

Inability of Sphingosine and Calmodulin to Control Ryanodine Receptor in Malignant Hyperthermia

Romeo Betto, Alessandra Teresi⁽¹⁾, Manuela Duca⁽¹⁾, Federica Turcato⁽¹⁾, Daniela Danieli-Betto⁽²⁾ and Roger Sabbadini⁽³⁾

C.N.R. Institute of Neuroscience, Unit for the study of Neuromuscular Biology and Physiopathology, (1) Department of Biomedical Sciences, (2) Department of Human Anatomy and Physiology, University of Padova, Padova, Italy and (3) Department of Biology and Molecular Biology Institute, San Diego State University, San Diego, USA

Abstract

Sarcoplasmic reticulum Ca^{2+} release of malignant hyperthermia-susceptible (MHS) skeletal muscle is hypersensitive to both agonists and antagonists of the ryanodine receptor channel. This study examined whether sphingosine and calmodulin, endogenous modulators of the channel, play a role in the abnormal responsiveness of pathological muscle. Sphingosine caused a dose dependent inhibition of [^3H]ryanodine binding to MHS and normal terminal cisterns (TC) membranes of the sarcoplasmic reticulum (SR). The sphingosine concentration capable of inhibiting 50% of the binding (IC_{50}) was 1.5-times higher for MHS compared to membranes isolated from normal animals. Calmodulin also caused a dose dependent inhibition of [^3H]ryanodine binding. However, at variance with sphingosine and other inhibitors, the calmodulin effect was incomplete in that 1 μM calmodulin inhibited only 50% of the [^3H]ryanodine binding to MHS membranes. Sphingosine's inhibitory action was particularly effective at activating pCa levels (5 to 4) in normal membranes, while in MHS membranes the ability of sphingosine to block ryanodine receptor was minimal. Similarly, Calmodulin inhibition of [^3H]ryanodine binding was maximal in normal membranes at pCa 4 but less potent in MHS membranes. Both sphingosine and calmodulin exert the highest inhibitory action to the ryanodine receptor at activating pCa levels in normal membranes. Similarly, at the same pCa values, both drugs are less effective in blocking the mutated channel. i.e., particularly at pCa levels that maximally activate muscle cells. These results indicate that the lower sensitivity to inhibition of the MHS Ca^{2+} -release channel by endogenous antagonists, such as sphingosine and calmodulin, may play an important role in the abnormal response of the mutated channel.

Key words: calmodulin, malignant hyperthermia, ryanodine receptor, sarcoplasmic reticulum, sphingosine.

Basic Appl Myol 12 (4): 159-166, 2002

Malignant hyperthermia (MH) is an inherited skeletal muscle disorder caused in humans by a number of point mutations in the sarcoplasmic reticulum ryanodine receptor (RyR) [17] and in the pig by an Arg⁶¹⁵ to Cys⁶¹⁵ point mutation [12]. MH episodes are triggered in susceptible patients and animals by volatile anaesthetics such as halothane, and depolarising muscle relaxants. The disorder is characterised by prolonged uncontrolled contracture, hyperthermia, and death if not promptly treated with the ryanodine receptor blocker, dantrolene [14]. At variance with the normal channel, the altered RyR is both hypersensitive to stimulation by agonists such as μM calcium, ATP, and caffeine [10, 19, 21] and less sensitive to antagonists such as mM calcium, mag-

nesium, and ruthenium red [20, 21]. Halothane triggers MH crisis by causing a rapid myoplasmic calcium rise that precedes metabolic and clinical signs [26].

Sphingosine is a potent inhibitor of both RyR1 and RyR2, the isoforms of the channel expressed in skeletal and cardiac sarcoplasmic reticulum, respectively. [^3H]Ryanodine binding studies [7, 27, 31], recordings of the channel reconstituted into planar bilayer membranes [22], and Ca^{2+} release measurements [2, 7, 27] have provided evidence for a direct action of sphingosine on the RyR. Importantly, sphingosine is endogenous to skeletal and cardiac muscle membranes and is present at concentrations shown to be relevant to the activity of the RyR [7, 28]. In addition, [^3H]ryanodine binding studies performed

on receptor fragments indicate that sphingosine binds directly to the portion of RyR1 between Arg⁴⁴⁷⁵ and the C-terminus [22]. This makes sphingosine candidate for modulating the RyR in muscle and it has been proposed that sphingosine may play a physiological role in maintaining the channel closed in the resting state [29].

Calmodulin is an ubiquitous calcium binding protein involved in the regulation of several cellular processes [4]. In skeletal and cardiac muscles, calmodulin modulates calcium homeostasis by acting on a number of key proteins [1], including the RyR [18, 32]. Calmodulin directly activates the RyR at submicromolar calcium concentrations, whereas it inhibits the channel at higher calcium concentrations [11, 34]. This direct action is supported by the presence of calmodulin binding sites in the RyR protein [35].

Because of the reduced sensitivity to antagonists of the MHS RyR1, the aim of the present study was to evaluate the possible contribution of sphingosine and calmodulin to the abnormal responsiveness of the mutated channel.

