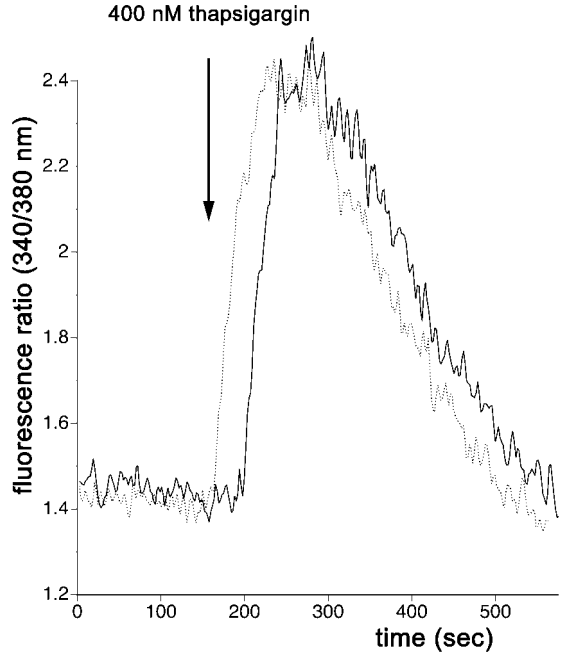

FIG. 4. **The thapsigargin-sensitive $[Ca^{2+}]_i$ stores of EBV-immortalized cells from control and MHS V2168M patients are not significantly different.** Conditions as described in Fig. 3. Where indicated, 400 nM thapsigargin were added. Continuous trace, control cells; dotted trace cells carrying the V2168M RYR1 gene mutation. The traces are representative of experiments carried out at least 11 times.

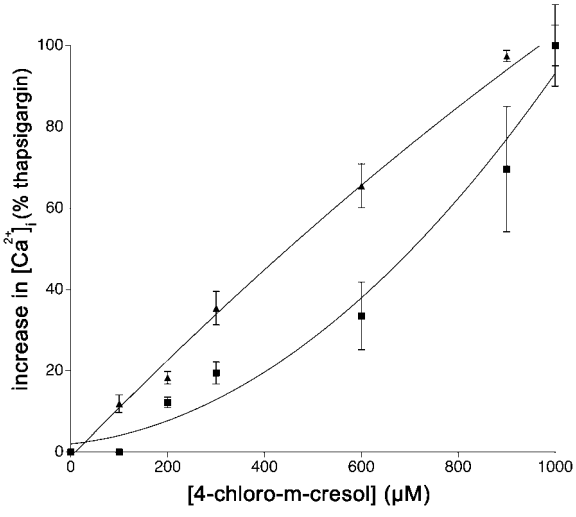

FIG. 5. **EBV-immortalized lymphocytes from MHS individuals carrying the V2168M RYR1 gene mutation are more sensitive to 4-chloro-*m*-cresol than EBV lymphocytes from control individuals.** The increases in $[Ca^{2+}]_i$ induced by the indicated concentrations of 4-chloro-*m*-cresol were calculated as a percentage of the maximal amount which could be released by thapsigargin. Results are expressed as mean (±SD of $n = 4$–12). The EC_{50} of 4-chloro-*m*-cresol-induced increase in $[Ca^{2+}]_i$ for EBV-cells from controls was 750 μM whereas that from MHS individuals with the V2168M mutation was 450 μM.

