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Enhanced Ca^{2+}-induced calcium release by isolated sarcoplasmic reticulum vesicles from malignant hyperthermia susceptible pig muscle

James R. Mickelson, Julie A. Ross, Brian K. Reed and Charles F. Louis

Department of Veterinary Biology, 295 Animal Science / Veterinary Medicine Building, University of Minnesota, 1988 Fitch Avenue, St. Paul, MN 55108 (U.S.A.)

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To further define the possible involvement of sarcoplasmic reticulum calcium accumulation and release in the skeletal muscle disorder malignant hyperthermia (MH), we have examined various properties of sarcoplasmic reticulum fractions isolated from normal and MH-susceptible pig muscle. A sarcoplasmic reticulum preparation enriched in vesicles derived from the terminal cisternae, was further fractionated on discontinuous sucrose density gradients (Meissner, G. (1984) J. Biol. Chem. 259, 2365–2374). The resultant MH-susceptible and normal sarcoplasmic reticulum fractions, designated $F_0 - F_4$, did not differ in yield, cholesterol and phospholipid content, or nitrendipine binding capacity. Calcium accumulation (0.27 μmol Ca / mg per min at 22°C), Ca^{2+}-ATPase activity (0.98 μmol P_i / mg per min at 22°C), and calsequestrin content were also similar for MH-susceptible and normal sarcoplasmic reticulum fraction F_3. To examine sarcoplasmic reticulum calcium release, fraction F_3 vesicles were passively loaded with ^{45}Ca (approx. 40 nmol Ca / mg), and rapidly diluted into a medium of defined Ca^{2+} concentration. Upon dilution into 1 μM Ca^{2+}, the extent of Ca^{2+}-dependent calcium release measured after 5 s was significantly greater for MH-susceptible than for normal sarcoplasmic reticulum, 65.9 ± 2.8% vs. 47.7 ± 3.9% of the loaded calcium, respectively. The $C_{1/2}$ for Ca^{2+} stimulation of this calcium release (5 s value) from MH-susceptible sarcoplasmic reticulum also appeared to be shifted towards a higher Ca^{2+}-sensitivity when compared to normal sarcoplasmic reticulum. Dantrolene had no effect on calcium release from fraction F_3, however, halothane (0.1–0.5 mM) increased the extent of calcium release (5 s) similarly in both MH-susceptible and normal sarcoplasmic reticulum. Furthermore, Mg^{2+} was less effective at inhibiting, while ATP and caffeine were more effective in stimulating, this Ca^{2+}-dependent release of calcium from MH-susceptible, when compared to normal sarcoplasmic reticulum. Our results demonstrate that while sarcoplasmic reticulum calcium-accumulation appears unaffected in MH, aspect(s) of the sarcoplasmic reticulum Ca^{2+}-induced calcium release mechanism are altered. Although the role of the Ca^{2+}-induced calcium release mechanism of sarcoplasmic reticulum in situ is not yet clear, our results suggest that an abnormality in the regulation of sarcoplasmic reticulum calcium release may play an important role in the MH syndrome.

Abbreviations: MH, malignant hyperthermia; MHS, malignant hyperthermia-susceptible; SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Mes, 4-morpholineethanesulphonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

Correspondence: Dr. J.R. Mickelson, Department of Veterinary Biology, 295 Animal Science/Veterinary Medicine Building, University of Minnesota, 1988 Fitch Avenue, St. Paul, MN 55108, U.S.A.

Introduction

Malignant hyperthermia (MH) is a hypermetabolic myopathy, characterized by increased muscle metabolism, rapidly rising temperature, and muscle rigidity [1]. Physiological and biochemical evidence supports the hypothesis that MH results from a defect in skeletal muscle calcium regulation (for review see Ref. 2). An MH episode is usually initiated in susceptible individuals by exposure to volatile anesthetics, such as halothane [3], and can be prevented or reversed by the skeletal muscle relaxant dantrolene [4]. Porcine MH has been very useful in the study of the closely related human syndrome [5].

The sarcoplasmic reticulum (SR), by responding to surface membrane depolarization via the transverse tubule system, releases and subsequently resequesters calcium, thus playing a major role in the regulation of the contraction-relaxation cycle of muscle. Many investigations into the primary defect of MH have, therefore, focused on calcium accumulation and release by the SR [6–22]. Studies to date comparing the calcium-uptake capability of SR fractions isolated from MH-susceptible (MHS) and normal muscle have yielded contradictory data. Although reports of either diminished [6–9] or enhanced [10,11] MHS SR calcium-accumulating ability have appeared, other studies have demonstrated no difference in this aspect of SR activity between MHS and normal muscle [12–18]. The physiological mechanism by which the SR releases calcium is poorly understood at present (for review see Refs. 23 and 24), however, several studies agree in principle that some aspect(s) of the calcium-release mechanism is altered in MHS SR. Many groups of investigators have reported that MHS muscle biopsies are more sensitive than normal to halothane- or caffeine-induced contractures [25–29]. By using single skinned-fibers, Endo et al. [20] observed that the MHS SR Ca^{2+}-dependent (Ca^{2+}-induced) calcium release mechanism was both more sensitive to Ca^{2+}, and released calcium at a greater rate than normal SR. Halothane increased the Ca^{2+}-sensitivity as well as the rate of calcium release in both MHS and normal fibers [20]. Kim et al. [18], utilizing an isolated SR preparation, found no difference in the Ca^{2+}-sensitivity of MHS and normal SR calcium release, but reported an increased rate of MHS SR calcium release induced by halothane, Ca^{2+}, and ionic replacement, when compared to normal SR. These authors report that calcium release induced by ionic replacement is transverse-tubule mediated [30]. Other evidence for an abnormality associated with MHS SR calcium release is provided by a lower SR intravesicular calcium threshold for calcium release by isolated SR [17,19], and a lower mechanical threshold [31] as well as an enhanced twitch to tetanus ratio [32,33] of intact MHS muscle bundles.

