Anesthesiology 2006; 104:1191–201

Characterization of Ryanodine Receptor–mediated Calcium Release in Human B Cells

Relevance to Diagnostic Testing for Malignant Hyperthermia

Leslie C. McKinney, Ph.D., Thomas Butler, Ph.D.,† Shawn P. Mullen, Ph.D.,† Michael G. Klein, Ph.D.‡*

Background: **Mutations in the ryanodine type 1 receptor (RyR1) are causative for malignant hyperthermia. Studies in human B lymphocytes have shown that measurement of RyR1-mediated intracellular Ca^{2+} (Ca^{2+}_i) release can differentiate between normal and malignant hyperthermia–susceptible individuals. The authors have further developed the B-cell assay by pharmacologically characterizing RyR1-mediated Ca^{2+} release in two normal human B-cell lines and demonstrating increased sensitivity of lymphocytes to the RyR1 agonist 4-chloro-*m*-cresol (4-CmC) in the porcine model of MH.**

Methods: **Ca^{2+}_i was measured fluorometrically using fura-2 in populations of cells in suspension or with fluo-4 in single cells using confocal microscopy. The Dakiki and PP normal human B cell lines were used, as well as lymphocytes obtained from normal and malignant hyperthermia–susceptible pigs. 4-CmC was used to elicit RyR1-mediated Ca^{2+} release; all experiments were performed in the absence of external Ca^{2+}.**

Results: **EC_{50} values for 4-CmC were 0.98 and 1.04 mM for Dakiki and PP cells, respectively, demonstrating reproducibility. The 4-CmC–induced increase in Ca^{2+}_i was eliminated by thapsigargin and was unaffected by xestospongin C. The Ca^{2+}_i increase was separable from mitochondrial stores and was inhibited by azumolene. Caffeine did not induce Ca^{2+}_i release, but ryanodine depleted intracellular stores by 50%. Lymphocytes from pigs carrying the Arg614Cys mutation in RyR1 showed increased sensitivity to 4-CmC (EC_{50} = 0.47 *vs.* 0.81 mM for cells derived from normal animals).**

Conclusions: **RyR1-mediated Ca^{2+} signals can be pharmacologically distinguished from other intracellular sources in human B cells, and alterations of RyR1 function can be successfully detected using Ca^{2+} release from intracellular stores as an end point.**

MALIGNANT hyperthermia (MH) is a genetically inherited disorder whose distinguishing feature is an abnormal response to volatile anesthetics and depolarizing muscle relaxants. Exposure to these agents during surgery triggers uncontrolled Ca^{2+} release from the sarcoplasmic reticulum through the ryanodine receptor (RyR), inducing a cascade of biochemical events that results in muscle rigidity, rhabdomyolysis (muscle breakdown), cardiac arrhythmia, and lethal hyperthermia. Mutations in the skeletal muscle ryanodine type 1 receptor (RyR1) have been linked to more than 50% of human MH cases.[1,2] To date, more than 40 mutations in RyR1 have been shown to be causal for the disease.[3,4]

Definitive diagnosis of MH is made by means of the caffeine–halothane contracture test (CHCT) on biopsied leg muscle. Muscle fibers from MH-susceptible persons are markedly more sensitive to halothane[5] and to the RyR "agonists" caffeine and 4-chloro-*m*-cresol (4-CmC).[6,7] The latter compound is more potent than caffeine and has been shown to selectively induce release of intracellular Ca^{2+} (Ca^{2+}_i) in several skeletal muscle preparations and isolated sarcoplasmic reticular vesicles.[8–13] It is the reproducible shift in the dose sensitivity of muscle contraction to these drugs that has led to use of the CHCT as *the* diagnostic indicator of MH.[14] However, because of the invasive nature of the test, only approximately 10% of those experiencing "suspicious" responses to anesthesia elect to have it (estimated from referrals to the Malignant Hyperthermia Hotline). Therefore, there is a pressing need for a less invasive diagnostic test in addition to a better understanding of the pharmacology of MH.

An alternative way to test for MH is to assay RyR1-mediated Ca^{2+} release directly. The rationale behind this approach is that Ca^{2+}_i release is a sensitive indicator of RyR1 function and that mutations in RyR1 that lead to abnormally high Ca^{2+}_i release in skeletal muscle will also lead to aberrant Ca^{2+}_i release in other cell types expressing RyR1. This approach is currently being tested in two tissues, myotubes[15–20] and B lymphocytes.[21–23]

In myotubes derived from MH patients with identified mutations in RyR1, Ca^{2+}_i release induced by 4-CmC or caffeine was increased compared with controls; specifically, the EC_{50} for 4-CmC was reduced from 203 μM to 98 μM, and that for caffeine was reduced from 3.8 mM to 1.8 mM.[24] The increased Ca^{2+}_i response in myoblasts segregated well with the MH phenotype. However, this assay, although promising, still requires surgical biopsy and myotube culture and thus is not a significant improvement over the current CHCT.

* Research Assistant Professor, Department of Anesthesiology, Uniformed Services University of the Health Sciences. † Post-doctoral Fellows, ‡ Associate Professor, Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, Maryland.

Received from the Department of Anesthesiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland. Submitted for publication April 15, 2005. Accepted for publication January 4, 2006. Supported by grant No. HO-80AF (to Dr. McKinney) from the Uniformed Services University of the Health Sciences and grant No. AR44197 (to Dr. Klein) from the National Institutes of Arthritis, Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland. Drs. Butler and Mullen are supported by the Interdisciplinary Training Program in Muscle Biology at the University of Maryland School of Medicine, Baltimore, Maryland, and grant No. 5T32AR007592 from the National Institutes of Arthritis, Musculoskeletal and Skin Diseases, National Institutes of Health. Presented at the 46th Annual Biophysical Society Meeting, San Antonio, Texas, February 23–27, 2003; the Gordon Research Conference on Muscle: Excitation/Contraction Coupling, Colby Sawyer College, New London, New Hampshire, June 8–13, 2003; Experimental Biology (FASEB), Washington, D.C., April 17–24, 2004; and the Association of University Anesthesiologists 52nd Annual Meeting, Baltimore, Maryland, May 6–8, 2005.

Address correspondence to Dr. McKinney: Department of Anesthesiology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, Maryland 20814. Lmckinney@usuhs.mil.

In B cells, initial reports also indicated a correlation between increased Ca^{2+}_i responses to caffeine and 4-CmC and the MH phenotype. In the largest study to date (n = 13 patients), Ca^{2+}_i release was measured using flow cytometry in primary B cells freshly isolated from persons undergoing CHCT at the Uniformed Services University of the Health Sciences (Bethesda, Maryland) MH testing center.[23] Caffeine- or 4-CmC–induced Ca^{2+}_i release was slightly increased over controls, but population variability of the Ca^{2+}_i measurements was large, and methodologic problems weakened the interpretation of the data. First, measurements were made in the presence of normal (1 mM) extracellular Ca^{2+}, which means that a significant portion of the Ca^{2+} signal was likely due to influx of Ca^{2+} and not due to release of Ca^{2+} from ryanodine-sensitive stores. Second, the increased responsiveness of B cells derived from MH-susceptible patients to caffeine was detected only when an extremely high (50 mM) caffeine concentration was used. Third, most patients at the time of original study had not been genotyped, and subjects may have been included who were CHCT positive but did not carry RyR1 mutations. However, later work showed that the increased Ca^{2+}_i signal did segregate with the presence of RyR1 mutations in two MH families,[3,23] and a second laboratory independently confirmed that 4-CmC sensitivity was increased in Epstein-Barr virus–transformed B cells derived from an MH patient carrying the Val2168Met mutation in RyR1.[21] Taken together, these experiments lend considerable strength to the hypothesis that it is possible to differentiate between normal and MH patients on the basis of Ca^{2+}_i signals from B cells.

