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Abnormal uptake and release of Ca^{2+} ions from human malignant hyperthermia-susceptible sarcoplasmic reticulum

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Abstract

Malignant hyperthermia (MH) is a pharmacogenetic myopathy that occurs in humans and several other mammalian species. There has been limited investigation of Ca^{2+} transport by human heavy sarcoplasmic reticulum (HSR) vesicles despite the fact that mutations of the ryanodine receptor Ca^{2+} release channel have been linked to inheritance of MH. In this study, the Ca^{2+} release and uptake mechanisms in human MH-susceptible HSR (MHS) vesicles were investigated and the kinetics and sensitivity compared to normal vesicles. Alterations in Ca^{2+} regulation were thereby elucidated. HSR vesicles from 6 normal (MHN) and 5 MHS patients were compared using a dual-wavelength continuous Ca^{2+} flux assay in the presence of pyrophosphate. The loading capacity and loading rate of Ca^{2+} in MHS vesicles were reduced by almost 50%. These parameters were restored to normal when the Ca^{2+} channel blocker ruthenium red was added. Calcium-induced calcium release, halothane-induced calcium release, and trifluoperazine-induced calcium release were clearly elevated in MHS HSR vesicles compared to MHN vesicles. The results suggest that MH ryanodine receptors exist in a more open resting state than those in normal muscle.

Keywords: Malignant hyperthermia; Sarcoplasmic reticulum; Ca^{2+}-induced Ca^{2+} release; Ca^{2+} loading ability; Calsequestrin

1. Introduction

Malignant hyperthermia is an autosomal dominantly inherited myopathy that is triggered by inhalation anaesthetics and depolarising muscle relaxants such as halothane and succinylcholine. The manifestations include a pronounced skeletal muscle rigidity, a rapid rise in temperature, and severe hypermetabolism. The myopathy may be fatal unless treated immediately with the muscle relaxant dantrolene [1,2]. The frequency of occurrence is approximately 1:12,000 in children and 1:40,000 in adults [3], although the population frequency of MHS may be as high as 1:1000 [4]. Diagnosis of MH susceptibility is based on the hypersensitivity of biopsied muscle to halogenated anaesthetics and the Ca^{2+} release agonist caffeine [5,6]. Inheritance of MH is linked to over 18 different human point mutations in the skeletal muscle isoform of the ryanodine receptor gene [7]. MHS humans and stress syndrome-susceptible swine show similar response characteristics to anaesthetics [8]. A mutation in the *RYR*1 of the pig (specifically Arg615Cys) was identified in all susceptible porcine strains [9,10]. This mutation was shown to be responsible for the increased sensitivity to agonists such as halothane and caffeine [11].

The majority of studies on Ca^{2+} transport by the ryanodine receptor (Ca^{2+} release channel) protein in MH have used porcine muscle HSR, but few data are available on human HSR. Although porcine HSR is a useful model for studying the molecular aetiology of MH, the defect that leads to MH in humans may not be the same. MH in swine is a recessively inherited disorder, whereas the human form is dominantly inherited. There is only one mutation associated with the disease in pigs, whereas a large number of mutations may give rise to the myopathy in humans [12]. Therefore, any attempt to directly correlate results obtained from swine with those from humans may be fundamentally flawed.

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Abbreviations: CICR, Ca^{2+}-induced Ca^{2+} release; HEK-293, human embryonic kidney; HSR, heavy sarcoplasmic reticulum; IVCT, *in vitro* caffeine halothane contracture test; MH, malignant hyperthermia; MHS, malignant hyperthermia-susceptible; MHN, malignant hyperthermia normal; MOPS, 3-[*N*-Morpholino]propanesulphonic acid; *RYR*1, ryanodine receptor skeletal muscle gene; and TFP, trifluoperazine.

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Within the small body of published data on human HSR, there is considerable inconsistency regarding the functional properties of MHS vesicles. Some published papers investigating human HSR Ca^{2+} transport indicate no differences between MHS and MHN muscle, despite the evidence implicating ryanodine receptor Ca^{2+} leakage in the aetiology of MH [13,14]. In earlier experiments measuring Ca^{2+} uptake and release in HSR, the chosen *in vitro* experimental conditions may not have been optimal for reproducing the differences between MHS and MHN vesicles that may exist *in vivo*. Most earlier studies were performed in the absence of pyrophosphate and measured passive Ca^{2+} uptake into HSR [15,16]. In the absence of pyrophosphate, the Ca^{2+}-loading ability of HSR is decreased by 96–98%, making a comparison of the Ca^{2+} capacity and kinetics difficult [17].