The understanding of the primary defect in MHS muscle would be greatly enhanced by further studies of calcium transport by well-characterized purified SR fractions. Recently, a technique has been developed to isolate terminal-cisternae-derived SR preparations that are highly enriched in calcium-releasing activity [34]. The use of this SR isolation procedure has several advantages in the study of calcium release in that the vesicles can be passively loaded with calcium to significant levels, allowing the independent effects of various modulators of the calcium release process to be examined at a constant initial SR calcium-load. Furthermore, calcium release is rapid and can be chemically quenched. We now report the use of this SR isolation techniqiue [34] to prepare calcium-releasing SR fractions from MHS and normal pig muscle. Our data suggests that although calcium accumulation by MHS and normal SR appears identical, the initial phase of Ca^{2+}-induced calcium release from MHS SR releases more calcium, and has a different sensitivity to Ca^{2+}, Mg^{2+}, ATP and caffeine than does normal SR. Thus, an important role for abnormal SR calcium release in the initiation of MH and in the functioning of MHS muscle is indicated.

Materials and Methods

Materials. Experimental animals were obtained from the University of Minnesota Experimental Farm, where they were part of a swine genetics herd maintained by Dr. William E. Rempel, of the Department of Animal Science, for genetic studies of MH inheritance. The pigs were tested for susceptibility to MH by a halothane-challenge test (5

min exposure to 3% halothane) at least three weeks prior to use. MHS animals were of the Pietrain breed, which reacts positively to the halothane-challenge test by demonstrating limb muscle rigidity [35]. Normal animals were of the Yorkshire breed, which were non-reactors during the halothane-challenge test.

Reagents of the highest purity available were obtained from Sigma Chemical Co., or purchased elsewhere at analytical grade. Water was redistilled from glass. $^{45}CaCl_2$ and [^{3}H]nitrendipine were from New England Nuclear. Calmodulin was prepared from bovine testes by the method of Gopalakrishna and Anderson [36]. Halothane was from Halocarbon Laboratories, Inc., Hackensack, NJ. Dantrolene was a gift of Norwich-Eaton Pharmaceuticals, Norwich, NY, and nitrendipine was a gift of Miles Laboratories Inc., New Haven, CT.

Isolation of SR membranes. Animals were anesthetized by intravenous infusion of sodium thiamylal, placed on a respirator, and ventilated with room air. Further doses of thiamylal were then administered to maintain surgical anesthesia (usually longer than 30 min). During deep anesthesia, the longissimus dorsi muscle was removed, and immediately placed in ice. The procedure was designed to ensure that an MH episode, with damaging effects on membrane properties [21,22], had not been triggered before or during the muscle dissection. This was confirmed by the demonstration of homogenate pH values which remained steady, in the range 6.7–6.8, throughout the membrane isolation procedure.

SR membranes were isolated from the longissimus dorsi by the procedure of Meissner [34]. Briefly, the muscle was minced and homogenized in 5 vols. (w/v) of 0.1 M NaCl, 5 mM Tris-maleate (pH 6.8) for 60 s in a Waring blender. After centrifugation of the homogenate for 30 min at $2600 \times g$, the pellet was discarded and the resultant supernatant was centrifuged for 30 min at $10000 \times g$. This pellet was resuspended, treated with 0.6 M KCl [34], and centrifuged at $130000 \times g$. The pellets were resuspended in 10% sucrose (w/v), 0.4 M KCl, 20 μM $CaCl_2$, 5 mM Tris-Mes (pH 6.8) and placed on discontinuous sucrose gradients containing 0.4 KCl, 20 μM $CaCl_2$, 5 mM Tris-Mes (pH 6.8) in all layers. The tubes were centrifuged for 5 h at 25000 rpm in a Beckman SW27 rotor, and the material banding at the interfaces of the different sucrose layers was removed and processed as described [34]. Fractions were labelled according to their position in the gradient as described in Results, frozen in liquid nitrogen, and stored at $-70°C$.

Chemical determinations on membranes. The protein, cholesterol and phospholipid contents of the SR membrane fractions were determined as described previously [37].

Ca^{2+}-ATPase and calcium-uptake determination. SR ATPase activities were measured in 0.1 M KCl, 10 mM Pipes (pH 7.0), 10 μM ionophore A23187, 25 μg protein/ml at 22°C. The reaction was initiated by addition of 5 mM MgATP. Ca^{2+}-ATPase activity was defined as the difference between the ATPase activity measured in the presence of 0.1 mM $CaCl_2$, and that measured in the presence of 1 mM EGTA.

SR calcium uptake was measured in 0.1 M KCl, 10 mM Pipes (pH 7.0), 5 mM oxalate, 0.1 mM $^{45}CaCl_2$, 100 μg protein/ml at 22°C. The reaction was initiated by the addition of 5 mM MgATP, whereafter samples were taken at 30-s intervals and the SR calcium content determined by a Millipore filtration technique. The rate of calcium uptake was determined by linear regression analysis.