A, *lanes 1* and *3*) and 338 bp (*panel B*, *lanes 1* and *3*), respectively. Digestion of the PCR-amplified genomic DNA with *Msl*I gives rise to a band of ~192 bp (*panel A*, *lanes 2* and *4*). An extra band of ~196 bp (*) is present after digestion of the amplified genomic DNA from EBV-immortalized cells from a patient carrying the V2168M mutation (*panel A*, *lane 4*). Digestion of the PCR-amplified cDNA fragment with *Msl*I yielded no additional bands when RT-PCR was performed on cDNA obtained from EBV-immortalized cells from a healthy volun-

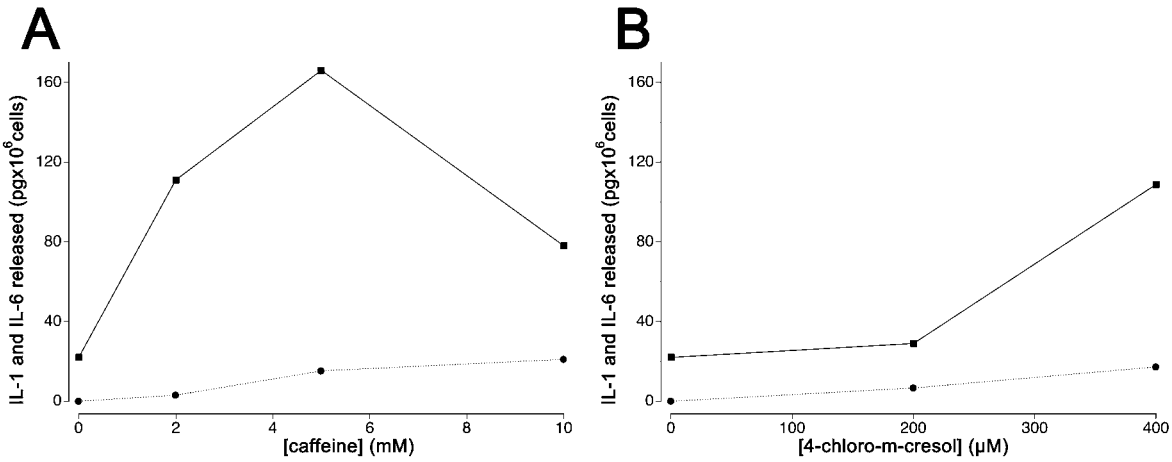

FIG. 6. **Caffeine and 4-chloro-*m*-cresol stimulate IL-1β release from isolated peripheral blood mononuclear leukocytes.** Cells from controls were incubated at 37 °C with the indicated concentrations of agonist. After 60 min cells were centrifuged and the amount of IL-1β and IL-6 released into the supernatant was determined by ELISA. Data represent the result of a typical experiment from a single individual carried out in triplicate. (■——■ IL-1β; ●·····● IL-6).

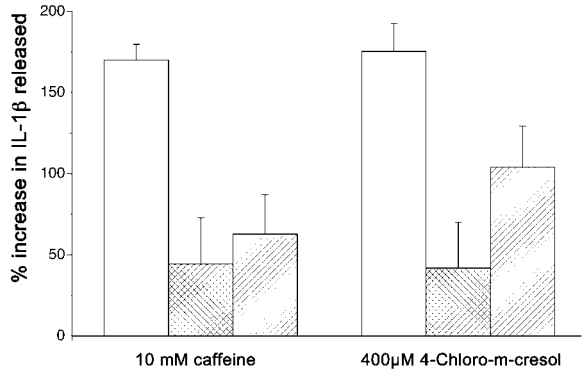

FIG. 7. **IL-1β release from peripheral blood mononuclear leukocytes is calcium-dependent.** Cells from 4 normal controls were incubated at 37 °C with 10 mM caffeine or 400 μM 4-chloro-*m*-cresol (empty boxes), with 10 mM caffeine or 400 μM 4-chloro-*m*-cresol after treatment with 50 μM BAPTA in Ca^{2+}-free medium (crossed boxes) or with 20 μM dantrolene plus 10 mM caffeine or 400 μM 4-chloro-*m*-cresol (hatched boxes). After 60 min cells were centrifuged and the amount of IL-1β released into the supernatant was determined by ELISA. Results are plotted as % increase (± S.E.) in IL-1β released. Mean spontaneous release by mononuclear leukocytes incubated at 37 °C for 60 min. with carrier alone was 20 pg/10^6cells and was considered 100%.

teer (*panel B*, *lane 2*). On the other hand, digestion of the PCR amplified cDNA obtained from EBV-immortalized cells from a patient carrying the V2168M, with *Msl*I, yielded two bands of ~146 and 192 bp (*panel B*, *lane 4*).