In this paper, we examined Ca^{2+} release kinetics in HSR vesicles from muscle biopsies obtained from MHS and MHN patients which were diagnosed according to the European MH Group protocol [5,6]. The defect in Ca^{2+} regulation in human MHS vesicles has not been characterised. However, it is possible that some or all of the Ca^{2+} regulatory proteins that play an integral part in the operation of the ryanodine receptor are involved [18]. For instance, calmodulin has a probable modulatory role in Ca^{2+} release from SR through the ryanodine receptor channel [19]. We therefore investigated the importance of altered protein–receptor interaction in the aetiology of MH.

We also showed the importance of including calpain inhibitors in investigations concerning human HSR. Calpain can partially degrade the cytosolic foot portion of the ryanodine receptor [20]. This results in increased channel open times and increases the probability of the channel being in a more open state. Calpain-digested HSR exhibits greater Ca^{2+} release through the ryanodine receptor [21]. In the absence of calpain inhibitors, proteolytic digestion of the ryanodine receptor may occur, masking possible differences in Ca^{2+} transport between MHS and MHN HSR vesicles. Consequently, the effects of proteolytic digestion on $CICR^{+}$ and halothane-induced Ca^{2+} release were established. However, to this effect it was necessary to use rat skeletal muscle HSR, since the quantity of human material was limited and unavailable for subsidiary investigations.

2. Materials and methods

2.1. Isolation of HSR from rat skeletal muscle

Heavy sarcoplasmic reticulum fraction was isolated from muscle taken from the hind limbs of male Wistar rats. After fat and connective tissue were removed, the muscle was minced and stored in ice-cold 0.9% saline. All stages of the preparation were performed on ice at 0–4°. Approximately 50 grams of muscle were homogenised in a blender for 7 × 15-second pulses in 4 volumes of homogenising medium (10 mM Tris–maleate, 0.1 M NaCl, 1 μg/mL of aprotinin, 1 μg/mL of leupeptin, 1 μg/mL of pepstatin, 5.8 mg/mL of benzamidine, pH 7.0). The homogenate was centrifuged at 1300 *g* for 5 min. The supernatant was filtered through eight layers of cheesecloth, and the concentrations of aprotinin, leupeptin, and pepstatin were each increased to 5 μg/mL. The homogenate was centrifuged at 10,000 *g* for 10 min. The supernatant was filtered through eight layers of cheesecloth and centrifuged at 28,000 *g* for 30 min. The resulting pellet was washed in homogenising medium containing 0.1 M KCl and re-centrifuged at 28,000 *g* for 30 min. The final pellet was resuspended in 0.1 M KCl to a final protein concentration of 15–20 mg/mL. Aliquots of suspension were flash-frozen in liquid nitrogen and stored at −70°. The protein concentration was determined by the method of Bradford [22] using ovalbumin as standard.

Table 1
IVCT data of MHS and MHN biopsied muscle used to prepare HSR

Patient number	Threshold halothane concentration (mM)	Amplitude of halothane contracture at 0.54 mM (mN)	Threshold caffeine concentration (mM)	Amplitude of caffeine contracture at 2 mM (mN)
MHS1	0.13	43	0.5	29
MHS2	0.27	19	1.5	13
MHS3	0.54	59	0.5	30
MHS4	0.13	55	1.5	13
MHS5	0.13	17	2.0	4
MHN1–6	0.0	0.0	0.0	0.0

HSR was obtained from 5 MHS and 6 MHN biopsies after dissection of muscle fibres required for IVCT. Muscle was deemed as MHS when ≤ 2% (v/v) halothane or ≤ 2 mM caffeine generated ≥ 2 mN force [6]. Each of the 6 MHN samples generated no detectable tension at the respective halothane and caffeine thresholds.

2.2. Isolation of HSR from human skeletal muscle

In vitro contracture testing of biopsied *vastus lateralis* muscle for susceptibility to MH was performed according to the European MH Group protocol [5,6]. HSR was obtained from 5 MHS and 6 MHN biopsies after dissection of muscle fibres required for IVCT. This was carried out with the express permission of each patient and approved by the Ethics Committee of Cork University Medical School. The diagnostic data are shown in Table 1. The muscle biopsies were washed in cold 0.9% (w/v) saline, finely minced, and homogenised in a loosely fitting potter homogeniser with 4 volumes of cold 10 mM Tris, 0.1 M NaCl, 0.3 M sucrose, 5 μg/mL of aprotinin, 5 μg/mL of leupeptin, and 5 μg/mL of pepstatin, pH 7.0. The homogenate was centrifuged for 5 min at 1300 *g*. The supernatant was retained, filtered through cheesecloth, and centrifuged for 10 min at 6000 *g*. The supernatant was again centrifuged for 45 min at 32,000 *g*. The pellet was washed in the resuspension medium (10 mM Tris, 0.1 M NaCl, 0.1 M KCl, 5 μg/mL of aprotinin, 5