Materials and methods

Materials

9,21-[³H]ryanodine (60-75 Ci/mmol) was purchased from DuPont New England Nuclear (Köln, Germany). Unlabeled high purity ryanodine was from Calbiochem (San Diego, CA, USA). Calmodulin was from Sigma (St. Luis, MS, USA). *Taq* polymerase and *Asp* HI restriction endonuclease were obtained from Boehringer (Mannheim, Germany). Sense and antisense primers 5'-TCCAGTTTGCCACAGGTCCTACCA-3' and 5'-ATTCACCGGAGTGGAGTCTCTGAG-3' were from Pharmacia Biotech (Milano, Italy). Sphingosine and all other chemicals were obtained from Calbiochem.

Animals

Longissimus dorsi muscles from normal Yorkshire and from Belgian Landrace pigs, homozygous for the gene causing MHS, were utilized for all experiments. MH susceptibility was tested preliminarily by halothane challenges. After three weeks, selected pigs were sacrificed and muscles were rapidly excised, cut in about 20 g pieces, frozen in liquid nitrogen, and stored at -80°C. Pigs were genotyped for the demonstration of the Arg⁶¹⁵ to Cys⁶¹⁵ mutation of the RyR1 using the polymerase chain reaction/restriction endonuclease test described elsewhere [24].

Sarcoplasmic reticulum membrane preparation

Sarcoplasmic reticulum terminal cisterns (TC) membranes were purified by the procedure of Saito et al. [30] with modifications described by Damiani and Margreth [6]. Briefly, muscle specimens were homogenized in a medium containing 0.3 M sucrose, 5 mM imidazole, pH 7.4, 5 µg/ml leupeptin and 100 µM PMSF. After the first 10 min centrifugation at 7,500 *g*, the pellet was re-suspended in the same medium and centrifuged at 15,000 *g* for 20 min. Total membranes were then pel-

leted by centrifuging at 120,000 *g* for 90 min and the next pellet re-suspended in the above medium and then overlaid on a discontinuous sucrose gradient [30]. The fraction at the interface between 38-45% sucrose was collected and diluted with 5 mM imidazole, pH 7.4 and centrifuged at 110,000 *g* for 60 min. Final TC membranes were re-suspended in 0.3 M sucrose, 5 mM imidazole, pH 7.4 and stored at -80°C.

Purified membrane proteins were resolved by SDS-PAGE on 5-10% polyacrylamide linear gradients, stained by Coomassie brilliant blue and photographed.

[³H]Ryanodine binding assays

Equilibrium binding studies were performed essentially as previously described [3,27]. Sarcoplasmic reticulum TC membranes (at a protein concentration of 0.1 mg/ml) were incubated with [³H]ryanodine for 90 min at 37°C in a final volume of 0.5 ml. To determine the binding constants (*K*_d and *B*_{max}) of the high-affinity binding site, equilibrium experiments were performed in a binding buffer containing 0.1 M KCl, 20 mM Hepes, pH 7.0 in the presence of 1-50 nM [³H]ryanodine and 10 mM ATP, respectively. The effects of sphingosine were investigated in the presence of various concentrations of each inhibitor. The action of calmodulin was determined by incubating TC membranes (at a protein concentration of 0.1 mg/ml) with 20 nM [³H]ryanodine in 0.5 ml of 0.1 M KCl, 20 mM Hepes, pH 7.0 and 20 µM free calcium for 90 min at 37°C. pCa dependence of the binding of [³H]ryanodine to TC membranes was measured at selected pCa values obtained by the addition of CaCl₂ alone or, below pCa 4, by buffering with different concentrations of EGTA, as calculated on the basis of the stability constants published by Fabiato [9]. Samples were filtered onto Whatman GF/B filters, and the filters were then washed with three 5-ml cold 0.1 M KCl, 20 mM Hepes, pH 7.0 buffer. Radioactivity was measured by liquid scintillation counting. Specific [³H]ryanodine binding was determined by subtracting from total binding the nonspecific binding obtained in the presence of 10 µM cold ryanodine. Equilibrium binding constants in the presence and absence of inhibitor were determined by using the iterative curve fitting program Table Curve (SSPS, Chicago, IL).

Analysis of data

Data analysis was performed with the program Sigma Plot (SSPS, Chicago, IL). Scatchard analysis was used to determine the dissociation constant (*K*_d) and maximal binding capacity (*B*_{max}) from equilibrium binding data. Data are expressed as means ± SEM. Paired or unpaired Student *t* tests were used for evaluation of the mean values. A value of *p* < 0.05 was considered to be statistically significant.

Results

Sphingosine and calmodulin are endogenous inhibitors of sarcoplasmic reticulum RyR [22, 27, 31]. To

asses the contribution of these inhibitors in the altered Ca^{2+} release from MHS sarcoplasmic reticulum, we isolated TC membranes from normal and MHS muscles and examined the ability of the inhibitors to affect [^3H]ryanodine binding. Sarcoplasmic reticulum TC membranes purified from normal and MHS pig muscles showed a protein pattern almost identical, with highly comparable RyR levels (Figure 1). Equilibrium binding analysis showed that the maximal number of binding sites (B_{max}) was 12.9 ± 1.7 ($n = 5$) and 12.3 ± 2.9 ($n = 4$) in normal and MHS membranes, respectively, confirming that equivalent levels of protein were expressed by both normal and MHS pig tissue.