Nitrendipine binding. Nitrendipine binding was determined at room temperature in 50 mM Tris (pH 7.4), 5 nM [^{3}H]nitrendipine, and less than 0.5% PEG 500 as solvent. Membranes were collected on Whatman GFB filters and immediately washed with 5 ml ice-cold 200 mM choline chloride, 20 mM Tris (pH 7.4). Nonspecific binding was determined in the presence of 1 μM nitrendipine.

Passive calcium-loading of SR membranes and the determination of calcium release. The procedure was essentially that described by Meissner [34]. SR vesicles to be passively loaded with ^{45}Ca were first incubated on ice for 30 min in 400 μl of 0.1 M KCl, 10 mM Pipes (pH 7.0), 20 μM $CaCl_2$, at a protein concentration of approx. 15 mg/ml. The vesicles were collected by centrifugation in a Beckman Airfuge ($100000 \times g$, 15 min), resuspended in a minimal volume of water, and the protein concentration of a small aliquot was determined. The

remainder was added to an Eppendorf tube containing 0.1 M KCl, 10 mM Pipes (pH 7.0), 5 mM $CaCl_2$, 0.5 mCi/ml ^{45}Ca, (final concentrations). The SR protein concentration of the loading medium was approx. 10 mg protein/ml. Passive calcium-loading was allowed to proceed for more than 2 h at room temperature, after which time the tube was placed on ice until use.

The dilution medium for calcium release was contained in either polystyrene test tubes, or for halothane experiments, 1 ml glass Reacti-vials (Pierce) with Teflon-lined septum and screw on caps. In the standard protocol, the calcium release medium (22°C) consisted of 0.1 M KCl, 10 mM Pipes (pH 7.0), and either 10 mM EGTA plus 10 mM $MgCl_2$, or a defined Ca^{2+} concentration that was established with a Ca-EGTA buffer [38]. The total $CaCl_2$ concentration of the release medium was held constant at 1 mM, which included that $CaCl_2$ carried over with the SR from the calcium-loading medium (5% of total $CaCl_2$). 200 μl of the dilution medium was placed in the appropriate vessel, a 2 μl aliquot of ^{45}Ca-loaded SR was smeared on the side of the tube, and calcium release initiated by rapid vortexing, to mix the SR with the dilution medium. At indicated times after mixing (usually 5 s), SR calcium release was stopped by the addition of 20 μl of a 0.1 M KCl, 110 mM EGTA, 110 mM $MgCl_2$, 10 mM Pipes (pH 7.0) solution. The SR calcium content was then determined following filtration on 0.45 μm Millipore filters, and rapid washing with 5 ml ice-cold 0.1 M KCl, 10 mM Pipes (pH 7.0), 5 mM $MgCl_2$, 1 mM EGTA. The per cent calcium release from the SR was expressed as the SR calcium content under the experimental condition, relative to the calcium content upon dilution into 10 mM EGTA plus 10 mM $MgCl_2$, a condition which essentially completely inhibited SR calcium release [34]. In experiments in which halothane was present during calcium release, after application of the ^{45}Ca-loaded SR, the glass vial was sealed. Halothane from a saturated aqueous solution (20 mM) was then added to the dilution media, through the septum, by way of a Hamilton syringe.

Electrophoretic analysis. SR membrane samples (50 μg protein in 100 μl 1% SDS) were heated to 90°C for 10 min, when 25 mM dithiothreitol (final) was added. These samples were electrophoresed on 5–20% gradient polyacrylamide gels, in the presence of 0.1% (w/v) SDS [39]. Gels were stained with Coomassie Blue and then analyzed in a gel-scanning densitometer at 540 nm (E-C Apparatus Corp.). The molecular weights of SR proteins were determined with the use of molecular mass markers purchased from Sigma. The position of calsequestrin was confirmed by using Stains-All [40].

Statistical analysis. All comparisons of mean values of MHS and normal populations were analyzed by a Student's *t*-test.

Results

Characterization of SR fractions $F_0 - F_4$ from porcine skeletal muscle

The membranes obtained by differential centrifugation between $2600 \times g$ and $10\,000 \times g$ were subsequently fractionated on a discontinuous sucrose gradient. Material collected at each of the five interfaces, labelled $F_0 - F_4$ (Table I); only infrequently was a pellet observed. The greatest amount of material collected in the F_3 band, with lesser amounts of material in the F_0, F_1, F_2 and F_4 layers (Table I). In proceeding from fractions F_0 (lightest) to F_4 (heaviest), the relative distribution of the surface membrane markers total cholesterol content and nitrendipine binding decreased, as did the phospholipid content (Table I). The data also demonstrate that there was no difference in yield, cholesterol and phospholipid content, or nitrendipine binding between fractions derived from MHS and normal muscle.

SDS-polyacrylamide gel electrophoretograms of SR fractions F_0–F_4 (not shown), demonstrated a protein distribution typical of SR membranes. The major component of all fractions was the Ca^{2+}-ATPase (M_r of approx. 105 000). The other major component of these fractions was the calcium binding protein, calsequestrin (M_r 64 000), which increased in relative distribution proceeding from F_0 to F_4.