To determine whether the B-cell assay can be made specific enough to use as a diagnostic tool for MH, it is necessary to prove that Ca^{2+}_i release can be a sensitive indicator of RyR1 function. In addition, because there are multiple intracellular pools of Ca^{2+}, including inositol trisphosphate (IP_3)–sensitive stores and mitochondria, it is also necessary to demonstrate that RyR1-mediated Ca^{2+}_i release can be selectively measured. In this work, we characterize the pharmacologic properties of Ca^{2+}_i release in two human B-cell lines (Dakiki and PP), demonstrating that a RyR1-sensitive component can be isolated. We then show that the Ca^{2+}_i release is specific to RyR1 and that lymphocyte sensitivity to 4-CmC is increased in the porcine model of MH.

Materials and Methods

Cell Culture

Experiments were performed on the Epstein-Barr virus–transformed human lymphoblast Dakiki cell line (American Type Culture Collection, Manassas, VA) and on a normal human line generated at Uniformed Services University of the Health Sciences designated PP. The Dakiki line was preferentially used because RyR1 has been partially characterized and is reliably expressed in these cells.[25] A few experiments were performed on a mixed population of elutriated primary human lymphocytes (obtained from the National Institutes of Health [Bethesda, Maryland] blood bank). Cell lines were cultured in RPMI 1640 (Gibco Invitrogen, Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (Gibco Invitrogen). Cells were maintained in log phase growth at a density of 1 to 3 million cells/ml and regularly assayed for CD19 expression by flow cytometry using mouse anti-human CD19 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Individual batches were expanded from frozen stocks every 3 months and were routinely greater than 90% $CD19^+$.

Isolation of Porcine Lymphocytes

Pietrain pigs carrying the Arg615Cys RyR1 mutation were obtained from University of Minnesota Experimental Farm (Minneapolis, MN). Yorkshire *sus scrofa* control pigs were obtained from Archer Farms (Darlington MD). A mixed lymphocyte preparation was prepared from whole blood using endotoxin-free Ficoll/Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden). The buffy coat (5 ml), which was usually not well defined, was dispensed into 30 ml Ca^{2+}-free, Mg^{2+}-free Hanks balanced salt solution (HBSS) and centrifuged. The pellet was resuspended in hypotonic HBSS (50%) for 10 min to lyse contaminating erythrocytes, spun, and resuspended in HBSS. Further purification of B cells from this preparation was not attempted at this time to conserve cell numbers.

Ca^{2+}_i Measurements

Intracellular Ca^{2+} measurements were made using Ca^{2+}-sensitive fluorescent indicator dyes in two ways: (1) from populations of cells in suspension, using a cuvette-based system, and (2) from single cells, using confocal microscopy.

(1) Cells in suspension were loaded with 3 μM fura-2 AM in HBSS plus 0.1% endotoxin-free bovine serum albumin (Sigma Chemical Co., St. Louis, MO) for 30–45 min at 37°C, washed, and maintained at room temperature (approximately 22°C) in HBSS until use. Cells were spun and resuspended at a density of 2×10^6/ml in a 3-ml cuvette volume at 37°C and continuously stirred. Pharmacologic agents were added directly to the cuvette, and fluorescence measurements were taken approximately every 0.5 s using a dual excitation wavelength Ratiomaster fluorometer (Photon Technology, Inc., Monmouth, NJ). Duration of the measurements usually did not exceed 20 min.

Data are presented as emission ratios. Occasionally, ratio values were converted to $[Ca^{2+}_i]$ using *in vitro* calibration of the K^+ salt of fura-2 in defined Ca^{2+} buff-

ers (Molecular Probes, Eugene, OR). The *in vitro* fluorescence signal was linear from 0.7 to 7.7, corresponding to 0–350 nM Ca^{2+}. Because caffeine has been reported to affect the dissociation constant (Kd) of fura-2 for Ca^{2+},[26] we tested the effect of 1 mM 4-CmC on the Kd of fura-2 for Ca^{2+} and found no significant difference. Fully saturated dye in cells permeabilized with 1 μM ionomycin in HBSS gave a ratio of 25. Steady state fluorescence ratios were obtained by averaging values for at least 10 s. Estimation of the amount of Ca^{2+} released from intracellular stores (in arbitrary units) was obtained by integrating baseline subtracted fluorescence transients. We measured the extent of fura-2 sequestration by sequentially permeabilizing the plasma and intracellular membranes by 20 μM digitonin and 1% Triton-X, respectively, in the absence of external Ca^{2+}.[27] For a 1-h loading period at 37°C, Dakiki and PP cells respectively showed 29 ± 3% (n = 13) and 27 ± 3% (n = 4) of the dye sequestered. Porcine lymphocytes showed 14 ± 1% (n = 4). No correction for sequestration was applied to the data.

The extent of fura-2 leak was determined as described in Nuccitelli.[27] Leak was approximately 40%/h (rate coefficient 0.0075 ± 0.0003 min^{-1}, n = 3, for Dakiki cells), somewhat slower for PP cells (0.0041, n = 1), and was completely inhibited by reducing the temperature from 37°C to room temperature (data not shown). Because total time at 37°C usually did not exceed 20 min, measurements were not corrected for leak.

Each cuvette experiment included the following controls: verification of adequate fura-2 loading: cells were permeabilized with 1 μM ionomycin in HBSS to fully saturate the fura-2; ratios less than 15 were considered unacceptable; measurement of the total releasable Ca^{2+}_i: cells were permeabilized with 1 μM ionomycin in Ca^{2+}-free HBSS; benchmark response to 4-CmC: cells were exposed to 1 mM 4-CmC in Ca^{2+}-free HBSS.

(2) Ca^{2+}_i measurements from single cells were made using the nonratiometric dye fluo-4 on a confocal system consisting of an Olympus IX-70 (Olympus, Inc., Melville, NY) inverted microscope with a Bio-Rad Radiance confocal scan head controller and laser (Bio-Rad, Inc., Hercules, CA).[28] Cells were loaded with fluo-4 for 30 min at 37°C, washed, and maintained at room temperature for approximately 30–90 min until use. Aliquots of cells were plated onto coverslips, allowed to adhere for approximately 20 min, washed, and mounted on a microscope stage on a vibration-isolation table. Cells were vigorously perfused to remove nonadherent cells. Pharmacologic agents were applied by perfusion, and fluorescence measurements were taken every 1–2 s. In some experiments, cells were permeabilized with 1 μM ionomycin at the end of the experiment to determine maximum fluorescence intensity values.