The binding of [^3H]ryanodine is dependent upon the functional state of the channel, and consequently, it represents a useful tool to analyse the action of modulators of Ca^{2+} release channel function [5, 15]. The effects of sphingosine on the [^3H]ryanodine binding to TC membranes isolated from normal and MHS skeletal muscles is shown in Figure 2. Sphingosine produced a dose dependent inhibition of [^3H]ryanodine binding to normal pig skeletal muscle membranes with IC_{50} of about 20 μM . In contrast to normal TC membranes, the action of sphingosine in the MHS muscle membranes was less effective yielding an IC_{50} value of about 33 μM . These results clearly indicate that higher concentrations of sphingosine were needed in MHS in respect to normal membranes to produce similar levels of inhibition.

We also investigated the ability of calmodulin to distinguish the behaviour of MHS vs. normal membranes. To

exclude any possible phosphorylation-dependent inhibition of RyR activity [8, 33], the effects of calmodulin on [^3H]ryanodine binding were studied in the absence of ATP and in the presence of 20 μM calcium. The removal from the medium of ATP, powerful stimulator of [^3H]ryanodine binding [18], also had the effect of reducing the maximal binding capacity to both normal and MHS membranes (compare Figures 2 and 4 with Fig. 3 and 5, respectively). Calmodulin caused a dose dependent inhibition of the binding of [^3H]ryanodine to both normal and MHS TC membranes (Figure 3). However, calmodulin inhibition of the normal RyR was incomplete, since only about 75% of the binding was inhibited at the higher calmodulin concentrations. For MHS membranes, the action of calmodulin was even less effective, because the binding was inhibited only to the 50% level. The inability of calmodulin to block all of the available [^3H]ryanodine binding is unique, since sphingosine (Figure 2) and all other known inhibitors are able to completely block ryanodine binding to the channel [18].

In order to better understand the inhibitory action of sphingosine on normal and MHS RyR, we examined the pCa dependence of [^3H]ryanodine binding in the absence and presence of sphingosine. The [^3H]ryanodine binding at different pCa values exhibited a bell shaped curve both with normal and MHS membranes (Figure 4). Sphingosine caused about 90% inhibition of [^3H]ryanodine binding in normal membranes over almost all pCa values tested, whereas in MHS membranes, the inhibitory action of sphingosine was dramatically reduced (30-40%) but not eliminated.

In order to evaluate the physiological concentration and, thus, the putative action of sphingosine in pig muscles, we measured its level by high performance liquid chromatography [28]. Normal pig muscles exhibited levels of sphingosine (14.8 ± 0.3 pmol/g of fresh tissue, $n = 3$) comparable to those of the rat [28]. Interestingly, the endogenous sphingosine level of MHS muscles was lower (6.2 ± 0.2 pmol/g of fresh tissue, $n = 3$) than that of normal muscle.

Given that calmodulin inhibition is calcium dependent [11, 34], the effect of 1 μM calmodulin on [^3H]ryanodine binding to normal and MHS membranes was studied at pCa varying from 8 to 3 (Figure 5). It can be noted that in the absence of calmodulin, [^3H]ryanodine binding was maximal at pCa 5 in both MHS and normal TC membranes. Calmodulin (1 μM) shifted to the left the bell-shaped curve of the pCa-dependence of [^3H]ryanodine binding in both MHS and normal TC membranes, with the maximum stimulation of binding shifted to pCa 6. However, inhibition of [^3H]ryanodine binding in the pCa range varied between 6 and 3 and it was significantly less potent in MHS than in normal membranes. For example, calmodulin inhibition of [^3H]ryanodine binding to normal membranes was about 75-80%, while in MHS membranes was about 50% at pCa 4 (Figure 5).

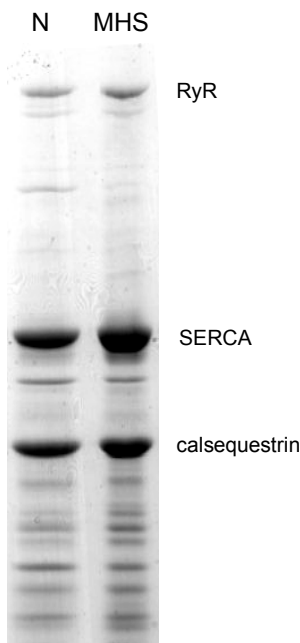


Figure 1. SDS-PAGE analysis of normal (N) and MHS sarcoplasmic reticulum TC membranes. Sample proteins (40 μg each) were stained with Coomassie Brilliant Blue. The ryanodine receptor (RyR), the Ca^{2+} pump (SERCA) and calsequestrin proteins are indicated.