We next tested the sensitivity of EBV-immortalized B-cell lines from healthy controls and from the 4 MHS subjects carrying the V2168M RYR1 gene mutation, to the RYR-activator 4-chloro-*m*-cresol. This compound has been shown to activate type 1 and 2 RYR in isolated muscle vesicles but has no effect on type 3 RYR (20, 29–31). Fig. 3*A* shows that the addition of 300 μM 4-chloro-*m*-cresol causes an increase in the $[Ca^{2+}]_i$ of the B-cells. This effect is abolished by pre-treatment with thapsigargin an inhibitor of SERCA type CaATPase, indicating that the intracellular stores that are endowed with the RYR also contain this ATPase (Fig. 3*B*). We also tested the specificity of 4-chloro-*m*-cresol by adding it to HL60 cells, a human myelomonocytoid cell line. In this case treatment with 4-chloro-*m*-cresol failed to elicit release of Ca^{2+} from intracellular stores (Fig. 3*C*), confirming the specificity of this agonist and the lack of functional RYR1 calcium release channels in this myelomonocytoid cell line.

Malignant hyperthermia has been shown to affect the functional characteristics of the skeletal muscle calcium release channel; in particular, most mutations in the RYR1 gene cause a shift in the dose-response curve to RYR agonists, to a lower agonist concentration. Therefore, we examined the dose-response curve of 4-chloro-*m*-cresol-induced calcium release from intracellular stores of EBV-immortalized lymphocytes from the 4 MHS individuals carrying the V2168M mutation in the RYR channel.

We first calculated the size of the thapsigargin-sensitive calcium pools; the mean (± S.D.) change in fluorescence was 0.87 (± 0.19, $n = 11$) fluorescence units, for controls and 0.74 (± 0.13, $n = 20$) fluorescence units for cells carrying the V2168M mutation. Fig. 4 shows a representative trace of the peak $[Ca^{2+}]_i$ induced by 400 nM thapsigargin. Considering the small day-to-day variations in the experimental conditions and potential differences linked to the use of different cell lines however, we normalized our results by calculating the mean increase in $[Ca^{2+}]_i$ induced by different 4-chloro-*m*-cresol concentrations, as a percentage of the total amount of calcium released by 400 nM thapsigargin. We next performed a dose-response curve to 4-chloro-*m*-cresol by averaging the mean amount of calcium released from each of the cell lines established from the 4 patients harboring the V2168M mutation and from the 4 healthy volunteers. Our results show that the capacity of 4-chloro-*m*-cresol to induce Ca^{2+} release was significantly different in the EBV-immortalized B-cells from control and MHS individuals (Fig. 5; repeated measurement ANOVA, $p = 0.018$) and the EC_{50} for 4-chloro-*m*-cresol was shifted from 750 μM in cells from control individuals to 450 μM in cells from MHS individuals.

We next carried out two sets of experiments: first, we examined whether stimulation of RYR by caffeine and 4-chloro-*m*-cresol caused the release of IL-1β and IL-6 from the EBV-immortalized B-cell lines. Under our experimental conditions, the amount of cytokines released by these cells was barely detectable. Thus, we examined the effect of RYR agonists on IL-1β and IL-6 release from peripheral blood mononuclear leukocytes. We also examined whether the effect of the RYR agonist could be blocked (i) by dantrolene, an inhibitor of the type 1 RYR (32, 33) and (ii) by chelating intracellular calcium ions with BAPTA. We only investigated "early" cytokine release (after 60 min) as the symptoms of an MH crisis almost always occur within the first hour after contact with trigger agents. Experiments were first performed on whole peripheral blood mononuclear leukocytes from control individuals. Fig. 6 shows