Further characterization of SR fraction F_3

We chose to further characterize fraction F_3, because of its excellent calcium-releasing ability, to be discussed in detail below. Determination of

TABLE I

CHARACTERIZATION OF SUCROSE GRADIENT FRACTIONS F_0–F_4 [a]

Fraction [b]		Yield (mg protein/100 g muscle)		Cholesterol (μg/mg protein)		Phospholipid (μg P/mg protein)		Nitrendipine binding (pmol/mg protein)	
		MHS (5)	Normal (5)	MHS (3)	Normal (3)	MHS (3)	Normal (3)	MHS (3)	Normal (3)
F_0	10.0%–21.6%	1.6± 0.6	2.2±0.8	167±15	192±49	25.3±4.5	29.5±4.8	8.5±1.9	6.6±1.0
F_1	21.6%–32.6%	5.1± 0.7	8.4±2.9	91±13	107± 8	20.5±0.9	20.4±0.4	6.5±1.1	5.5±0.4
F_2	32.6%–36.4%	11.8± 3.6	9.8±2.8	33± 4	24± 1	15.1±1.7	15.3±2.2	2.8±1.3	2.6±0.7
F_3	36.4%–40.4%	26.5±10.9	26.3±5.7	18± 8	22±12	15.0±1.2	17.7±2.9	3.5±1.6	4.0±0.8
F_4	40.4%–44.3%	7.5± 1.0	7.6±1.0	12± 5	17± 9	10.4±0.9	10.7±2.7	2.1±0.4	2.6±0.2

[a] Membrane fractions were prepared and characterized as described in Material and Methods. All values are reported as means ± S.E. The number of preparations examined is given in parenthesis.

[b] Fractions were labelled according to their migration in a discontinuous sucrose gradient. The interface of the indicated sucrose concentrations (w/v) at which the various fractions collected is indicated.

the Ca^{2+}-ATPase activity of SR fraction F_3, 0.968 ± 0.084 and 0.994 ± 0.223 μmol P_i/mg per min (22°C) for five MHS and normal preparations, respectively, confirmed a high content of SR vesicles in this fraction. Calcium uptake activities, 0.252 ± 0.029 and 0.291 ± 0.048 μmol Ca/mg per min (22°C), for five MHS and normal F_3 fractions, respectively, were stimulated approximately 2-fold by the inclusion of 0.5 μM ruthenium red. There was no difference in any aspect of the calcium-accumulating function of SR fraction F_3 vesicles between MHS and normal muscle.

Fig. 1 demonstrates that the protein distribution of SR fraction F_3 preparations derived

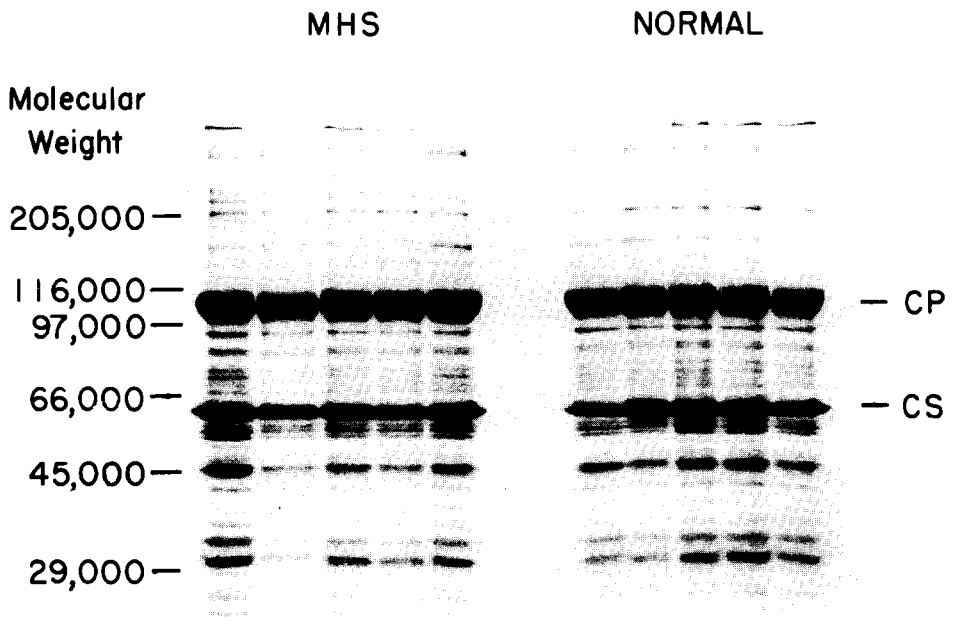

Fig. 1. Electrophoretic analysis of SR fraction F_3. SR fractions were prepared and electrophoresis was performed as described in Materials and Methods. The positions of molecular weight markers are given on the left, and the positions of the Ca^{2+}-ATPase (CP) and calsequestrin (CS) are indicated on the right.

from five different MHS or normal pigs was similar. Densitometric analysis of the Coomassie-blue stained gel showed that the relative content of calsequestrin did not differ significantly between MHS and normal fractions (4.2 ± 0.2 vs. 4.6 ± 0.3 units, respectively, means $\pm$ S.E., $P > 0.10$).

Calcium release by SR fraction F_3

The ability of the porcine SR fractions to release calcium was determined following passive loading of the membrane vesicles with ^{45}Ca [34]. Under our loading conditions, SR fraction F_3 vesicles contained approx. 40 nmol Ca/mg protein, with no significant difference noted between MHS and normal preparations. Upon dilution of the calcium-loaded F_3 vesicles into various media, calcium was released from the SR (Fig. 2). The rate of calcium release from the vesicles was quite slow throughout the 60 s time-course when the dilution medium contained 10 mM EGTA plus 10 mM $MgCl_2$. The ability of EGTA plus $MgCl_2$ to inhibit the SR calcium release was utilized to inhibit further calcium release during the sampling procedure. When the SR dilution medium contained Ca^{2+} concentrations of 0.01 or 1.0 μM, a significant portion of the loaded calcium was released rapidly (within 5 s), after which calcium release continued at a much reduced rate (Fig. 2). We have chosen to express the extent of SR calcium release under varying experimental conditions as a percentage of the SR calcium content of an identical calcium-loaded SR aliquot 5 s after dilution into 10 mM EGTA plus 10 mM $MgCl_2$; the latter condition is reported as zero calcium release. It is evident from Fig. 2 that calcium release by MHS and normal SR fraction F_3 vesicles in the EGTA plus $MgCl_2$ dilution media did not differ greatly. However, when calcium release was initiated by dilution of the calcium-loaded SR into either 0.01 or 1.0 μM Ca^{2+}, MHS SR released