μg/mL of leupeptin, 5 μg/mL of pepstatin). All procedures were carried out on ice at 0–4°. The final pellet was resuspended to a protein concentration of 15–20 mg/mL in the preceding medium. HSR was stored at −70° and used within one month of preparation. The protein concentration was determined by the method of Bradford [22] using ovalbumin as standard.

2.3. Ca^{2+} uptake and release measurements

Ca^{2+} uptake and release by HSR were measured by dual-wavelength spectrophotometry based on the method of [17] using anti-pyralazo III Ca^{2+} chelometric dye. HSR (57 μg/mL) was added to medium containing 19 mM MOPS, 93 mM KCl, 7.5 mM sodium pyrophosphate, 1 mM MgATP, 5 mM creatine phosphate, 20 μg/mL of creatine kinase, and 250 μM anti-pyrylazo III in a total volume of 2 mL at pH 7.0 using a ground glass-stoppered glass cuvette. Ca^{2+} flux was followed continuously on a Hewlett Packard 8452A diode-array spectrophotometer operating at a measuring wavelength of 710 nm and a reference wavelength of 790 nm. The spectrophotometer was equipped with a magnetic cuvette stirrer and a thermostatted cuvette holder that maintained the temperature at 37° ± 0.1. Ca^{2+} pulses of 20 nmol were added to the medium using a Hamilton dispensing syringe (Hamilton Inc.). The uptake of each Ca^{2+} pulse was followed spectrophotometrically. Saturation of rat and human HSR was achieved once an increment of added Ca^{2+} failed to return to the baseline absorbance value. The Ca^{2+} pulses used to load the HSR were also used to calibrate the Ca^{2+} concentration in the solution. Initial rates of release were determined using a linear regression analysis program in the Hewlett Packard kinetics software. The rate constant of rapid Ca^{2+} uptake was approximated as $2(\ln 2)/(t_O - t_X)$, where t_O is the time at which Ca^{2+} was added and t_X is the time taken to return to baseline [23]. The rate of Ca^{2+} uptake was calculated from the first seven 20-nmol additions of Ca^{2+}. An enhancement of the rate of Ca^{2+} loading by ruthenium red was calculated in the same manner. The concentration of halothane in the cuvette was measured by gas chromatography (Hewlett Packard 5890 Series II Gas Chromatograph) with electron capture detection using a chromasorb-Poracil C column (Supelco) running isothermally at 110° and with an injector and detector temperature of 150°. The halothane stock solution was prepared daily in ethanol (538 mM) and the concentrations in solution were calculated using Henry's Law [24]. Appropriate dilutions of the stock solution were used to achieve final aqueous cuvette concentrations of 0.14, 0.28, 0.55, 0.825, 1.1, 1.375, and 1.65 mM, which are equivalent to 0.5%, 1%, 2%, 3%, 4%, 5%, and 6% (v/v) halothane, respectively. Control experiments showed no effect of ethanol at the concentrations used (<0.5%).

Table 2
Ca^{2+} uptake capacity of MHS and MHN HSR vesicles

	Ca^{2+} uptake capacity (mean ± SEM) (nmol Ca^{2+}/mg HSR protein)	
	−Ruthenium red	+Ruthenium red
MHN	3070 ± 175	3070 ± 149
MHS	1579 ± 105	2982 ± 140

HSR (57 μg/mL) was loaded to saturation with Ca^{2+} in 20-nmol additions. This was measured in the presence and absence of ruthenium red (10 μM); N = 5. In the absence of ruthenium red, the difference between the MHN and MHS vesicular Ca^{2+} uptake capacities is significant at $P < 0.001$. There was no significant difference when ruthenium red was added ($P > 0.05$).

2.4. Statistical methods

Differences between means were evaluated using an unpaired Student's *t*-test. The differences were considered significant if $P \leq 0.05$.