Because fluo-4 signals are not independent of dye concentration, all increases in fluorescence must be referred to the individual baseline of each cell. Data are thus presented as raw fluorescence (arbitrary units) or increased fluorescence as a percentage of the baseline (Δf/f). The analysis of Ca^{2+}_i transients was performed off-line, using custom routines written in IDL (Research Systems, Inc., Boulder, CO) running on Silicon Graphics, Inc. (Mountain View, CA) workstations. The time course of Ca^{2+}_i change was plotted for each cell in the field. Cells were discarded if resting fluorescence exceeded a threshold determined for each experiment to eliminate cells with spuriously high Ca^{2+}_i. Excluded cells did not exceed 10% of the total. The data from each cell was then pooled to give an average response for the population of cells in that field.

Test Solutions

Test solutions were as follows:

1. Normal HBSS (Gibco Invitrogen).
2. Normal HBSS plus 5 mM ethylene glycol bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA high purity; Fluka, Basel, Switzerland).
3. Ca^{2+}- and Mg^{2+}-free HBSS plus 1 mM EGTA. In the text, these solutions will be referred to as normal Ca^{2+}, low-Ca^{2+}, and Ca^{2+}-free HBSS, respectively, where Ca^{2+} = 1 mM, 35 nM, and 0.1 nM at 37°C, pH 7.25 (Ca^{2+} derived from the free software program Webmaxlite version 1.15§).
4. Caffeine-containing solutions: Hanks solutions were prepared with isosmotic substitution of caffeine for NaCl. These solutions consisted of 5.4 mM KCl; 95, 120, or 140 mM NaCl; 10 mM HEPES buffer; and 50, 25, or 5 mM caffeine, pH = 7.3.
5. As controls for the caffeine-containing solutions, Hanks solutions were prepared with isosmotic substitution of sucrose or *N*-methyl glucamine · Cl for NaCl. The solutions were made as above, with sucrose or *N*-methyl glucamine · Cl instead of caffeine. All reagents were from Sigma Chemical Corp.

Reagents

4-Chloro-*m*-cresol, ionomycin, and thapsigargin (Calbiochem, San Diego, CA) were solubilized in dimethyl sulfoxide and stored at −20°C. Caffeine, 3-deaza-cADPribose (Sigma), and ryanodine (Calbiochem) were solubilized in distilled H_2O. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; Sigma) was solubilized in EtOH and stored at −20°C. Xestospongin C (Calbiochem) was solubilized in dimethyl sulfoxide and used the same day. Azumolene (gift of Jerry Parness, M.D., Department of Anesthesiology, University of Pittsburgh Medical School, Pittsburgh, Pennsylvania) was solubilized in dimethyl sulfoxide and stored at 4°C.

§ Available at: www.stanford.edu/cpatton/maxc.html. Accessed February 7, 2006.

Statistics

All averaged data are reported as mean ± SEM. Tests for significant differences between means were performed using the Student *t* test with Graph Pad Instat software (Graphpad Software, Inc., San Diego, CA) Dose–response data were fit by nonlinear regression to a variable slope sigmoid curve y = min + (max − min)/(1 + 10^((log EC_{50} − x) * slope)) using Graph Pad Prism software.

Results

Resting Ca^{2+}_i

Ratios for resting Ca^{2+}_i for Dakiki cells bathed in normal, low, and Ca^{2+}-free HBSS were 1.62 ± 0.03; n = 72), 1.23 ± 0.02 (n = 70), and 1.16 ± 0.008 (n = 69), which corresponded to 39, 20, and 16 nM Ca^{2+}_i, respectively. Values for PP cells in normal and Ca^{2+}-free HBSS were 1.62 ± 0.02 (n = 10) and 1.16 ± 0.006 (n = 37), which corresponded to 39 and 16 nM, respectively. Values for MH and normal pigs in HBSS were 1.48 ± 0.1 (n = 17) and 1.49 ± 0.035 (n = 18), corresponding to 32 and 33 nM Ca^{2+}_i, and values in Ca^{2+}-free HBSS were 1.16 ± 0.007 (n = 50) and 1.17 ± 0.01 (n = 31), corresponding to 16 and 17 nM Ca^{2+}_i.

Characterization of RyR-mediated Ca^{2+}_i Release

We used five criteria to show that a specific Ca^{2+}_i release signal from RyR-sensitive stores could be isolated: (1) dose-dependent, reversible Ca^{2+}_i release by the RyR agonist 4-CmC in the absence of external Ca^{2+}; (2) elimination of the 4-CmC–induced Ca^{2+}_i signal by depletion of smooth endoplasmic reticular (SER) Ca^{2+}_i stores with thapsigargin; (3) sensitivity to other pharmacologic agents that affect RyR; (4) 4-CmC-induced Ca^{2+}_i release in the presence of xestospongin C, a selective inhibitor of the IP_3 receptor; and (5) demonstration that 4-CmC–induced Ca^{2+}_i release is not affected by depletion of mitochondrial Ca^{2+} stores with FCCP.

4-CmC–induced Ca^{2+}_i Release in the Absence of External Ca^{2+}. Dose-response curves for 4-CmC were determined by measuring the peak fluorescence value as a result of Ca^{2+}_i release during exposure to each drug concentration. The magnitude of the 4-CmC response was dependent on external Ca^{2+}. Shown in figure 1 are dose–response curves obtained from cells bathed in normal (A), low-Ca^{2+} (B), and Ca^{2+}-free (C) HBSS. Note that baseline fluorescence was lower in solutions of decreased external Ca^{2+}. In normal HBSS (fig. 1A), 4-CmC induced large Ca^{2+}_i signals that reached steady state after approximately 1 min. At steady state, the Ca^{2+}_i signal is presumed to result from a mixture of 4-CmC-induced release of Ca^{2+} from intracellular stores, Ca^{2+}-induced Ca^{2+} release from intracellular stores, influx of Ca^{2+} from the extracellular space, and extrusion/reuptake of Ca^{2+}_i from the cytoplasm by either the plasma membrane or SER Ca^{2+}-adenosine triphosphatase. In low-Ca^{2+} HBSS (fig. 1B), the peak signal was reduced, and above 1 mM 4-CmC, the signal peaked and then declined. This was even more pronounced in the complete absence of external Ca^{2+} (fig. 1C). Therefore, removal of external Ca^{2+} eliminated the component of Ca^{2+} influx across the plasma membrane and left only the component released from intracellular stores.