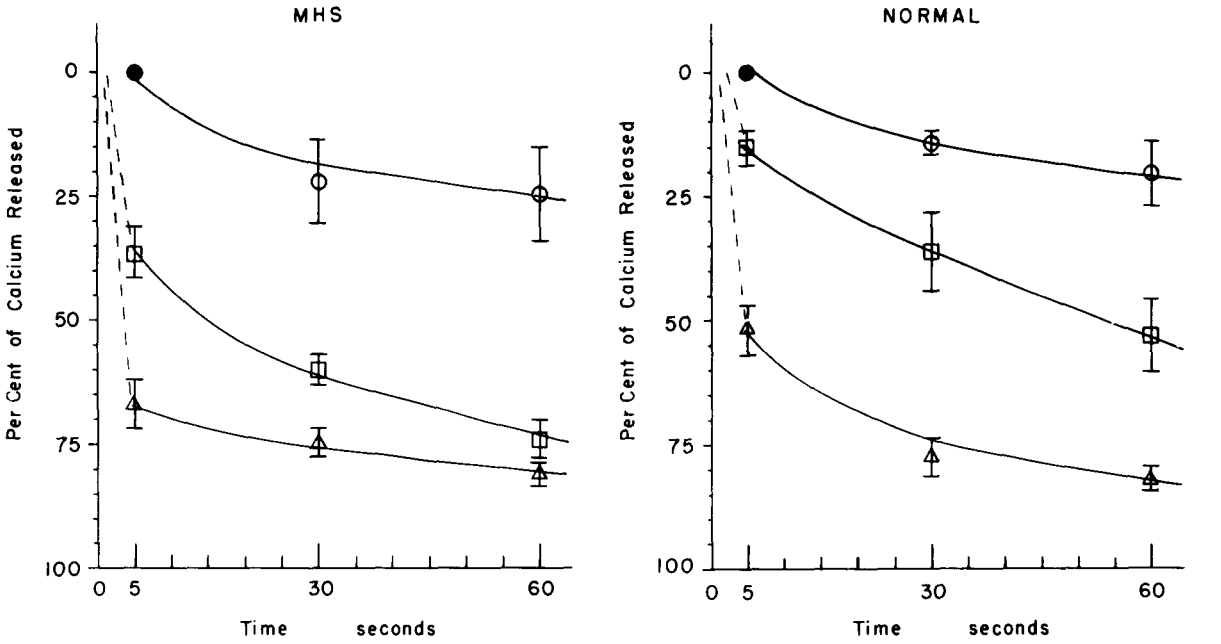

Fig. 2. Time-course of calcium release by SR fraction F_3. SR Fraction F_3 (approx. 10 mg/ml) was passively loaded with ^{45}Ca in the presence of 0.1 M KCl, 10 mM Pipes (pH 7.0), 5 mM $^{45}CaCl_2$, as described in Materials and Methods. Calcium-loaded SR was rapidly diluted 100-fold into a medium containing 0.1 M KCl, 10 mM Pipes (pH 7.0), and 10 mM EGTA plus 10 mm $MgCl_2$ (○), 10^{-8} M Ca^{2+} (□), or 10^{-6} M Ca^{2+} (△). Ca^{2+} concentrations were established by Ca^{2+}-EGTA buffers. At the indicated time points, EGTA plus $MgCl_2$ was added to stop calcium release (final concentrations of 10 mM) and SR calcium content was determined by Millipore filtration. Per cent calcium release is expressed as the SR calcium content relative to the calcium content 5 s after dilution into 10 mM EGTA plus 10 mM $MgCl_2$ (filled circle). Data for MHS SR fraction F_3 is given on the left and normal SR fraction F_3 on the right. All points are means $\pm$ S.E. of duplicate determinations on three different preparations.

significantly more calcium after 5 s than did normal SR at the same dilution-medium Ca^{2+} concentration. 60 s after dilution into 1 μM Ca^{2+}, both MHS and normal SR had released approx. 80% of the loaded calcium. The marked difference in the early rapid phase (5 s) of calcium release between MHS and normal fraction F_3 SR is explored further below.

Calcium release by SR fractions F_2–F_4 was examined after dilution of the calcium-loaded SR into 1 μM Ca^{2+} (Table II). The data demonstrate that MHS SR fractions F_2–F_4 released significantly more calcium after 5 s than did the respective normal SR fraction ($P < 0.05$). Whether the SR preparations were derived from MHS or normal muscle, SR fraction F_2 released less calcium than did F_3 or F_4; F_3 and F_4 had approximately equal calcium-releasing ability. Since fraction F_3 was obtained in greatest yield (Table I), and demonstrated nearly maximal calcium-releasing ability (Table II), this SR fraction was chosen for all further studies.