3. Results

The Ca^{2+} uptake capacity of MHN and MHS HSR was measured by maximally loading the vesicles with Ca^{2+}. A 47% reduction in Ca^{2+} capacity was observed for MHS vesicles ($P < 0.001$) compared to MHN vesicles (Table 2). The rate constant for Ca^{2+} uptake in MHS vesicles was reduced by 45% ($P < 0.001$) compared to MHN vesicles (Table 3). The capacity and uptake rate of MHS vesicles were restored to those of MHN vesicles in the presence of the specific Ca^{2+} channel blocker ruthenium red (10 μM). There was no significant difference in MHN vesicular Ca^{2+} uptake in the presence and absence of ruthenium red.

Using rat skeletal muscle HSR, the significance of calpain inhibition for CICR and halothane-induced Ca^{2+} release was shown (Fig. 1a). Proteolysis of the ryanodine receptor resulted in an increase in both Ca^{2+} and halothane-induced Ca^{2+} release rates from HSR. The extraluminal Ca^{2+} concentration threshold for CICR was reduced 4-fold from 50 μM (intact HSR) to 12.5 μM (proteolysed HSR).

Table 3
Rate constant of Ca^{2+} uptake

	Rate constant of Ca^{2+} uptake (min^{-1})	
	−Ruthenium red	+Ruthenium red
MHN	$2.34 \times 10^{-2} \pm 1.40 \times 10^{-3}$	$2.01 \times 10^{-2} \pm 1.05 \times 10^{-3}$
MHS	$1.28 \times 10^{-2} \pm 1.05 \times 10^{-3}$	$2.24 \times 10^{-2} \pm 1.49 \times 10^{-3}$

The rate constant of rapid Ca^{2+} uptake was approximated as $2(\ln 2)/(t_O - t_X)$ [23]. This was measured in the presence and absence of ruthenium red (10 μM); N = 5. In the absence of ruthenium red, the difference between the MHN and MHS vesicular Ca^{2+} uptake rates is significant at $P < 0.001$. There was no significant difference when ruthenium red was added ($P > 0.05$).

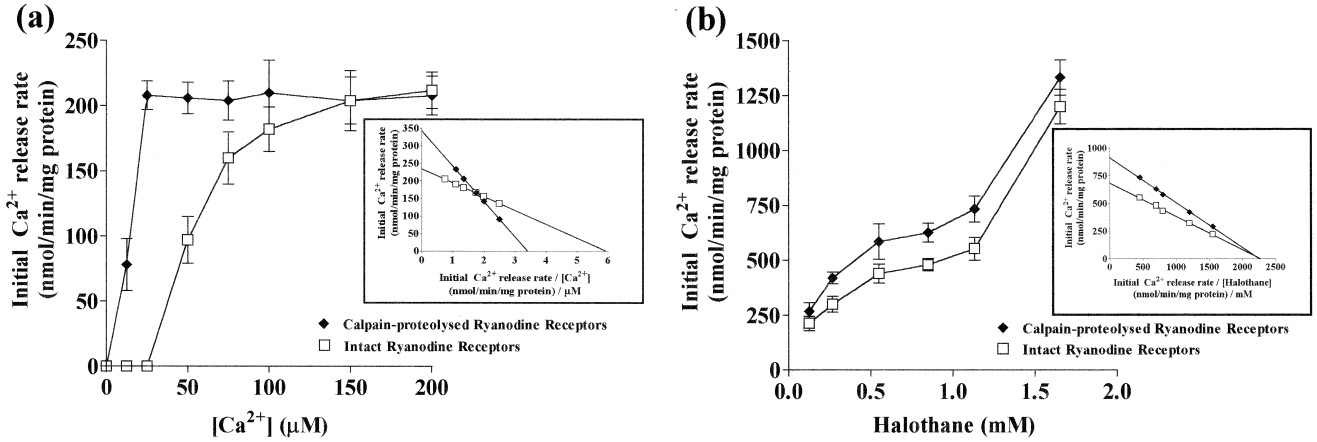

Fig. 1. Effect of calpain proteolysis on rates of (a) CICR and (b) halothane-induced Ca^{2+} release. Rat HSR (30 μg/mL) was maximally preloaded with 320 ± 20 nmol (mean ± SD) Ca^{2+} prior to stimulation of Ca^{2+} release. HSR was prepared in the presence (intact ryanodine receptors) or absence (proteolysed ryanodine receptors) of the calpain inhibitor leupeptin. Each assay represents the mean ± SEM of assays performed in duplicate on 3 separate preparations of HSR. Inset: Woolf–Augustinsson–Hofstee plots of the data.