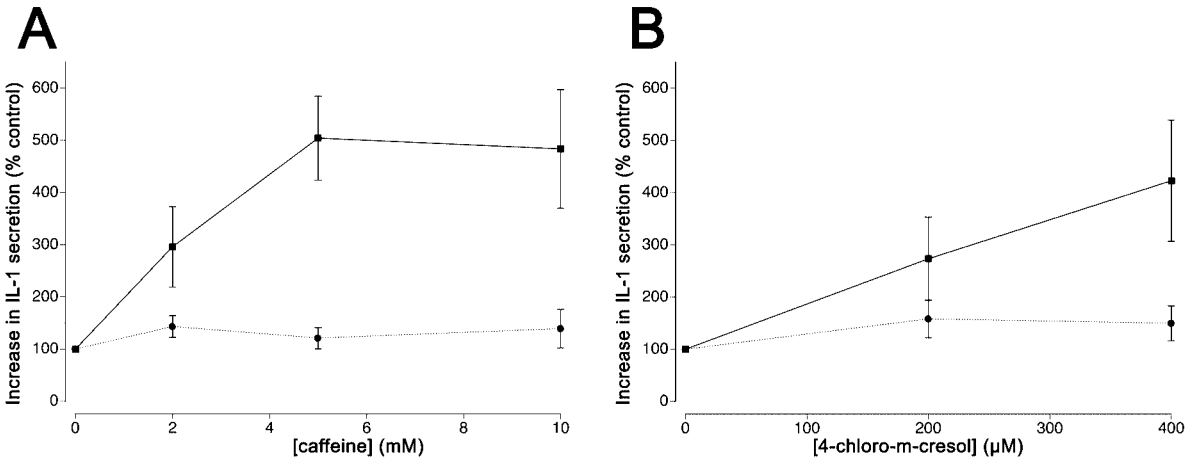

FIG. 8. **Peripheral blood mononuclear leukocytes from MHS individuals carrying the V2168M mutation release more IL-1β than mononuclear leukocytes from controls after treatment with caffeine and 4-chloro-*m*-cresol.** 2×10^5 cells were incubated at 37 °C for 60 min with either caffeine (*panel A*) or 4-chloro-*m*-cresol (*panel B*); the amount of IL-1β released into supernatant was determined by ELISA. Values are expressed as % increase in IL-1β released by cells treated with carrier alone for 60 min at 37 °C. Experiments were carried out in triplicate in cells isolated from 4 MHS and 4 MHN individuals (caffeine) or 3 MHS and 4 MHN individuals (4-chloro-*m*-cresol) and are given as mean ± SD (●·····● MHN and ■——■ MHS).

that under our experimental conditions both caffeine (*panel A*) and 4-chloro-*m*-cresol (*panel B*) caused a dose-dependent increase in interleukin production in cells from control individuals; the amount of IL-1β released was ~5-fold higher than that of IL-6 released. This increase appears to be calcium-dependent because depletion of intracellular calcium stores with BAPTA inhibited the release of IL-1β induced by 10 mM caffeine and 400 μM 4-chloro-*m*-cresol (Fig. 7, *crossed boxes*). Furthermore, 20 μM dantrolene abolished the stimulation of IL-1β release by 10 mM caffeine and 400 μM 4-chloro-*m*-cresol, supporting the involvement of the RYR Ca^{2+} channel in this process (Fig. 7, *hatched boxes*).

Finally, we compared the amount of IL-1β released after addition of the two RYR agonists by peripheral blood mononuclear leukocytes from 4 healthy donors and 4 individuals carrying the RYR1 MH-linked mutation. Fig. 8 shows the percentage increase in IL-1β released, induced by treating leukocytes with the indicated concentrations of caffeine (*panel A*) and 4-chloro-*m*-cresol (*panel B*) for 60 min at 37 °C. There was considerable between-subject variability in the amount of IL-1β released into the medium. We thus normalized the values by calculating the amount of IL-1β released after incubating the leukocytes from each individual, for 60 min at 37 °C with carrier alone; this value was considered 100%, and the increase in interleukin released by caffeine and 4-chloro-*m*-cresol was calculated. Repeated measurements ANOVA revealed significant differences in the amount of IL-1β released after addition of caffeine and 4-chloro-*m*-cresol, between individuals carrying the V2168M mutation and the control group ($p = 0.0034$ and $p = 0.0026$, respectively).