Sphingosine, calmodulin and malignant hyperthermia

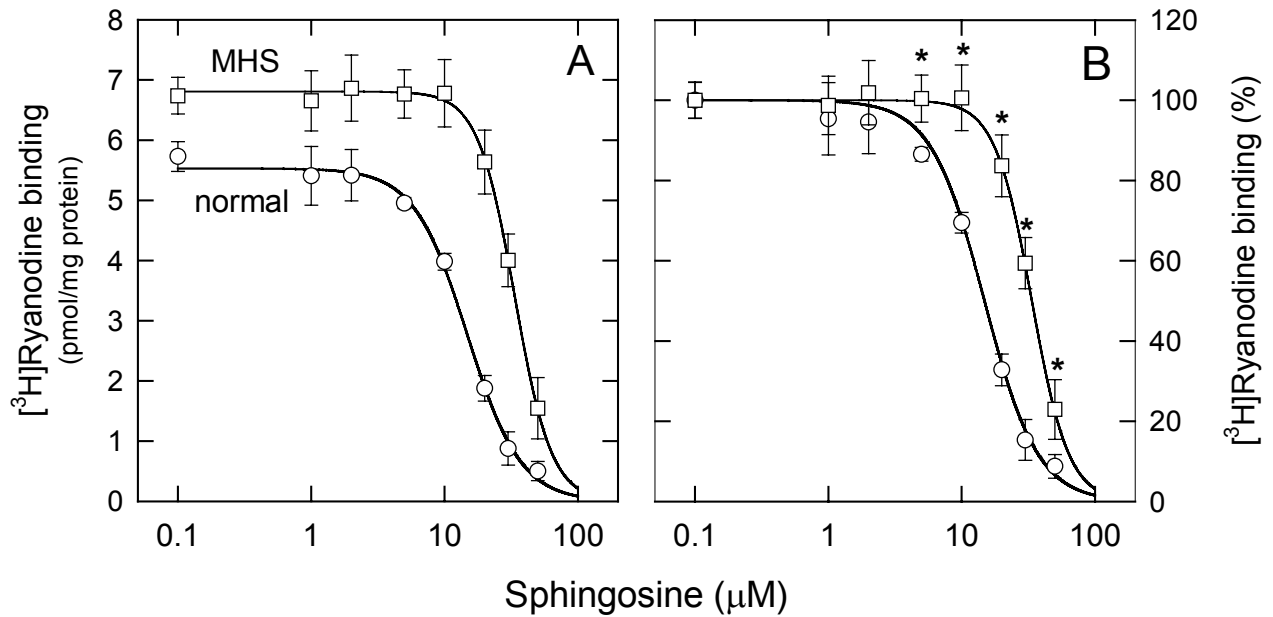


Figure 2. Dose dependence of sphingosine effect on the $[^3\text{H}]$ ryanodine binding to normal (○) and MHS (□) TC membranes (50 μg). $[^3\text{H}]$ ryanodine binding was measured in a buffer containing 20 nM $[^3\text{H}]$ ryanodine, 0.1 M KCl, 20 mM Hepes, pH 7.0, 300 μM Ca^{2+} and 10 mM ATP. (A) Effect of sphingosine on the absolute $[^3\text{H}]$ ryanodine binding. (B) Same data as in (A) expressed as percent of maximal binding. Symbols represent the means $\pm$ SEM of four experiments performed in triplicate. Asterisks indicate data significantly different at least at $p < 0.05$ (Student's t test).

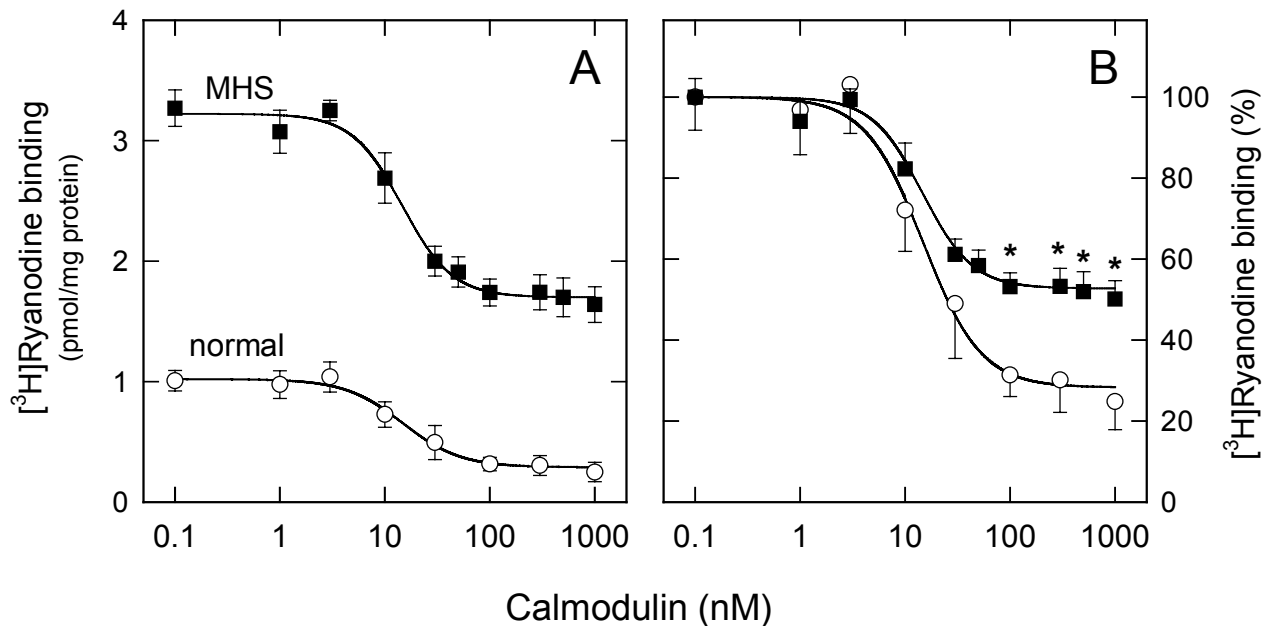


Figure 3. Dose dependence of calmodulin effect on the $[^3\text{H}]$ ryanodine binding to normal and MHS TC membranes. $[^3\text{H}]$ ryanodine binding was measured in a medium containing 50 μg of TC membranes, 20 nM $[^3\text{H}]$ ryanodine, 0.1 M KCl, 20 mM Hepes, pH 7.0 and 20 μM Ca^{2+} . (A) Effect of calmodulin on the absolute binding. (B) Same data as in (A) expressed as percent of maximal binding. Each data point is the mean $\pm$ SEM of four separate experiments performed in triplicate. Asterisks indicate data significantly different at least at $p < 0.05$ (Student's t test).