The maximum rate of Ca^{2+} release (V_{max}) was 237.3 ± 11.3 nmol Ca^{2+}/min/mg protein for intact HSR and 345.5 ± 29.5 nmol Ca^{2+}/min/mg protein for proteolysed HSR (mean ± SD). The Ca^{2+} concentration that triggered 50% of the maximum rate of Ca^{2+} release ($K_{0.5}$) from intact HSR was 100.4 ± 6.0 μM Ca^{2+} compared to 19.7 ± 2.6 μM Ca^{2+} for proteolysed HSR (mean ± SD). The release profiles for halothane-induced Ca^{2+} release from intact and proteolysed HSR were similar, with no significant difference between the $K_{0.5}$ values of 0.34 ± 0.03 mM halothane (intact) and 0.32 ± 0.02 mM halothane (proteolysed) (mean ± SD) (Fig. 1b). However, the maximum initial rates of Ca^{2+} release in both types of HSR were significantly different at all halothane additions with the exception of 1.65 μM halothane. The maximum rates of Ca^{2+} release (V_{max}) from intact HSR (682.5 ± 32.6 nmol Ca^{2+}/min/mg protein [mean ± SD]) and proteolysed HSR (916.0 ± 32.3 nmol Ca^{2+}/min/mg protein [mean ± SD]) were significantly different ($P < 0.001$).

CICR from MHS and MHN HSR vesicles was measured after the vesicles were preloaded with 180 nmol Ca^{2+}/mg protein (MHS and MHN vesicles) or 350 nmol Ca^{2+}/mg protein (MHN vesicles). The former represented maximum saturation of MHS vesicles and approximately half-saturation of MHN vesicles, while the latter represented maximum saturation of MHN vesicles. CICR was stimulated by adding 50–150 μM Ca^{2+}. At this Ca^{2+} loading, CICR from MHS vesicles was concentration-dependent. It did not occur at the submaximal Ca^{2+} loading in MHN vesicles (results not shown), but was triggered when these were maximally saturated with 350 nmol Ca^{2+} (Fig. 2). The total calcium loading was 0 to 474 nmol (saturated MHS, unsaturated MHN) and 0 to 720 nmol (saturated MHN) (Fig. 3). The Ca^{2+} threshold for CICR was reduced by approximately 46% in saturated MHS vesicles compared to saturated MHN vesicles. There was no overlap in the CICR thresholds of the MHS and MHN patients.

The initial rate of halothane-induced Ca^{2+} release from HSR vesicles at clinically relevant halothane concentrations (0.5–6% v/v) was measured after preloading the vesicles with 1.58 μmol Ca^{2+}/mg HSR protein. Both MHN and MHS vesicles exhibited concentration-dependent halothane-induced Ca^{2+} release (Fig. 4). MHS vesicles demonstrated a 6-fold lower halothane threshold for halothane-induced Ca^{2+} release compared to MHN vesicles (0.14 mM compared to 0.83 mM). Rates of release were significantly greater in HSR from each MHS patient compared to HSR from normal patients for all concentrations of halothane tested.

The effect of the calmodulin antagonist TFP on Ca^{2+} release was examined. TFP-induced Ca^{2+} release from MHS HSR vesicles was elevated compared to that in MHN HSR vesicles (Fig. 5). The ED_{50} (effective dose of TFP that triggered 50% of maximal Ca^{2+} release) values for TFP were 19.4 ± 1.0 μM and 14.5 ± 1.0 μM (mean ± SD; N = 6) for MHN and MHS vesicles, respectively; these values were significantly different ($P < 0.001$). Hill plots of the data yielded Hill coefficients of 4.8 ± 0.6 and 4.3 ± 0.5 for MHN and MHS vesicles, respectively, which were not significantly different ($P > 0.05$). The maximum rate of Ca^{2+} release, calculated from the Hill plot, was 148.9 ± 8.3 nmol Ca^{2+}/min/mg HSR protein for MHN and 276.5 ± 9.3 nmol/min/mg HSR protein (mean ± SD; N = 6) for MHS. These values were significantly different ($P < 0.001$).

4. Discussion

The agonist thresholds for initiation of Ca^{2+} release from HSR were clearly reduced in MHS individuals. This was previously observed in investigations that used heterologous expression of recombinant DNA in HEK-293 cells [25,26]. A significant increase in sensitivity to agonists including halothane and Ca^{2+} was demonstrated in cells expressing