DISCUSSION

The clinical signs of an impending MH crisis are highly variable and include muscle rigidity, metabolic acidosis, rhabdomyolysis, tachycardia, and an increase in body temperature (1, 2). Investigations into the molecular mechanism of MH have led to the hypothesis that this is a disorder of skeletal muscle excitation-contraction coupling and that the clinical signs are a result of the hypermetabolic state caused by alterations in the mechanism regulating the myoplasmic calcium concentration. The aim of the present report was to establish whether MH-associated mutations in the RYR1 gene could also be associated with an increase in body temperature via an alteration of thermoregulatory mechanisms. Body temperature is controlled by the thermoregulatory centers localized in the anterior hypothalamus. Increases in body temperature may be caused either (i) by a "peripheral" mechanism, involving an increase of thermogenesis by skeletal muscle contraction (shivering) and/or a decrease of thermodissipation, or (ii) by a central mechanism mediated by endogenous pyrogens. Phagocytic cells produce and release the major endogenous pyrogens IL-1, IL-6, tumor necrosis factor-α, and interferon-α, although endothelial cells, fibroblasts, myoblasts, and B-lymphocytes are also capable of producing, among others, IL-α and IL-β.

We first carried out a set of experiments to confirm the results of Sei *et al.* (23) and unequivocally demonstrate the expression of type 1 RYR in EBV-immortalized B-cell lines. RT-PCR analysis revealed the presence of the mRNA encoding skeletal muscle type 1 RYR in B-lymphocytes. The presence of mRNA strongly suggests but does not necessarily prove the existence of the protein product. This issue was confirmed by immunoblotting, which revealed a protein band referable to the RYR in the microsomal fraction of B-lymphocytes. The expression of type 1 RYR in B-cells was also confirmed by genotype analysis of patients carrying the V2168M mutated RYR1 allele in their B-lymphocytes. The EBV-immortalized B-lymphocytes thus offer an interesting tool to investigate the functional effects of "natural" mutations in the RYR1 gene. To trigger calcium release from the intracellular store of EBV-immortalized B-cells, we used 4-chloro-*m*-cresol, a RYR type 1-specific agonist, which has been used to characterize MH-linked mutation (34–37). Our results show that EBV-immortalized lymphocytes from normal donors are sensitive to 4-chloro-*m*-cresol, and that the presence of the V2168M mutation in the RYR1 gene decreases the EC_{50} of 4-chloro-*m*-chresol-induced calcium release ~2-fold (750 μM *versus* 450 μM for control and MHS-EBV cells, respectively). We would like to point out that the EC_{50} of 4-chloro-*m*-cresol-induced calcium release in EBV-transformed lymphocyte is similar to that described previously in other experimental models (34–37). Thus, treatment with Epstein-Barr virus does not dramatically interfere with the pharmacological sensitivity of the RYR.

The most interesting result of the present report concerns the involvement of the RYR on IL-1β release from the peripheral blood mononuclear leukocytes of MHS and control individuals. Caffeine and 4-chloro-*m*-cresol are considered specific activators of RYR1, although the latter compound has been

shown to be specifically active on RYR type 1 and 2 (20, 29–31). We believe that caffeine and 4-chloro-*m*-cresol are eliciting IL-1β release by stimulating the RYR of circulating B-lymphocytes. However, because cells of the immune system work in an integrated network and communicate with each other via cytokine production, both *in vivo* and under our experimental conditions, we cannot exclude the possibility that activated B-lymphocytes may in turn activate other cell types (monocytes, endothelial cells, fibroblasts, etc.) to also release IL-1β. We also assayed whether RYR agonists affect the release of other cytokines such as interferon-γ or tumor necrosis factor-α, but the amounts of these cytokines present in the supernatant of stimulated peripheral blood leukocytes was barely detectable (results not shown). As to the EBV-immortalized cell lines, the amounts of IL-1β released was barely detectable, a result consistent with the fact that synthesis and production of cytokines by B-lymphocytes may be restricted to specific stages of differentiation (38).