Discussion

The abnormal calcium handling of skeletal muscles from MHS animals is attributed to altered sensitivity to modulators of the mutated receptor channel [10, 17, 19,

20, 21]. The mutated channel is especially sensitive to the inhalant anaesthetic, halothane, thus increasing its intrinsic altered sensitivity to other modulators that might also be present [17, 21]. In the current study, we examined the inhibitory action of sphingosine and

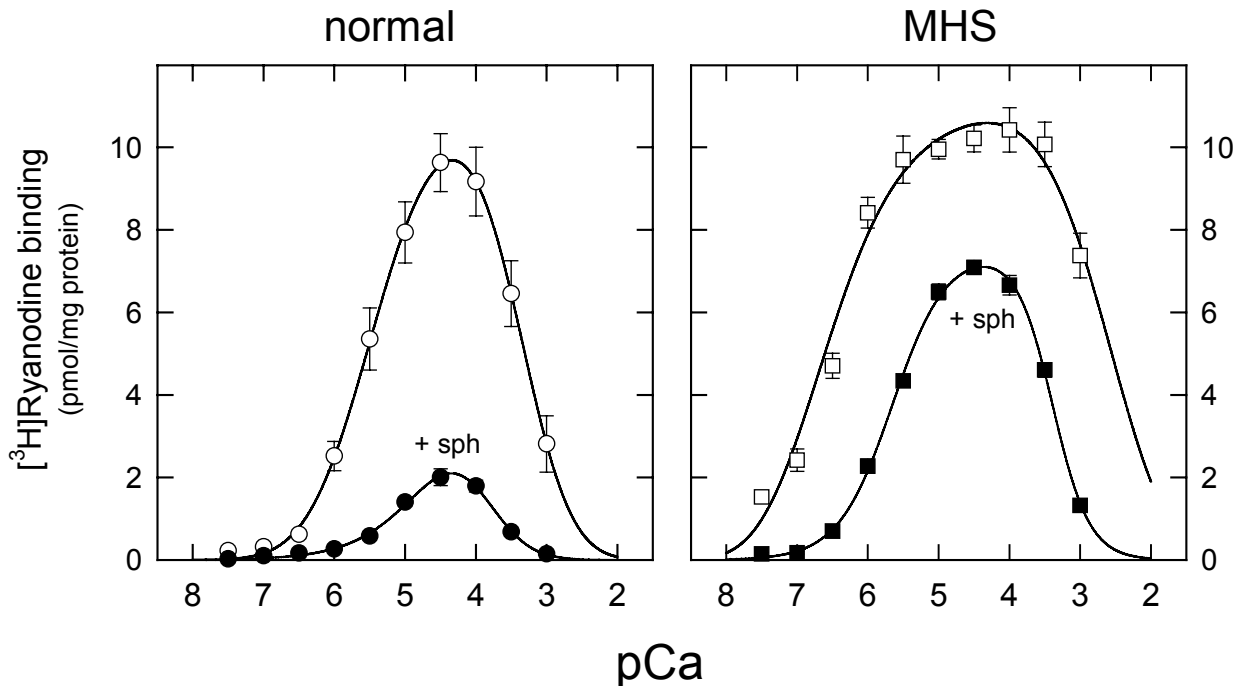


Figure 4. *pCa* dependence of sphingosine effect on the [^3H]ryanodine binding to normal and MHS sarcoplasmic reticulum TC membranes. [^3H]ryanodine binding was measured in a medium containing 50 μg of TC membranes, containing 20 nM [^3H]ryanodine, 0.1 M KCl, 20 mM Hepes, pH 7.0, containing 20 nM [^3H]ryanodine, 0.1 M KCl, 20 mM Hepes, pH 7.0, 300 μM Ca^{2+} , 10 mM ATP and in the absence (open symbols) or in the presence of 10 μM sphingosine (+ sph, closed symbols). *pCa* values were obtained by the addition of CaCl_2 alone or, below *pCa* 4, by buffering with different concentrations of EGTA, as calculated on the basis of the stability constants published by Fabiato [9]. Symbols represent the means $\pm$ SEM of four experiments performed in triplicate.

calmodulin using the [^3H]ryanodine binding assay with the recognized assumption that the binding of ryanodine to the receptor is high with the channel in the open state and low in the closed state [5, 15]. We demonstrated that these endogenous physiological antagonists of RyR that would otherwise inhibit the channel, are unable to do so effectively in the MHS model.

Sphingosine is a sphingomyelin metabolite known as a potent modulator of skeletal [27] and cardiac [7] RyR isoforms, and it has been demonstrated that skeletal muscle possess the enzymatic signalling machinery required to produce sphingosine [28]. For example, skeletal muscle T-tubule membranes are a rich source of the neutral sphingomyelinase [13]. Importantly, we previously demonstrated that sphingosine is endogenous to rat skeletal [28] and dog cardiac [7] muscles. Given that the terminal cisterns of the sarcoplasmic reticulum containing the RyR are in close proximity to the junctional T-tubule membranes, sphingosine is a good candidate for modulating the RyR in skeletal muscle and it has been proposed that sphingosine may play a physiological role in maintaining the channel closed in the resting state [29].