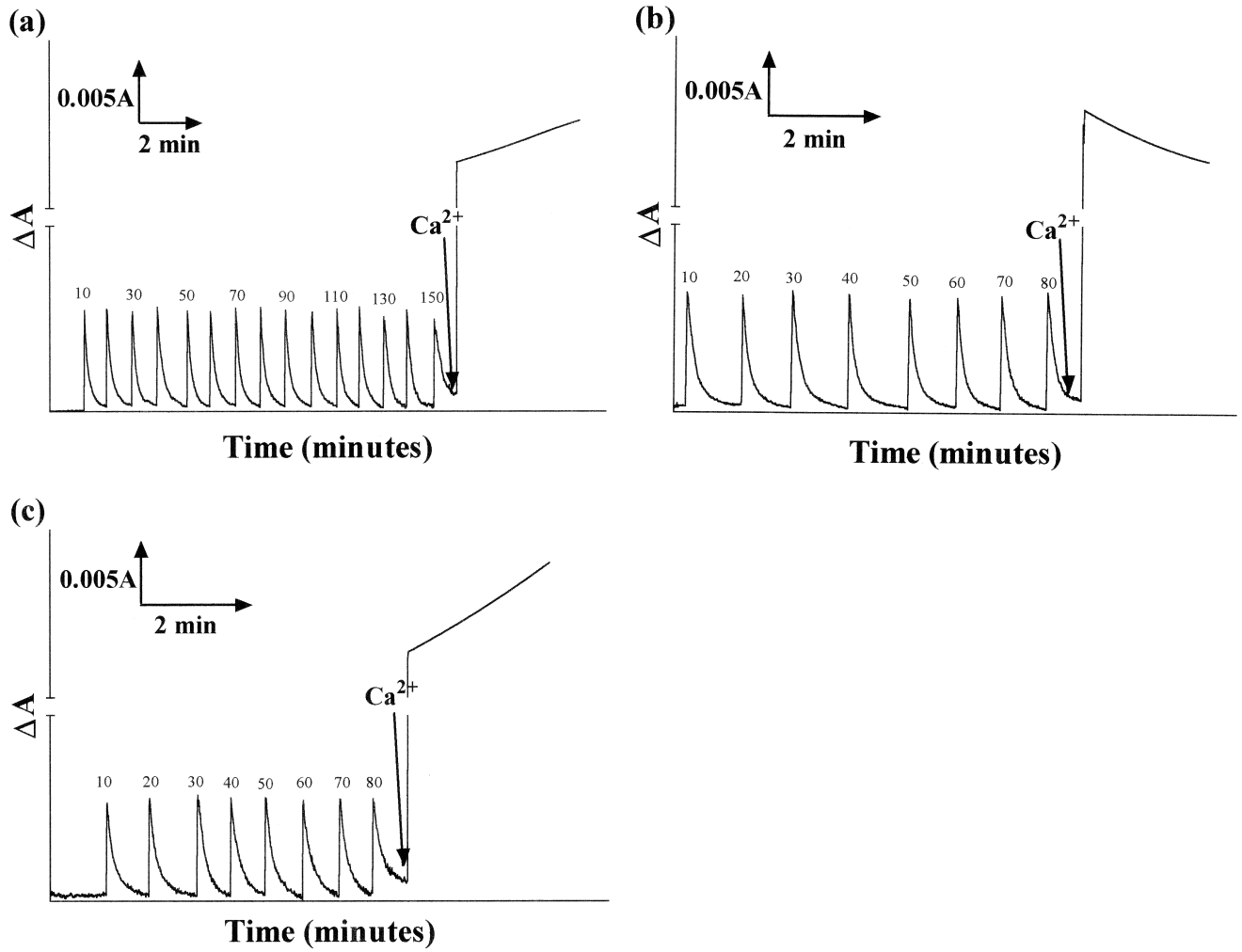

Fig. 2. Profiles of Ca^{2+} uptake and Ca^{2+}-induced Ca^{2+} release from (a) saturated MHN HSR, (b) unsaturated MHN HSR, and (c) saturated MHS HSR. HSR (2 mL; 57 μg/mL), prepared from MHN muscle, required the addition of 320 ± 20 nmol (mean ± SD) Ca^{2+} to achieve saturation. HSR (2 mL; 57 μg/mL) from MHS muscle required 160 ± 12 nmol (mean ± SD) to achieve saturation. This Ca^{2+} load corresponds to approximately half-saturation in MHN HSR. The labels above the peaks represent the concentration of added Ca^{2+} (μM).

the ryanodine receptor mutations compared to cells expressing wild-type receptors or receptors with other non-MH mutations. However, the expression system, which used rabbit *RYR*1 cDNA transfected into HEK-293 cells, is artificial and lacks the full biochemical environment of the native HSR used in these studies. Data obtained in the alien milieu of HEK-293 cells is potentially more prone to artefacts than data obtained directly on human HSR. Nevertheless, the increased sensitivity of MHS HSR vesicles to agonists and the altered Ca^{2+} loading of these vesicles are indicative of a greater channel open probability of the MH ryanodine receptor and are in general agreement with the results obtained from the HEK-293 cell model. Indeed, the reduction in the Ca^{2+} threshold for channel activation is strongly indicative of a leaky channel [26]. The observed increase in the sensitivity of MHS vesicles to halothane is consistent with the hypersensitivity of biopsied MHS muscle to contracture by halothane [27] and the increased sensitivity of human MHS channels to halothane in single-channel recordings [28].