Peak fluorescence ratios (minus baseline) for the full range of 4-CmC concentrations are plotted in figure 1D. Saturation of the dose–response curve was observed near 2 mM 4-CmC in Ca^{2+}-free HBSS. In normal or low-Ca^{2+} HBSS, peak fura-2 ratios saturated at concentrations above 4 mM. These ratios were similar to those obtained by exposure to 1 μM ionomycin (not shown), suggesting that concentrations of 4-CmC above 4 mM were toxic,

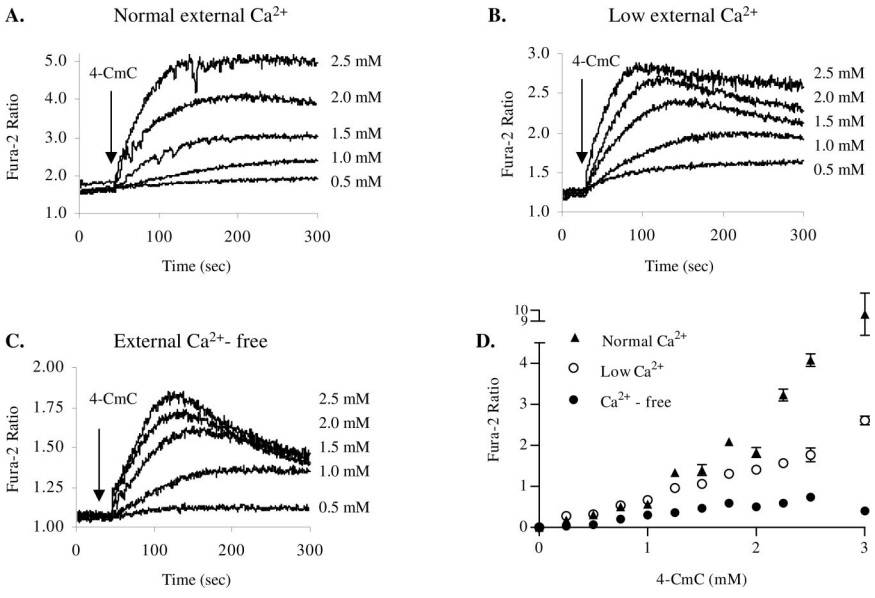

Fig. 1. Time course and dose dependence of 4-chloro-*m*-cresol (4-CmC)–induced intracellular Ca^{2+} release. Dakiki cells in suspension bathed in Hanks balanced salt solution (HBSS) (*A*), low-Ca^{2+} HBSS (*B*), or Ca^{2+}-free HBSS (*C*). Traces show fura-2 ratios resulting from addition of 0.5, 1.0, 1.5, 2.0, or 2.5 mM 4-CmC at 30–50 s. Estimated intracellular Ca^{2+} concentration for a fura-2 ratio of 5 was 206 nM. Dimethyl sulfoxide controls showed no effect on Ca^{2+} release. (*D*) 4-CmC dose–response. Dakiki cells bathed in HBSS (▲), low-Ca^{2+} HBSS (○), or Ca^{2+}-free HBSS (●). Y values are the mean ± SEM of peak minus baseline fluorescence ratios for specified concentrations of 4-CmC. In HBSS, n = 3–6 for each concentration; in low-Ca^{2+} HBSS, n = 3–10; in Ca^{2+}-free HBSS, n = 5–23 for all concentrations except 1 mM 4-CmC, where n = 75.

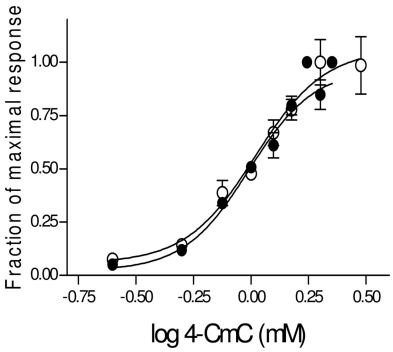

Fig. 2. Normalized 4-chloro-*m*-cresol (4-CmC) dose–response curves for two cell lines: Data from Dakiki (●) and PP (○) cell lines were normalized to the maximum fluorescence value for that curve. n = 3–5 for PP. Data were fit as described in Materials and Methods.

either directly or indirectly, by causing excessive Ca^{2+} influx. For that reason, we limited the dose–response analysis to concentrations less than 2.5 mM and used only data obtained in Ca^{2+}-free HBSS.

Figure 2 shows the normalized dose–response curves for 4-CmC in Ca^{2+}-free HBSS for the Dakiki and PP cell lines. For each cell line, the curve saturated near 2 mM, with EC_{50} values of 0.98 ± 0.02 and 1.04 ± 0.03 mM (mean ± SEM), respectively. The EC_{50} value was similar (1.3 mM) when 4-CmC responses were plotted differently, as a fraction of total ionomycin-induced Ca^{2+} release.

To determine whether Ca^{2+} release was occurring from the entire population of cells and not just a subset, confocal microscopy was used to measure the response of single cells to 4-CmC. The fluorescent Ca^{2+}_i signal was both uniform across the field and robust (typical Δf/f = 2; Δf/f = (peak – baseline)/baseline fluorescence). Shown in figure 3 are three traces illustrating the dose-dependent response to 0.5, 1.0, and 1.5 mM 4-CmC in Ca^{2+}-free HBSS. 4-CmC was applied at 60 s and washed off at 150 s, thus demonstrating reversibility. A similar

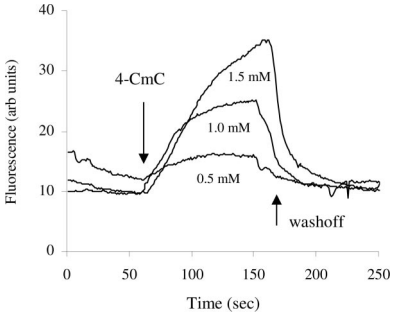

Fig. 3. 4-Chloro-*m*-cresol (4-CmC)–induced intracellular Ca^{2+} release in single cells. Averaged tracings from single Dakiki cells using confocal microscopy. Cells were perfused in Ca^{2+}-free Hanks balanced salt solution, then with 0.5 (*lower*), 1.0 (*middle*), or 1.5 (*upper*) mM 4-CmC, respectively. *Y-axis* is Δf/f in arbitrary units. Each trace represents the averaged response of 37–43 cells gathered from a single field and is representative of at least three fields.

response was obtained from primary human lymphocytes (mixed population; data not shown).

Depletion of Intracellular Stores Eliminates 4-CmC–induced Ca^{2+} Release. Intracellular stores can be depleted in two ways: by treatment with ionomycin, which nonselectively permeabilizes all intracellular membranes, and by thapsigargin, which inhibits the SER Ca^{2+}-adenosine triphosphatase and selectively depletes SER stores.[29,30] Figure 4A shows the time course of Ca^{2+} release after application of thapsigargin in Ca^{2+}-free solution. Thapsigargin, 100 nM, led to a transient increase in Ca^{2+}_i that declined to resting levels over a time course of minutes. 4-CmC applied after thapsigargin yielded no further Ca^{2+}_i release, whereas subsequent application of 1 μM ionomycin induced residual Ca^{2+}_i release from non-SER stores. Figure 4B shows the converse experiment. Application of thapsigargin after 1 mM 4-CmC still