As previously shown in rabbit skeletal muscle membranes [27], sphingosine caused a dose-dependent inhibition of [^3H]ryanodine binding to normal pig TC membranes. Importantly, significantly higher concentrations of sphingosine were needed in MHS to produce equiva-

lent levels of inhibition. The inability of sphingosine to control the closing state of the mutated channel of MHS membranes was particularly evident when we examined the *pCa* dependence of [^3H]ryanodine binding. Consistent with previous results [19], we demonstrate that MHS-derived membranes exhibit a typical bell-shaped *pCa* for ryanodine binding (Figure 4). The exaggerated total binding capacity is suggestive that the MHS membranes are hypersensitive even to calcium requirement, even though the general character of the bell-shaped curve indicates the lower propensity to be closed at high or at low *pCa* values, respectively, as seen in the normal condition. Importantly, sphingosine was unable to inhibit the [^3H]ryanodine binding to MHS membranes to the extent that it does in normal membranes, at all *pCa* values tested. In addition, we report that normal pig muscles contain endogenous levels of sphingosine comparable to those of the rat [28]; Moreover, MHS muscle sphingosine levels are lower than that of normal muscle. Altered mechanisms for fatty acid turnover have been consistently reported in MHS swine [25]. Our results suggest that in addition to the lower ability of sphingosine to control the mutated ryanodine receptor plus the lower content of the lipid in MHS muscles most likely contribute in concert to worsen the phenomenon.

Calmodulin is a ubiquitous calcium binding protein known to modulate several cellular activities both indi-

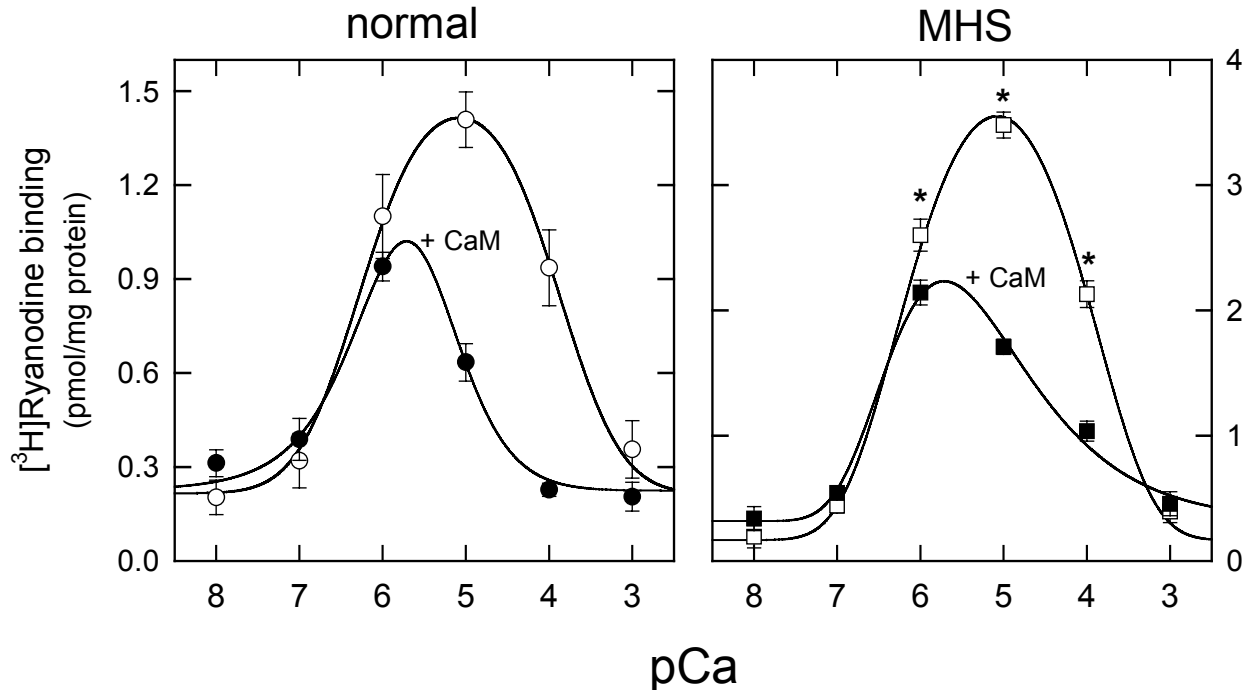


Figure 5. *pCa* dependence of calmodulin effect on the [3 H]ryanodine binding to normal and MHS sarcoplasmic reticulum TC membranes. [3 H]ryanodine binding was measured in a medium containing 50 μ g of TC membranes, 20 nM [3 H]ryanodine, 0.1 M KCl, 20 mM Hepes, pH 7.0, in the absence (○) or in the presence (●) of 1 μ M calmodulin (+ CaM). *pCa* values were obtained by the addition of CaCl_2 alone or, below *pCa* 4, by buffering with different concentrations of EGTA, as calculated on the basis of the stability constants published by Fabiato [9]. Each data point is the mean $\pm$ SEM of four separate experiments, performed in triplicate. Asterisks indicate data significantly different at least at $p < 0.05$ (Student's *t* test).

rectly, through its activation of specific protein kinases [8, 33], and/or directly, by its action on the calcium release channels [18, 32]. The direct action of calmodulin on calcium release channels has been validated by the identification of calmodulin binding sites in the SR ryanodine receptor sequence [35]. Moreover, it is suggested that the binding of calmodulin to RyR can either activate or inhibit the channel depending on the transition from a calcium free to a calcium bound state, in the resting and in the activated muscle, respectively [11, 34].