The dependence of the extent of SR fraction F_3 calcium release after 5 s on the Ca^{2+} concentration of the dilution medium is shown in Fig. 3. Calcium release was optimal at 1 μM Ca^{2+} and declined slightly at higher Ca^{2+}-concentrations. Maximal calcium release after 5 s was 65.9 ± 2.8 and 47.7 ± 3.9 per cent of the calcium load for MHS and normal SR, respectively (mean $\pm$ S.E. for five MHS and five normal preparations). This difference in extent of calcium release was highly significant ($P < 0.005$). MHS SR released significantly more calcium after 5 s than did normal SR throughout the entire range of Ca^{2+} concentrations (Fig. 3). Furthermore, MHS SR calcium release was not completely inhibited at the lowest Ca^{2+}-concentration examined (0.01 μM).

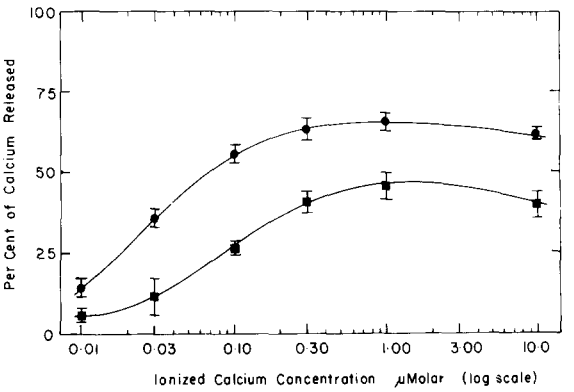

Fig. 3. Ca^{2+} dependence of SR fraction F_3 calcium release. SR fraction F_3 was loaded with calcium, and calcium release after 5 s at various Ca^{2+} concentrations was determined as described in Materials and Methods, and in the Legend to Fig. 2. All points are the means $\pm$ S.E. of replicates of two different experiments on each of five different MHS (●) and five normal (■) preparations. Per cent calcium release by MHS SR was significantly greater than by normal SR throughout the entire range of Ca^{2+} concentrations ($P < 0.005$).

Because our techniques could not measure the initial rate of calcium release, calculation of the K_m for Ca^{2+} activation of MHS and normal SR calcium release was not possible. However, from the 5-s time point data, it appears that the $C_{1/2}$ for the Ca^{2+} sensitivity of MHS SR calcium release was lower than that of normal SR, approx. 0.03 μM and 0.08 μM Ca^{2+} for MHS and normal SR, respectively (Fig. 3). If initial rates of calcium release were obtained, it is possible that the Ca^{2+} dependence of SR calcium release would appear different from that observed in Fig. 3.

TABLE II

CALCIUM RELEASE BY SR FRACTIONS F_2–F_4 [a]

Fraction F_2		Fraction F_3		Fraction F_4	
MHS	Normal	MHS	Normal	MHS	Normal
$46.2 \pm 2.7\%$	$34.3 \pm 4.2\%$ [b]	$66.8 \pm 4.9\%$	$51.6 \pm 5.2\%$ [b]	$72.0 \pm 2.1\%$	$55.3 \pm 4.2\%$ [b]

[a] Calcium release was determined 5 s after dilution into 1 μM Ca^{2+}, as described in Materials and Methods. All values are reported as the per cent of the loaded calcium which was released at 1 μM Ca^{2+}, relative to a control which was released into 10 mM EGTA plus 10 mM $MgCl_2$. Means $\pm$ S.E. of three MHS and three normal preparations.

[b] Significantly different at $P < 0.05$.

Effects of various modulators of SR fraction F_3 calcium release

No effect of 20 μM dantrolene on either MHS or normal SR fraction F_3 calcium release was noted at any Ca^{2+} concentration present in the dilution medium (determined on three different MHS or normal preparations, not shown). Halothane, however, significantly stimulated calcium release from both MHS and normal SR fraction F_3 (Fig. 4). The stimulation of MHS and normal SR calcium release (5 s) by both 0.1 mM and 0.5 mM halothane was significantly different from controls ($P < 0.05$) at all Ca^{2+} concentrations less than 0.10 μM. In addition, enhancement of SR calcium release by 0.5 mM halothane was significantly greater than that due to 0.1 mM halothane ($P < 0.05$) for Ca^{2+} concentrations of 0.01–0.10 μM but not for 0.003 μM. The data indicate that as the halothane concentration was increased, the $C_{1/2}$ for Ca^{2+} stimulation of calcium release was shifted toward lower Ca^{2+} concentrations (Fig. 4). However, from these data it was not possible to accurately determine the effect of halothane on the $C_{1/2}$ for Ca^{2+} of either MHS or normal SR calcium release. That more calcium release occurred at 0.003 μM than 0.010 μM Ca^{2+} could be due to an artifact of the single time point assay, or of inefficient Ca^{2+}-buffering by EGTA at this low Ca^{2+}-concentration.

The effects of other modulators of SR fraction F_3 calcium release are compared in Table III for either a maximally or minimally effective Ca^{2+} concentration present in the release medium. When determined at 1 μM Ca^{2+}, calcium release by normal SR was inhibited by 0.6 mM $MgCl_2$ to a greater extent than was MHS SR, 0.8 ± 0.5 vs. 18.3 ± 4.5 per cent calcium release, respectively. Also, in the presence of 1.0 mM ATP, 0.6 mM free Mg^{2+} (1.6 mM total $MgCl_2$) was unable to inhibit MHS SR calcium release, while it significantly inhibited normal SR calcium release (Table III). A slight inhibitory effect of calmodulin on both MHS and normal SR calcium release, in the presence of $MgCl_2$ and ATP, was observed.