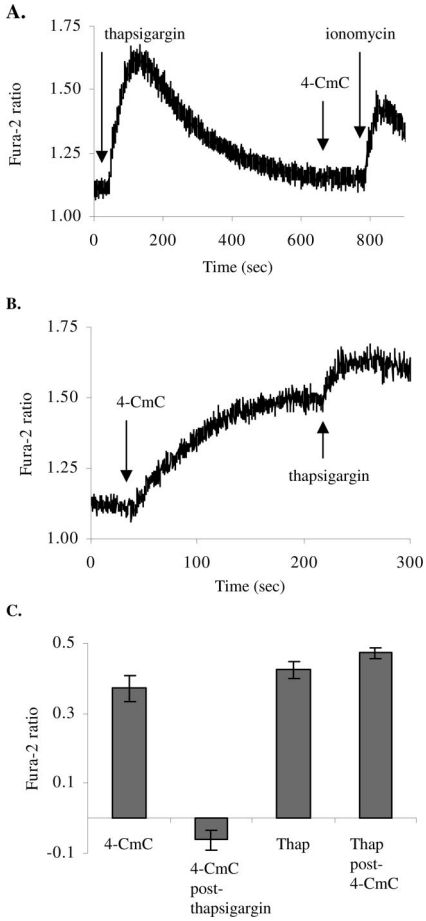

Fig. 4. Predepletion of intracellular stores eliminated 4-chloro-*m*-cresol (4-CmC)–induced intracellular Ca^{2+} release. Dakiki cells in suspension in Ca^{2+}-free Hanks balanced salt solution. (*A*) Representative trace showing response to 100 nM thapsigargin applied at 30 s, 1 mM 4-CmC at 630 s, 1 μM ionomycin at 800 s. (*B*) 4-CmC, 1 mM, at 40 s followed by 100 nM thapsigargin at 225 s. (*C*) *Y-axis* is peak fura-2 ratio minus baseline. Values are given as mean ± SEM. *First bar*: 4-CmC alone, n = 8; *second bar*: 4-CmC applied after thapsigargin, no increase in intracellular Ca^{2+} observed, n = 6; *third bar*: thapsigargin alone, n = 10; *fourth bar*: thapsigargin applied after 1 mM 4-CmC, same maximal levels achieved, n = 5.

produced an increase in Ca^{2+}_i, to the same absolute level as with thapsigargin alone. This indicated that 1 mM 4-CmC did not fully deplete the Ca^{2+}_i pool but that 4-CmC followed by thapsigargin released all the available Ca^{2+}_i. Figure 4C summarizes data from two experiments (multiple traces in each) demonstrating that predepletion of Ca^{2+}_i stores eliminates the ability of 4-CmC to induce a Ca^{2+}_i signal.

The size of the intracellular stores (in arbitrary units) was estimated by integrating the area of the Ca^{2+}_i transient. Thapsigargin released, on average, 58% of the total ionomycin-releasable stores. Maximal levels of 4-CmC (2 mM) released 65% of the total releasable Ca^{2+}_i, comparable to the amount released by thapsigargin (integrals measured or extrapolated for 500-s interval; multiple traces for each condition).

Sensitivity to Other Pharmacologic Agents That Affect RyR.

Azumolene. The effect of the membrane permeant RyR inhibitor azumolene was tested on 4-CmC–induced release. Azumolene has increased potency (EC_{50} = 20 μM in skeletal muscle) and solubility over its analog dantrolene.[31] However, because azumolene has a spectral sensitivity that overlaps fura-2, these experiments could be performed only on cells loaded with fluo-4 using confocal microscopy. Azumolene, 10 mM, was diluted into a cell suspension at a final concentration of 100 or 400 μM just after fluo-4 loading and washing were complete. Cells were then plated on coverslips, and 4-CmC–induced Ca^{2+}_i release was measured. Therefore, the total time of exposure to azumolene before application of 4-CmC was 30–90 min.

Figure 5 shows the Ca^{2+}_i signal resulting from application of 1.5 mM 4-CmC for control (upper trace) and azumolene-treated (lower trace) cells for two concentrations of azumolene, 100 μM (A) and 400 μM (B). The percent inhibition of the peak Ca^{2+}_i response was 23% and 50%, respectively (table 1). Because the sensitivity of Dakiki cells to azumolene was low, we verified the potency of this batch of azumolene by testing its effect on single frog skeletal muscle fibers, where 10 μM reversibly inhibited voltage-induced Ca^{2+}_i release by 70% (data not shown). Unexpectedly, in Dakiki cells, azumolene also affected the rate of decline after 4-CmC wash-off. The time constant (τ) for decline was increased 6.5-fold by 100 μM azumolene and almost 10-fold by 400 μM azumolene. The mechanism of this effect is not known.

Ryanodine. The effect of ryanodine on Ca^{2+}_i release and on 4-CmC–induced Ca^{2+}_i release was tested in Dakiki cells for both acute and long-term exposures over a wide range of doses. In skeletal muscle preparations, ryanodine is an agonist of RyR1 at low concentrations (nanomolars) and an antagonist of RyR1 at higher concentrations (micromolars).[32,33] Acute exposure of Dakiki cells to ryanodine (100 nM, 1 μM) had no significant

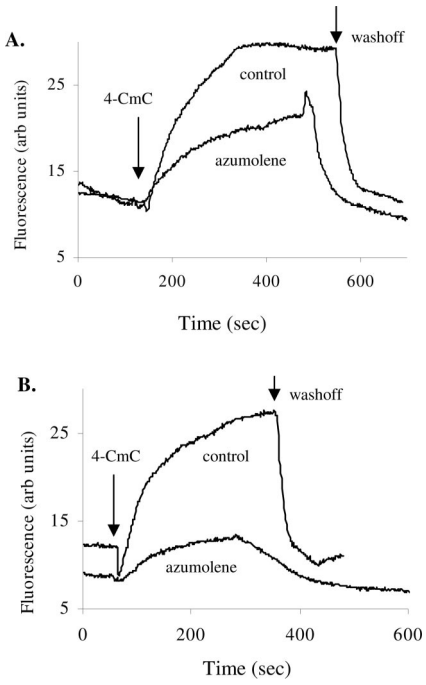

Fig. 5. Effect of azumolene on 4-chloro-*m*-cresol (4-CmC)–induced Ca^{2+}_i. Dakiki cells in 0 Ca^{2+} Hanks balanced salt solution plated for confocal microscopy. (*A*) *Upper trace*: 1.5 mM 4-CmC applied at 150 s and washed off at 550 s. *Lower trace*: 100 μM azumolene throughout; 1.5 mM 4-CmC applied at 150 s and washed off at 450 s. (*B*) *Upper trace*: 1.5 mM 4-CmC applied at 80 s and washed off at 380 s. *Lower trace*: 400 μM azumolene throughout; 1.5 mM 4-CmC applied at 80 s and washed off at 300 s. Each trace is the average of 43–59 single cell records taken from a single field for that condition.

effect on 4-CmC–mediated Ca^{2+}_i release for cells bathed in Ca^{2+}-free HBSS. A slight response (in the range of 0.1–0.2 ratio units) was observed for acute exposure to a very high concentration of ryanodine (1 mM) when cells were bathed in normal HBSS or low-Ca^{2+} HBSS but not Ca^{2+}-free HBSS.