We found that maximal stimulating concentrations of 4-chloro-*m*-cresol and caffeine caused the release of approximately 100 pg of IL-1β/10^6 cells. The amount of IL-1 produced by circulating B-lymphocytes in a normal individual could therefore be approximately 350 ng (0.7×10^9 cells × 5 liters of blood). Because IL-β is the most pyrogenic cytokine, inducing temperatures of 39 °C in response to doses as small as 1–10 ng/kg body weight (25), the theoretical quantity of IL-1 produced is well within the limit necessary to increase body temperature via a central mechanism.

MH reactions can be triggered by inhalative anesthetics, exercise, and stress, and several laboratories have reported that anesthetic agents, as well as sustained exercise activity, increase the plasma level of pro-inflammatory cytokines (39–42). Because human myoblasts have been shown to behave as immunologically active cells during inflammation, producing, among others, cytokines of the monocyte-macrophage cell lineage (43), it may be that in skeletal muscle cells cytokine secretion is influenced by elevations in the $[Ca^{2+}]_i$. The RYR1 calcium channel plays a central role in controlling the myoplasmic $[Ca^{2+}]$; thus, our results imply that the muscle cells from MHS individuals may also produce more IL-1, an additional factor that may contribute to the increase in body temperature. Dantrolene, an inhibitor of the RYR1, is a life-saving compound used by clinicians to revert MH reactions and neuroleptic malignant syndrome (21, 44, 45). This pharmacological agent has also been shown to decrease plasma and tissue concentrations of inflammatory cytokines in septic animals and improve their survival (46–49). Thus, by blocking the RYR, dantrolene may also block cytokine production by muscle cells and lymphocytes and, in this way, help re-establish physiological body temperature.

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B-lymphocytes from Malignant Hyperthermia-susceptible Patients Have an Increased Sensitivity to Skeletal Muscle Ryanodine Receptor Activators

Thierry Girard, Dario Cavagna, Elisabetta Padovan, Giulio Spagnoli, Albert Urwyler, Francesco Zorzato and Susan Treves

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Additions and Corrections

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B-lymphocytes from malignant hyperthermia-susceptible patients have an increased sensitivity to skeletal muscle by ryanodine receptor activators.

Thierry Girard, Dario Cavagna, Elisabetta Padovan, Giulio Spagnoli, Albert Urwyler, Francesco Zorzato, and Susan Treves

Page 48080, Fig. 6 legend and 48081, line 3: Cells were from an MHS individual and not from a control as indicated.

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Validated zinc finger protein designs for all 16 GNN DNA triplet targets.

Qiang Liu, ZhenQin Xia, Xiaohong Zhong, and Casey C. Case

Dr. Zhong's name was omitted from the list of authors. The corrected list is shown above.

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SYT associates with human SNF/SWI complexes and the C-terminal region of its fusion partner SSX1 targets histones.

Hiroyuki Kato, Agneta Tjernberg, Wenzhu Zhang, Andrew N. Krutchinsky, Woojin An, Tamotsu Takeuchi, Yuji Ohtsuki, Sumio Sugano, Diederik R. de Bruijn, Brian T. Chait, and Robert G. Roeder

Page 5498, footnote: The first line of the grant support footnote should read "This work was supported by Grants CA42567 (to R. G. R.) and RR00862 (to B. T. C.) from the National Institutes of Health."

Page 5501, lefthand column, line 26: "TPQPSSPMDQMGK (amino acids 236–261)" should read "TPQPSSPMDQMGK (amino acids 31–43)."