Both MHS and normal SR calcium release in the presence of 0.01 μM Ca^{2+} were stimulated by 0.1 mM ATP (Table III), however, MHS SR calcium release was maximally stimulated while normal SR calcium release was stimulated approximately half-maximally after 5 s (74.0 ± 3.4 versus 46.4 ± 7.7 per cent of the loaded calcium for MHS and normal SR, respectively). In the presence of 1 μM Ca^{2+}, 1 mM ATP maximally stimulated SR calcium release such that MHS and normal SR calcium releases were now equal (approx. 75 per cent calcium release). At 0.01 μM Ca^{2+}, caffeine was also an effective stimulator of both MHS and normal SR calcium release (Table

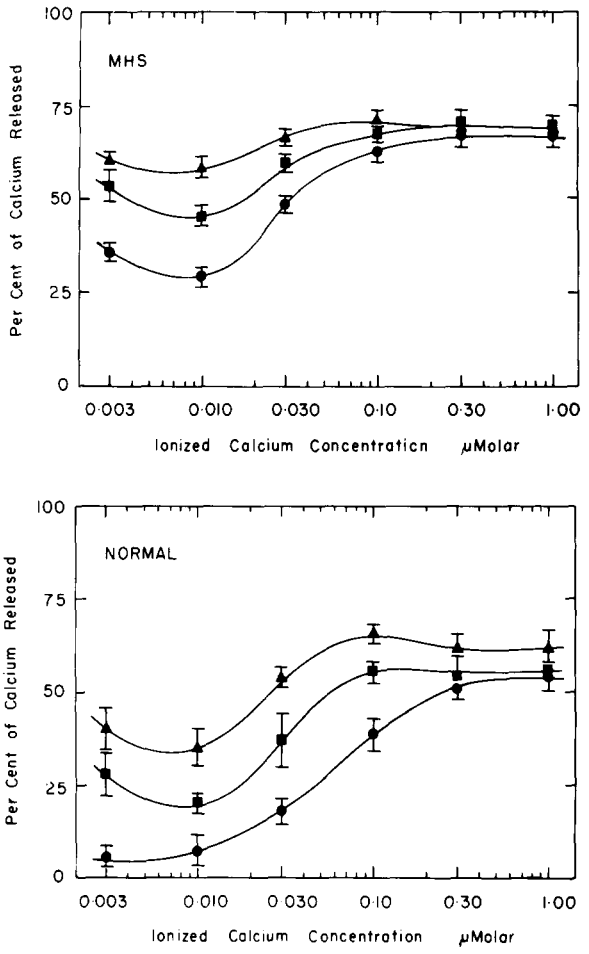

Fig. 4. Effect of halothane on SR fraction F_3 calcium release. SR fraction F_3 was loaded with calcium, and calcium release after 5 s at various Ca^{2+} concentrations was determined as described in Materials and Methods, and in the legend to Fig. 2. Either no halothane (●), 0.1 mM (■), or 0.5 mM halothane (▲) was present in the dilution medium. All points are the means ± S.E. of duplicate determinations on each of five different MHS (top) and five different normal (bottom) preparations.

TABLE III

MODULATORS OF CALCIUM RELEASE BY SR FRACTION F_3 [a]

Ca^{2+}	Modulator added	MHS	Normal
1 μM	None	68.2 ± 4.1%	52.2% ± 2.9% [b]
1 μM	0.6 mM $MgCl_2$	18.3 ± 4.5%	0.8% ± 0.5% [b]
1 μM	1.0 mM ATP	76.2 ± 2.1%	74.8% ± 1.6%
1 μM	1.6 mM $MgCl_2$ 1.0 mM ATP	73.7 ± 2.5%	35.8% ± 5.6% [b]
1 μM	1.6 mM $MgCl_2$ 1.0 mM ATP 1.0 μM calmodulin	67.9 ± 4.1%	27.2% ± 9.8% [b]
0.01 μM	None	21.4 ± 2.9%	4.4% ± 2.7% [b]
0.01 μM	0.1 mM ATP	74.0 ± 3.4%	46.4% ± 7.7% [b]
0.01 μM	5.0 mM caffeine	72.7 ± 2.6%	37.8% ± 3.0% [b]

[a] SR calcium release was determined as described in Materials and Methods, 5 s after dilution into the indicated Ca^{2+} concentration, and the presence or absence of various modulators. Means ± S.E. of five MHS and five normal preparations.

[b] Different from MHS at $P < 0.05$.

III). 5 mM caffeine induced a maximal release of calcium from the MHS SR, while causing an approximately half-maximal normal SR calcium release (72.7 ± 2.6 versus 37.8 ± 3.0 per cent for MHS and normal SR, respectively).

Discussion

The porcine SR fraction F_3 and F_4 vesicles sedimented at 10000 × *g*, migrated to a density in sucrose gradients typical of heavy SR [41] and triads [42], and contained a higher calsequestrin content than fractions F_0–F_2. This suggests that the majority of the porcine muscle calcium-releasing SR vesicles were derived from the SR terminal cisternae [34]. The maximal Ca^{2+}-ATPase and calcium-uptake activities, as well as the calsequestrin content of MHS and normal SR fraction F_3 did not differ, demonstrating that the calcium-accumulating function of the MHS and normal SR vesicles was identical. This result is in agreement with several other reports examining SR calcium-sequestering activity in MH [12–18].