Prolonged (1–2 h) exposure to ryanodine had several effects. At low concentration (100 nM), cells spontaneously released Ca^{2+}_i, although this effect was inconsistent and therefore not characterized. At high concentration (0.1–1 mM), it caused depletion of intracellular stores, which has been observed in other cell types[34] and is thought to be due to the ability of ryanodine to induce a partially open configuration in the RyR. We

Table 1. Effect of Azumolene on 4-CmC–induced Fluorescence Change

	Fluorescence Increase (Δf/f)	Time Constant of 4-CmC Wash-off (τ), s
Control	1.29 ± 0.07 (5)	12.02 ± 1.72 (5)
100 μm Azumolene	0.99 ± 0.11 (3)	77.20 ± 29.43(3)
400 μm Azumolene	0.65 ± 0.02 (3)	117.08 ± 26.8 (3)

Δf/f = (peak − baseline)/baseline value. Δf/f and τ both significantly different from control for each azumolene concentration; $P < 0.0001$. n = 3–5 separate experiments for each condition.

4-CmC = 4-chloro-*m*-cresol.

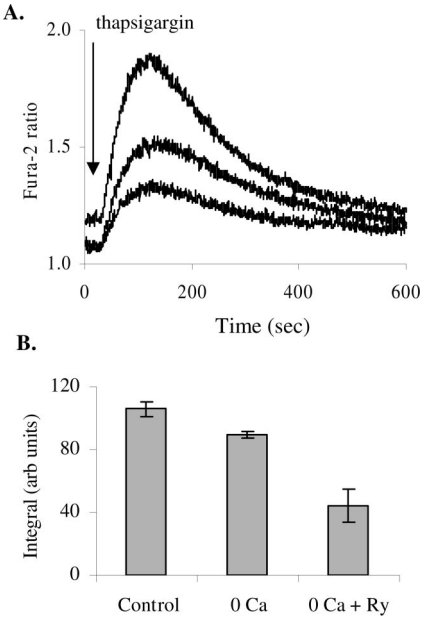

Fig. 6. Effect of ryanodine on intracellular Ca^{2+} stores. (*A*) Dakiki cells in suspension were preexposed to normal Hanks balanced salt solution (HBSS) (*upper trace*), Ca^{2+}-free HBSS (*middle trace*), or Ca^{2+}-free HBSS plus 1 mM ryanodine (*lower trace*) for approximately 1 h. Cells were washed and resuspended in intracellular Ca^{2+}–free HBSS, and 100 nM thapsigargin was applied at 30 s. Each trace represents a single experiment. (*B*) Measurement of thapsigargin-induced intracellular Ca^{2+} release. Integral values determined from individual tracings, then averaged to yield mean ± SEM for control, 0 Ca^{2+}, and 0 Ca^{2+} plus ryanodine. Values were 108 ± 5 (n = 23), 89 ± 2 (n = 3), and 44 ± 10 (n = 3), respectively. * Ryanodine-treated cells were significantly different from those in 0 Ca HBSS alone ($P = 0.0124$).

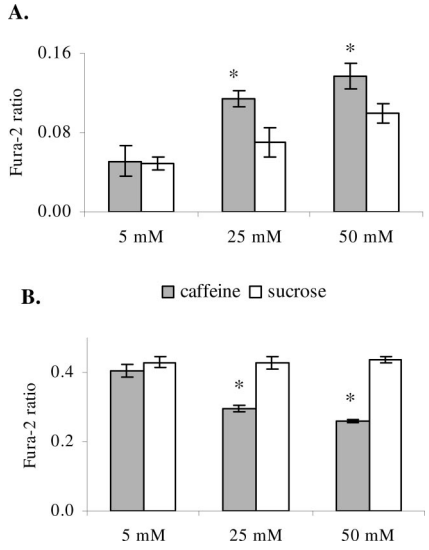

Fig. 7. Effect of caffeine on intracellular Ca^{2+} release. Dakiki cells in suspension in Ca^{2+}-free Hanks with Na^{+} isosmotically replaced by 5, 25, or 50 mM caffeine or sucrose. Cells were diluted directly into caffeine or sucrose containing Hanks after washing. (*A*) *Y-axis* represents the difference between the peak and initial baseline at T = 0 s. Values in caffeine were significantly different from sucrose for 25 ($P = 0.0413$) and 50 mM ($P = 0.0435$). n = 5 each for 5 and 25 mM caffeine and sucrose, n = 7 for 50 mM sucrose, and n = 8 for 50 mM caffeine. (*B*) 4-Chloro-*m*-cresol (4-CmC), 1 mM, applied at approximately 300 s. *Y-axis* represents the difference between the peak response to 4-CmC and the initial baseline at T = 0 s, and thus represents the total increase in response to caffeine or sucrose plus 4-CmC. n = 4 for each condition. Values in caffeine were significantly different from sucrose for 25 mM ($P = 0.0027$) and 50 mM ($P < 0.0001$).

assessed the extent of ryanodine-induced depletion by preexposing the cells to ryanodine under Ca^{2+}-free conditions, then applying thapsigargin (100 nM), and measuring the size of the resulting $Ca^{2+}{}_i$ transient. Figure 6A shows thapsigargin-induced $Ca^{2+}{}_i$ release under three conditions: control, no pretreatment (upper trace); cells exposed to Ca^{2+}-free HBSS for approximately 1 h (middle trace); and cells preexposed to Ca^{2+}-free HBSS plus 1 mM ryanodine for approximately 1 h (lower trace). By comparing the integrals of each $Ca^{2+}{}_i$ transient, it could be demonstrated that ryanodine reliably reduced the size of the thapsigargin-sensitive intracellular store by nearly half (fig. 6B). This effect was weakly dose dependent: 1 or 3 mM ryanodine reduced the $Ca^{2+}{}_i$ transient by half, and 100 or 500 μM reduced it by approximately 30% (n = 1–3 for each concentration; data not shown).

Caffeine. In Dakiki cells, we tested the ability of caffeine to directly induce $Ca^{2+}{}_i$ release as well as its effect on 4-CmC–induced $Ca^{2+}{}_i$ release (fig. 7). Because it had been previously reported that primary B lymphocytes were relatively insensitive to caffeine,[23,35] only high concentrations were tested (5–50 mM) on Dakiki cells. Ca^{2+}-free bathing solutions were prepared with caffeine or sucrose isosmotically substituted for NaCl. Control experiments performed with membrane impermeant *N*-methyl glucamine · Cl showed that 4-CmC–induced $Ca^{2+}{}_i$ release was unaffected by either partial or complete removal of NaCl (data not shown).

Acute exposure to caffeine (5, 25, 50 mM) in Ca^{2+}-free HBSS induced a slight, dose-dependent (0.05–0.14 ratio units) $Ca^{2+}{}_i$ release (fig. 7A). However, the increase was significantly different from that induced by isotonic substitution of sucrose for only 25 and 50 mM ($P < 0.05$). 4-CmC-induced $Ca^{2+}{}_i$ release was also somewhat affected by high concentrations of caffeine (fig. 7B). Preexposure (approximately 5 min) to caffeine caused a reduction in the subsequent 4-CmC–induced Ca^{2+} release that was significant at the 25- and 50-mM concentrations.