The present results demonstrate that calmodulin is unable to inhibit [3 H]ryanodine binding to MHS membranes to the extent that it does in membranes isolated from normal pig muscle. This finding suggests that the mutation Arg⁶¹⁵ to Cys⁶¹⁵ in the RyR protein [12] may influence one of the putative calmodulin binding sites. Recently, it has been reported that calmodulin, at low Ca^{2+} levels, activates [3 H]ryanodine binding to MHS vesicles to a greater extent than it does normal membranes, while no differences were noted in the inhibitory action of calmodulin at high Ca^{2+} levels [23]. In contrast with this finding, we reported a significant lower ability of calmodulin in inhibiting [3 H]ryanodine binding to MHS membranes. These different results could be due to the diverse [3 H]ryanodine binding protocols utilized. It has been recently reported that the affinity for calcium of calmodulin is reduced in the presence of halothane

[16]. Since the binding of calcium is essential to the activation by calmodulin [11, 34], this implies that, in addition to the observed reduced inhibitory effect of calmodulin on RyR of MHS muscles, the amount of activated calmodulin available to the channel may be decreased in the presence of halothane.

In conclusion, present results demonstrate that changes in sphingosine and calmodulin modulation of the ryanodine receptor may contribute to the altered behaviour of MHS skeletal muscle.

Acknowledgements

The contribution of Telethon Italy (grants n. 469) is gratefully acknowledged. The study was also supported by institutional funds from the Consiglio Nazionale delle Ricerche of Italy and by grants from "Cofinanziamento MURST 1999" and by a subcontract to NIH HL63903 to D. Danielli-Betto.

Address correspondence to:

Romeo Betto, C.N.R. Institute of Neuroscience, Unit for the Study of Neuromuscular Biology and Physiopathology, c/o Department of Biomedical Science, University of Padova, Viale Giuseppe Colombo 3, 35121 Padova, Italy, tel. +39 49 8276027, fax +39 49 8276040, Email betto@bio.unipd.it.