The ryanodine receptor is interdependent on the luminal Ca^{2+}-binding protein, calsequestrin. The binding of agonists to the ryanodine receptor causes a conformational change in calsequestrin, resulting in the dissociation of Ca^{2+} from the protein [29]. The resulting large concentration gradient across the HSR membrane facilitates the rapid efflux of Ca^{2+} from the HSR. The decreased loading ability exhibited by MHS HSR vesicles is indicative of a submaximal Ca^{2+}-binding state of calsequestrin [30]. This premature priming of calsequestrin for Ca^{2+} release is further evidence that the ryanodine receptor has a greater open probability in MHS muscle than in MHN muscle. The Ca^{2+} channel blocker ruthenium red restores the Ca^{2+}-loading ability of the vesicles by blocking the ryanodine receptor Ca^{2+} channel, indicating that calsequestrin returns to a maximal Ca^{2+}-binding conformation. The molecular defect

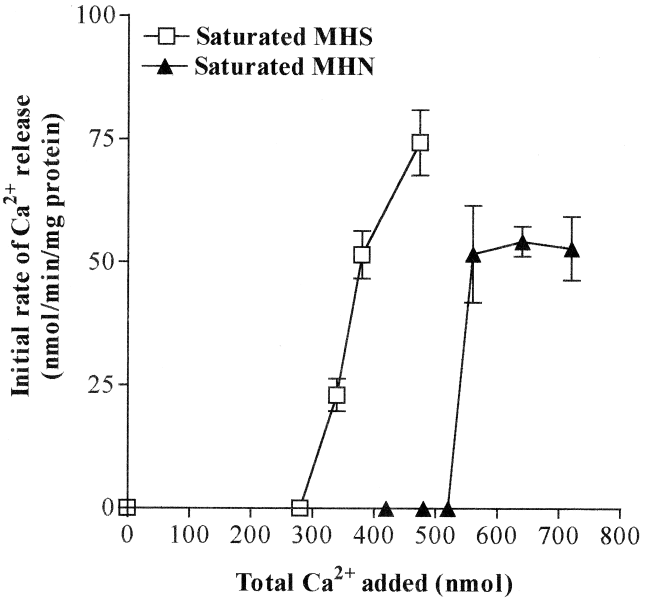

Fig. 3. Comparison of rates of CICR from saturated MHN and MHS HSR. HSR (2 mL; 57 μg/mL) was preloaded with 180 ± 12 nmol (mean ± SD) Ca^{2+} prior to stimulation of Ca^{2+} release. CICR was induced by adding $CaCl_2$ at the concentrations shown. Each assay represents the mean ± SEM of assays performed in duplicate on 3 separate preparations of HSR.