3-Deaza-cADPribose. cADPribose has been reported to be an endogenous activator of RyR in several preparations[36] and produces a larger than normal $Ca^{2+}{}_i$ release when injected into muscle fibers from MH-susceptible patients.[37] We tested the effect of the nonhydrolyzable cADPribose analog 3-deaza-cADPribose, which potently releases $Ca^{2+}{}_i$ in sea urchin egg homogenates (EC_{50} = 1 nM)[38] and has been shown to be effective when applied extracellularly in smooth muscle cells[39] and hemopoietic progenitor cells.[40] Acute exposure to 200 nM 3-deaza-cADPribose did not induce $Ca^{2+}{}_i$ release for cells bathed in either Ca^{2+}-free HBSS (n = 3) or Ca^{2+}-contain-

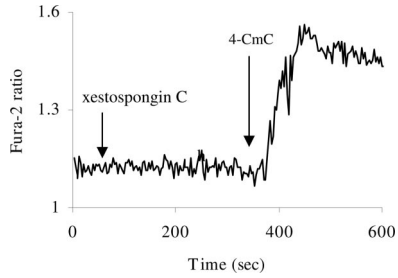

Fig. 8. Effect of xestospongin C on intracellular Ca^{2+} release. Dakiki cells in suspension in Ca^{2+}-free Hanks balanced salt solution. Xestospongin C, 1 μM, applied at 60 s; 1 mM 4-chloro-*m*-cresol (4-CmC) at 370 s. Representative of several experiments that include longer preincubation times (up to 20 min).

ing HBSS (n = 1) (data not shown). Likewise, preexposure for 5–40 min did not affect 4-CmC–induced Ca^{2+} release for 1 or 1.5 mM 4-CmC in either Ca^{2+}-free or normal Ca^{2+}-containing HBSS (n = 2–3 for each condition; data not shown).

Procaine. Last, procaine, a nonspecific RyR inhibitor,[41] was effective in inhibiting 4-CmC–induced $Ca^{2+}{}_i$ release by 60%, as measured in the single cell preparation (1 mM procaine tested with 1.5 mM 4-CmC on primary human lymphocytes; data not shown).

Effect of Xestospongin C.

IP_3R Inhibitors. Xestospongin C, a potent (EC_{50} = 358 nM), selective inhibitor of the IP_3R,[42–44] was tested for its effect on 4-CmC–induced $Ca^{2+}{}_i$ release. Figure 8 shows the time course of $Ca^{2+}{}_i$ release from cells exposed sequentially to xestospongin C and 4-CmC in Ca^{2+}-free HBSS. Xestospongin C alone did not produce any change, and the subsequent response to 1 mM 4-CmC was of similar amplitude to 4-CmC exposure alone (*cf.* fig. 1). Similar results were obtained for longer (5–65 min) preexposures to 1, 5, or 10 μM xestospongin C (n = 9 total).

Effect of FCCP. Mitochondria serve as a significant reservoir of Ca^{2+}, and there is evidence that they express RyRs on their outer membrane.[45] Because it was possible that 4-CmC–induced $Ca^{2+}{}_i$ release was occurring from mitochondria, it was necessary to demonstrate that depletion of mitochondrial Ca^{2+} did not affect the magnitude of 4-CmC–induced $Ca^{2+}{}_i$ release. We used FCCP, an uncoupler of oxidative phosphorylation that collapses the H^+ gradient and destroys the mitochondrial membrane potential, to selectively deplete mitochondrial Ca^{2+}. Control experiments showed that maximal release was accomplished with 2 μM FCCP (data not shown).

Application of 2 μM FCCP led to a small, reproducible release of $Ca^{2+}{}_i$ (fig. 9A) that did not fully recover to baseline. Subsequent addition of 4-CmC (1 mM) led to a typical response, indicating that depletion of mitochondrial Ca^{2+} did not eliminate 4-CmC–induced $Ca^{2+}{}_i$ release. In the converse experiment, FCCP added *after* 4-CmC also produced $Ca^{2+}{}_i$ release, but its size was more

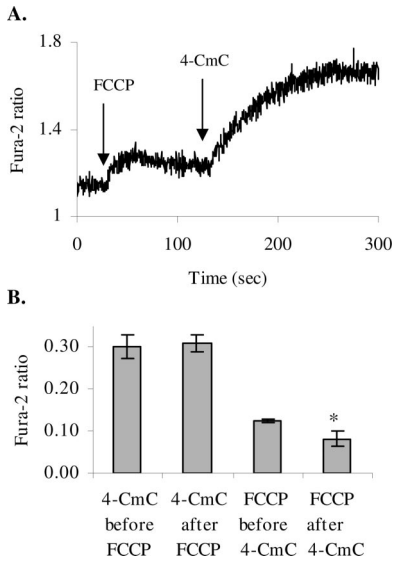

Fig. 9. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) does not affect 4-chloro-*m*-cresol (4-CmC)–induced intracellular Ca^{2+} release. Dakiki cells in suspension in Ca^{2+}-free Hanks balanced salt solution. (*A*) FCCP, 2 μM, applied at 30 s; 1 mM 4-CmC at 120 s. Tracing is from a single experiment. (*B*) *Y-axis* = peak minus baseline just before addition of drug. *Bars 1 and 2*: 4-CmC–induced intracellular Ca^{2+} signal before or after application of FCCP; means not significantly different. n = 13 and 15, respectively. *Bars 3 and 4*: FCCP-induced intracellular Ca^{2+} signal before or after 1 mM 4-CmC; means significantly different (P = 0.0085). n = 25 and 11, respectively.

variable and depended in part on when in the course of the 4-CmC–induced release the FCCP was added. A series of experiments are summarized in figure 9B, showing the magnitude of 4-CmC–induced $Ca^{2+}{}_i$ release before and after FCCP, and the magnitude of the FCCP response before and after 4-CmC.

Lymphocytes from MH Pigs Show Increased Sensitivity to 4-CmC

In the porcine model of MH, RyR1 carries an Arg615Cys mutation that confers hypersensitivity of muscle fibers to halothane and caffeine. We tested the sensitivity of porcine lymphocytes to 4-CmC to determine whether the lymphocyte population would show the increased $Ca^{2+}{}_i$ release expected for cells expressing a mutated RyR1. A number of control experiments established that the 4-CmC–induced $Ca^{2+}{}_i$ release was sensitive to external Ca^{2+} and was eliminated by pretreatment with thapsigargin (data not shown).

Normalized 4-CmC dose–response curves from normal and MH pigs are shown in figure 10. EC_{50} values (mean ± SEM) were 0.81 ± 0.08 and 0.47 ± 0.07 mM, respectively, demonstrating a nearly twofold increase in sensitivity. It was noted that the MH pig cells showed a lower maximal response to 4-CmC compared with cells from normal animals. Unfortunately, the size of the total ionomycin-sensitive stores was not measured in the MH lymphocytes, so we could not assess whether the lower maximal response was due to an intrinsically smaller

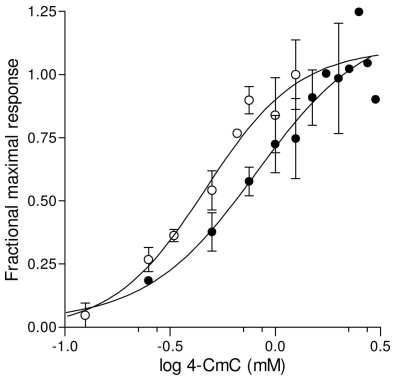

Fig. 10. 4-Chloro-*m*-cresol (4-CmC) dose response. Normal (●) and MH (○) pig lymphocytes in suspension bathed in Ca^{2+}-free Hanks balanced salt solution. Peak fura-2 ratios minus baseline values were normalized to the peak value for that curve and fit as described in Materials and Methods. *Data points* with *error bars* are means ± SEMs; n = 1–9 for each concentration.

store size. However, when EC_{50} values were derived from raw, unnormalized data, they showed the same shift. Therefore, the available data support increased dose sensitivity to 4-CmC in the animal model of MH.