SR calcium release occurring as a result of a change in the extravesicular Ca^{2+}-concentration has been termed Ca^{2+}-induced calcium release [23,24]. The major results of our study were that when purified heavy SR fractions of MHS and normal muscle were passively loaded with calcium to identical levels, the MHS SR demonstrated significantly greater Ca^{2+}-induced calcium release during the initial calcium release phase (Tables II and III, Figs. 2–4). At the optimal Ca^{2+} concentration for calcium release (1 μM), MHS and normal SR fraction F_3 released 65.9 and 47.7 per cent of the loaded calcium, respectively, after 5 s (Fig. 3). This mean difference was highly significant ($P < 0.005$). MHS SR also released significantly more calcium than normal SR (5 s) throughout the entire range of Ca^{2+} concentrations examined (Fig. 3). The extent of calcium release 60 s after dilution into 1 μM Ca^{2+} (Fig. 2), or 5 s after dilution into 1 μM Ca^{2+} plus 1 mM ATP (Table III), was approx. 80 per cent for both MHS and normal SR, suggesting that the maximal calcium-releasing ability of the MHS and normal SR preparations did not differ. Several other agents have been shown previously to influence the calcium release of isolated SR vesicles [17–19,23, 24,30,34,43–50]. In our studies, Mg^{2+} had a reduced ability to inhibit, while ATP and caffeine had an enhanced ability to stimulate the extent of MHS SR Ca^{2+}-induced calcium release (5 s) when compared to normal SR (Table III).

The skeletal muscle relaxant dantrolene (20 μM), which prevents or reverses MH [2,4], had no effect on the Ca^{2+}-induced calcium release of either the MHS or normal SR preparations. The mechanism and site of action of dantrolene are still not understood, with several reports yielding contradictory data [17,43,51–54]. Multiple sites of dantrolene effects may be indicated [54], and it is possible that such sites are extracted or inactivated during our SR purification procedure, or that dantrolene acts at a non-calcium-induced release mechanism [54]. Halothane, over a clinically-relevant concentration range (0.1–0.5 mM), significantly stimulated both MHS and normal SR Ca^{2+}-induced calcium release (Fig. 4). In so doing, halothane also appeared to shift the Ca^{2+} dependence of calcium release toward lower Ca^{2+} concentrations, i.e., increased the sensitivity of the SR Ca^{2+}-induced calcium release mechanism. That halothane enhances the SR Ca^{2+}-induced calcium release is in agreement with other studies [17,18,20,47,48]. It appears, therefore, that

the primary effect of halothane on the SR is a stimulation of calcium release from the terminal cisternae [47], as only at very high concentrations does halothane inhibit calcium uptake [7,15,47].

Endo et al. [20] have reported an increased rate and Ca^{2+}-sensitivity of MHS SR Ca^{2+}-induced calcium release, utilizing skinned fibers from a single MHS human patient. A more sensitive or enhanced Ca^{2+}-induced calcium release mechanism for MHS SR had been inferred previously, based on the increased caffeine-sensitivity of force production in MHS muscle biopsies [25–28]. Kim et al. [18] reported that the rate constants of calcium release induced by Ca^{2+}, halothane, Ca^{2+} plus halothane, and ionic replacement were always greater in MHS than normal porcine muscle heavy SR, while the Ca^{2+}-dependence of the rate constant and amount of SR calcium release did not differ from normal. Also, a lower intravesicular calcium threshold of isolated MHS SR for halothane- or Ca^{2+}-induced calcium release has been noted [17,19]. In our study, an enhanced rapid phase of MHS SR calcium release was observed under precisely defined conditions of Mg^{2+}, ATP, intra- and extravesicular calcium concentration. Although we were unable to kinetically determine a K_m for Ca^{2+}, the $C_{1/2}$ for Ca^{2+}-stimulation of MHS SR calcium release was lower than that of normal SR (Fig. 3), thus supporting the results of Endo et al. [20]. That caffeine increased isolated MHS SR calcium release more than normal SR calcium release (Table III), is also in agreement with the enhanced sensitivity of MHS muscle biopsies to caffeine-induced contracture [25–28]. Because the SR calcium release is very rapid [34], our measurement (5 s) also could not evaluate the possibility that the rate constant of MHS SR calcium release is greater than normal SR [18], although it appears likely. Since the rate of calcium release depends on the SR calcium content [30,34], the lower than normal intravesicular calcium threshold for MHS SR calcium release [17,19] could also be related to an altered regulation of the MHS SR Ca^{2+}-induced calcium release mechanism.

Recent studies have shown that many types of calcium release demonstrated with isolated SR are likely mediated by a calcium channel [44,45]. In intact muscle cells, the transverse tubule membrane potential regulates SR calcium release by an unknown mechanism, but it appears to be pharmacologically distinct from the Ca^{2+}-induced process [55]. It is possible, though, that the enhanced isolated MHS SR Ca^{2+}-induced calcium release indicates that in vivo MHS SR releases more calcium than normal SR in response to a given transverse tubule stimulation. An abnormality in MHS surface membrane control of SR calcium release has been demonstrated previously by a reduced mechanical threshold [31], an increased twitch : tetanus ratio of intact MHS muscle bundles [32,33], and an elevated resting Ca^{2+} concentration in the MHS sarcoplasm [56]. Halothane, by interacting with some component of the isolated SR appears to further sensitize both MHS and normal SR calcium release to stimulation. However, because the MHS SR calcium release is more sensitive to stimulation than is normal SR, the presence of halothane in vivo may cause an excessive stimulation, resulting in an uncontrolled calcium release and initiation of the MH response. The molecular basis of the proposed defect in MHS SR calcium release could conceivably involve an alteration in the structure of some component of the SR calcium channel. Whether this MHS SR defect alone is responsible for the characteristic responses of MHS muscle is unknown at this time. A complete understanding of the MH syndrome requires further definition of the roles of the several observed abnormalities, not only in MHS SR, but also in MHS mitochondria [57,58] and surface membranes [59–61].

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