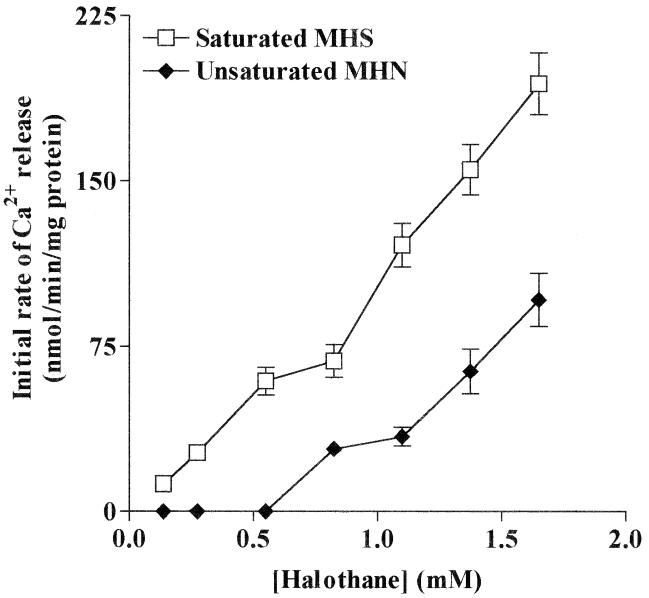

Fig. 4. Plot of rates of halothane-induced Ca^{2+} release from MHN and MHS HSR. HSR (2 mL; 57 μg/mL) was preloaded with 180 ± 12 nmol (mean ± SD) Ca^{2+}. Each value represents the mean ± SEM from 3 separate preparations of HSR, each determination performed in duplicate. Shown below are *t*-test values comparing MHN and MHS HSR initial release rates at the different halothane concentrations. Significant differences occurred between the MHS and MHN samples at each halothane concentration: 0.138 mM halothane, $P < 0.02$; 0.825 mM halothane, $P < 0.002$; 0.275 mM halothane, $P < 0.01$; 1.100 mM halothane, $P < 0.001$; 0.550 mM halothane, $P < 0.002$; and 1.375 mM halothane, $P < 0.001$.

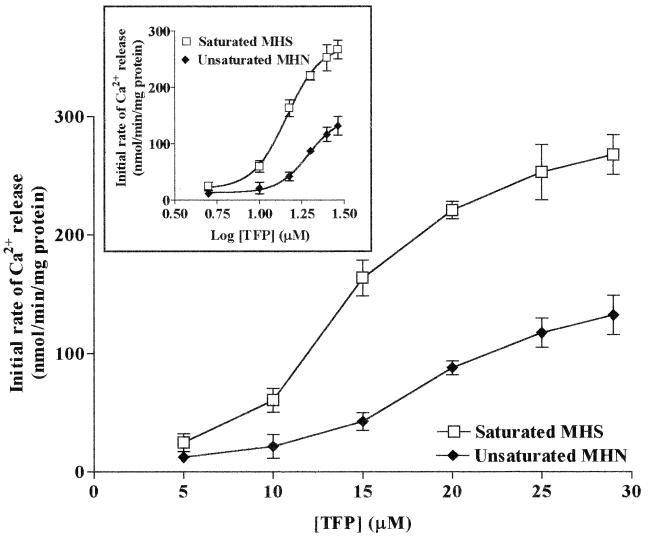

Fig. 5. Dose-response curve of TFP-induced Ca^{2+} release from MHN and MHS HSR. HSR (2 mL; 57 μg/mL) was preloaded with 180 ± 12 nmol (mean ± SD) Ca^{2+} prior to stimulation of release with TFP. Each value is the mean ± SEM from 3 different HSR preparations. Each determination was performed in duplicate. The initial Ca^{2+} release rates in MHN and MHS HSR at each TFP exposure were compared using a *t*-test. Significant differences occurred between the MHS and MHN samples at each TFP concentration ($0.001 < P < 0.05$). Inset: the Hill plots of the data.

in MH may therefore have agonist-independent as well as agonist-dependent components. MHS HSR vesicles will continuously recycle Ca^{2+}, because efflux from MH ryanodine receptors would be continuously elevated compared to normal even during relaxation. Some support for this comes from studies on whole muscle from MH-positive humans in which higher myoplasmic Ca^{2+} concentrations were detected using implanted Ca^{2+} electrodes [31]. Binding studies conducted on human MHS HSR vesicles concluded that the human MH ryanodine receptor has a higher [^{3}H]ryanodine-binding affinity, which is indicative of a more open state even in resting muscle [32].

Experiments performed on rat HSR in our laboratory clearly show a greater Ca^{2+} release through the ryanodine receptor in calpain-digested HSR exhibits. The interaction of Ca^{2+} and halothane with the ryanodine receptor is directly modulated by the state of the channel, as is shown in Fig. 1, a and b. This would explain why in previous studies conducted in the absence of calpain inhibitors, proteolytic digestion of the ryanodine receptor would have occurred, masking possible differences in Ca^{2+} transport between MHS and MHN vesicles. Therefore, the inclusion of calpain inhibitors in the HSR preparation has rendered it highly unlikely that the observed 'open state' of the ryanodine receptor is an artefact of channel proteolysis.

Finally, the effects of TFP on Ca^{2+} release are also indicative of an increased channel leakiness in MHS HSR. TFP-induced Ca^{2+} release was elevated for MHS vesicles compared to normal vesicles, with a nearly 2-fold difference in the maximum Ca^{2+} release rate achieved. It has been proposed that an enhanced sensitivity of the ryanodine re-

ceptor to calmodulin may contribute to elevated Ca^{2+} release from MHS vesicles [19]. However, the unchanged Hill coefficient for MHS vesicles compared to normal vesicles suggests that the enhanced sensitivity is due to the more open state of the Ca^{2+} release channel rather than an alteration in the co-operativity between the calmodulin-binding sites.

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