References

- [1] Berchtold MW, Brinkmeier H, Muntener M: Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease. *Physiol Rev* 2000; 80: 1215-1265.
- [2] Betto R, Teresi A, Fachechi-Cassano G, Salviati G, Sabbadini RA: Sphingosine inhibits calcium release from sarcoplasmic reticulum membranes. *Adv Exp Med Biol* 1992; 311: 403-404.
- [3] Betto R, Teresi A, Turcato F, Salviati G, Sabbadini RA, Krown K, Glembotski CC, Kindman LA, Dettbarn C, Pereon Y, Yasui K, Palade PT: Sphingosylphosphocholine modulates the ryanodine receptor/calcium-release channel of cardiac sarcoplasmic reticulum membranes. *Biochem J* 1997; 322: 327-333.
- [4] Chin D, Means AR: Calmodulin: a prototypical calcium sensor. *Trends Cell Biol* 2000; 10: 322-328.
- [5] Chu A, Diaz-Munoz M, Hawkes MJ, Brush K, Hamilton SL: Ryanodine as a probe for the functional state of the skeletal muscle sarcoplasmic reticulum calcium release channel. *Mol Pharmacol* 1990; 37: 735-741.
- [6] Damiani E, Margreth A: Subcellular fractionation to junctional sarcoplasmic reticulum and biochemical characterization of 170 kDa Ca^{2+} - and low-density-lipoprotein-binding protein in rabbit skeletal muscle. *Biochem J* 1991; 277: 825-832.
- [7] Dettbarn CA, Betto R, Salviati G, Palade PT, Jenkins GM, Sabbadini RA: Modulation of cardiac sarcoplasmic reticulum ryanodine receptor by sphingosine. *J Mol Cell Cardiol* 1994; 26: 229-242.
- [8] Dulhunty AF, Laver D, Curtis SM, Pace S, Haarmann C, Gallant EM: Characteristics of irreversible ATP activation suggest that native skeletal ryanodine receptors can be phosphorylated via an endogenous CaMKII. *Biophys J* 2001; 81: 3240-3252.
- [9] Fabiato A: Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods Enzymol* 1988; 157: 378-417.
- [10] Fill M, Coronado R, Mickelson JR, Vilven J, Ma J, Jacobson BA, Louis CF: Abnormal ryanodine receptor channels in malignant hyperthermia. *Biophys J* 1990; 50: 471-475.
- [11] Fuentes O, Valdivia G, Vaughan D, Coronado R, Valdivia HH: Calcium-dependent block of ryanodine receptor channel of swine skeletal muscle by direct binding of calmodulin. *Cell Calcium* 1994; 15: 305-316.
- [12] Fujii J, Otsu K, Zorzato F, de Leon S, Khanna VK, Weiler JE, O'Brien PJ, MacLennan DH: Identification of a mutation in porcine ryanodine receptor associated with malignant hyperthermia. *Science* 1991; 253: 448-451.
- [13] Ghosh N, Sabbadini RA, Chatterjee S: Identification, partial purification, and localization of a neutral sphingomyelinase in rabbit skeletal muscle. *Mol Cellular Biochem* 1998; 189: 161-168.
- [14] Harrison GG: The prophylaxis of malignant hyperthermia by oral dantrolene sodium in swine. *Brit J Anaesth* 1975; 47: 62-65.
- [15] Hawkes MJ, Nelson TE, Hamilton SL: [^3H]-Ryanodine as a probe of changes in the functional state of the Ca^{2+} -release channel in malignant hyperthermia. *J Biol Chem* 1992; 267: 6702-6709.
- [16] Levin A, Blanck TJJ: Halothane and isoflurane alter the Ca^{2+} binding properties of calmodulin. *Anesthesiology* 1995; 83: 120-126.
- [17] MacLennan DH: Ca^{2+} signaling and muscle diseases. *Eur J Biochem* 2000; 267: 5291-5297.
- [18] Meissner G: Ryanodine receptor/calcium release channels and their regulation by endogenous effectors. *Ann Rev Physiol* 1994; 56: 485-508.
- [19] Mickelson JR, Gallant EM, Litterer LA, Johnson KM, Rempel WE, Louis CF: Abnormal sarcoplasmic reticulum ryanodine receptor in malignant hyperthermia. *J Biol Chem* 1988; 263: 9310-9315.
- [20] Mickelson JR, Litterer LA, Jacobson BA, Louis CF: Stimulation and inhibition of [^3H]ryanodine binding to sarcoplasmic reticulum from malignant hyperthermia susceptible pigs. *Arch Biochem Biophys* 1990; 278: 251-257.
- [21] Mickelson JR, Louis CF: Excitation-contraction coupling, Ca^{2+} release channel, and cell Ca^{2+} regulation defects. *Physiol Rev* 1996; 76: 537-592.
- [22] Needleman DH, Aghdasi B, Seryshev AB, Schroepfer GJ, Hamilton SL: Modulation of skeletal muscle Ca^{2+} -release channel activity by sphingosine. *Am J Physiol Cell Physiol* 1997; 272: C1465-1474.
- [23] O'Driscoll S, McCarthy TV, Eichinger HM, Erhardt W, Lehmann-Horn F, Herrmann-Frank A: Calmodulin sensitivity of the sarcoplasmic reticulum ryanodine receptor from normal and malignant hyperthermia-susceptible muscle. *Biochem J* 1996; 319: 421-426.
- [24] Otsu K, Phillis MS, Khanna VK, de Leon S, MacLennan DH: Refinement of diagnostic assays for a probable causal mutation for porcine and human malignant hyperthermia. *Genomics* 1992; 13: 835-837.
- [25] Otten W, Iazzo PA, Eichinger HM: Effects of a high n-3 fatty acid diet on membrane lipid composition of heart and skeletal muscle in normal swine and in swine with the genetic mutation for malignant hyperthermia. *J Lipid Res* 1997; 38: 2023-2034.
- [26] Ryan JF, Lopez JR, Sanchez VB, Sreter FA, Allen PD: Myoplasmic calcium changes precede metabolic and clinical signs of porcine malignant hyperthermia. *Anesth Analg* 1994; 79: 1007-1011.
- [27] Sabbadini RA, Betto R, Teresi A, Fachechi-Cassano G, Salviati G: The effects of sphingosine on sarcoplasmic reticulum membrane calcium release. *J Biol Chem* 1992; 267: 15475-15484.

Sphingosine, calmodulin and malignant hyperthermia

- [28]Sabbadini RA, Mc Nutt W, Jenkins G, Betto R, Salviati G: Sphingosine is endogenous to cardiac and skeletal muscle. *Biochim Biophys Res Comm* 1993; 193: 752-758.
- [29]Sabbadini RA, Danieli-Betto D, Betto R: The role of sphingolipids in the control of skeletal muscle function: a review. *Ital J Neurol Sci* 1999; 20: 423-430.
- [30]Saito A, Seiler S, Chu A, Fleischer S: Preparation and morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. *J Cell Biol* 1984; 99: 875-885.
- [31]Sharma C, Smith T, Li S, Schroepfer GJ Jr, Needleman DH: Inhibition of Ca^{2+} release channel (ryanodine receptor) activity by sphingolipid bases: mechanism of action. *Chem Phys Lipids* 2000; 104: 1-11.
- [32]Smith JS, Rousseau E, Meissner G: Calmodulin modulation of single sarcoplasmic reticulum Ca^{2+} -release channels from cardiac and skeletal muscle. *Circ Res* 1989; 64: 352-359.
- [33]Suko J, Maurer-Fogy I, Plank B, Bertel O, Wiskovsky W, Hohenegger M, Hellmann G: Phosphorylation of serine 2843 in ryanodine receptor-calcium release channel of skeletal muscle by cAMP-, cGMP- and CaM-dependent protein kinase. *Biochim Biophys Acta* 1993; 1175: 193-206.
- [34]Tripathy A, Xu L, Mann G, Meissner G: Calmodulin activation and inhibition of skeletal muscle Ca^{2+} release channel (ryanodine receptor). *Biophys J* 1995; 69: 106-119.
- [35]Zorzato F, Fujii J, Otsu K, Phillips M, Green NM, Lai FA, Meissner G, MacLennan DH: Molecular cloning of cDNA encoding human and rabbit forms of the calcium release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. *J Biol Chem* 1990; 265: 2244-2256.