Discussion

This article extends the functional characterization of a 4-CmC–sensitive intracellular Ca^{2+} pool in the Dakiki human B-cell line. The fact that the pool was sensitive to both 4-CmC (thought to be selective for RyR1 and RyR2[46]) and azumolene (likely to be selective for RyR1 and RyR3[47]) indicates that release was mediated by RyR1, which is consistent with reverse transcriptase polymerase chain reaction data indicating that the Dakiki cell line expresses only the RyR1 isoform.[25] The 4-CmC–sensitive pool seemed to be the same as the thapsigargin-sensitive pool, because maximal concentrations of 4-CmC and thapsigargin released comparable amounts of $Ca^{2+}{}_i$. The pool could be pharmacologically distinguished from IP_3-sensitive stores and from mitochondrial stores. Therefore, we have demonstrated that, in a human B-cell line, $Ca^{2+}{}_i$ release can be a sensitive indicator of RyR1 function and that RyR1-mediated $Ca^{2+}{}_i$ release can be selectively measured.

To further develop this model system for use in diagnostic testing for MH, we tested whether $Ca^{2+}{}_i$ release measurements were sensitive enough to distinguish between normal and abnormal RyR1 function in the porcine model of MH. MH pigs carry the Arg614Cys mutation in their RyR1 receptors, which is causative for the disease and falls in the first so-called hot spot region on the RyR gene that is associated with MH mutations. Lymphocytes from MH pigs displayed an increased sensitivity to 4-CmC (EC_{50} decreased from 0.81 mM to 0.47 mM). The twofold magnitude of the shift was similar to that observed for 4-CmC–sensitive ^{3}H-ryanodine binding in MH porcine skeletal muscle.[9] Similar shifts in the dose sensitivity to 4-CmC have been demonstrated in human myocytes derived from patients carrying the Thr2206Met[24] and Ile2453Thr[18] mutations, and in human lymphocytes derived from a patient carrying the Val2168Met[21] mutation, all of which fall in the second hot spot region. Taken together, these findings support further testing of the lymphocyte preparation for use in diagnosing MH.

That said, there were a number of significant differences between the properties of the ryanodine-sensitive stores of the human B-cell lines and those of skeletal muscle. The EC_{50} for 4-CmC (approximately 1 mM), although similar to that reported by another laboratory for a human lymphocyte cell line (750 μM[21]), was much higher than that reported for human myotubes (203 μM[24]). Sensitivity of lymphocytes to azumolene, an analog of the RyR1 inhibitor dantrolene, was approximately 10-fold lower than for skeletal muscle. That azumolene was only a partial inhibitor of $Ca^{2+}{}_i$ release is consistent with the action of dantrolene, which also does not fully inhibit Ca^{2+} flux through isolated RyRs.[48]

Sensitivity of lymphocytes to ryanodine was also low, requiring exposure to 1 mM before store depletion was observed. This could be due to a reduced level of RyR1 expression, which is 1,000- to 10,000-fold less in Dakiki cells[25] than in skeletal muscle,[49] or could be due to differences in ryanodine binding. Unlike muscle, which has one high- (1–10 nM) and one low-affinity (1–10 μM) binding site for ^{3}H-ryanodine,[50] Dakiki cells have only a single, intermediate-affinity (110 nM) binding site.[25]

Dakiki cells exhibited a reduced sensitivity to caffeine in comparison to that of skeletal muscle. Caffeine was ineffective in inducing $Ca^{2+}{}_i$ release in the absence of external Ca^{2+}, which is in agreement with data obtained on primary B lymphocytes.[35] Even in the presence of external Ca^{2+}, caffeine is only a weak agonist in B cells[23] compared with skeletal muscle.

There are probably several reasons for the reduced sensitivity of Ca^{2+} release in lymphocytes to agents that bind to RyR1 as compared with skeletal muscle. One may be the variation in the conformation of RyR1 in the SER membrane, where it is diffusely arrayed, to that in the sarcoplasmic reticular membrane, where RyR1s are tightly packed in an ordered array and are precisely coupled to dihydropyridine receptors.

It is also likely that intracellular Ca^{2+} buffering affects the efficacy of at least two of the agents tested, caffeine and 3-deaza-cADPribose. The actions of each of these agents are enhanced by Ca^{2+}, and apparent sensitivity would be increased if there was either influx of Ca^{2+} across the plasma membrane or a robust amount of Ca^{2+}-induced Ca^{2+} release from the SER stores. This may, in fact, explain the findings of Sei *et al.*,[35] who showed that 50 mM caffeine induced $Ca^{2+}{}_i$ release from normal and MH-sensitive primary B lymphocytes in the presence of external Ca^{2+}. It is possible that very high

concentrations of caffeine (50 mM) activated a nonselective cation channel[51] that allowed Ca^{2+} influx,[52] leading indirectly to Ca^{2+}_i release.

In summary, our data indicate that it is feasible to use Ca^{2+}_i release as an assay for RyR1 function in human and porcine lymphocytes. The next steps in developing a diagnostic blood test for MH are (1) to show that Ca^{2+}_i release can reflect RyR1 function in primary human lymphocytes, which may not exclusively express RyR1,[53] and (2) to show that 4-CmC dose sensitivity correlates with positive CHCT for a large number of causative RyR1 mutations. These experiments will also contribute to our understanding of the role of ryanodine-sensitive Ca^{2+} stores in B-cell function, where it is poorly defined but is undoubtedly important. Two studies have already shown a potential role for RyRs in regulating interleukin-1β secretion[21] and B-cell activation,[54] and it is possible that RyRs will be found to have a role in shaping intracellular Ca^{2+} oscillations that can help to determine B-cell fate.[55]

The authors thank Yohsi Sei, M.D., Ph.D. (Department of Anesthesiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland), for supplying human B-cell lines; Said Bina, Ph.D., and Paul Mongan, M.D. (Department of Anesthesiology, Uniformed Services University of the Health Sciences), for supplying blood samples from normal and MH pigs; Mark Haigney, M.D. (Department of Medicine, Uniformed Services University of the Health Sciences), for supplying blood samples from normal pigs; Gerald Feldman, Ph.D. (Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland), for elutriated human lymphocytes; Edward J. Davis (Uniformed Services University of the Health Sciences) for excellent technical support; Susan Judge, Ph.D. (Department of Neurology, University of Maryland School of Medicine, Baltimore, Maryland), and John Capacchione, M.D. (Department of Anesthesiology, Uniformed Services University of the Health Sciences), for reviewing the manuscript; and Sheila Muldoon, M.D. (Department of Anesthesiology, Uniformed Services University of the Health Sciences), for invaluable discussion.

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