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An electron paramagnetic resonance study of skeletal muscle membrane fluidity in malignant hyperthermia

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Skeletal muscle sarcolemma (SL), transverse tubule (TT) and heavy sarcoplasmic reticulum (HSR) membranes were isolated from malignant hyperthermia susceptible (MHS) and normal pigs, and the rotational dynamics of lipid hydrocarbon chain motion was examined by electron paramagnetic resonance (EPR) spectroscopy. The stearic acid spin probe 16-SASL was incorporated into MHS and normal membranes and both the order parameter (S) and effective correlation time (τ_r) of probe motion were calculated from spectra recorded over the temperature range of 2 to 40 °C. At any given temperature, TT membranes exhibited significantly greater values for both the S and τ_r of probe motion than did SL, which exhibited significantly greater values than did HSR membranes. The order of decreasing S and τ_r values for 16-SASL mobility correlated with the decreasing cholesterol content of these membranes (TT > SL > HSR), however there was no difference in the S or τ_r values for a given membrane fraction isolated from both MHS and normal muscle. Arrhenius plots of 16-SASL mobility in SL, TT and HSR were linear from 2 to 40 °C, indicating no abrupt thermotropic change in the lipid hydrocarbon phase of any of the membrane types studied. Apparent activation energies (E_a), calculated from the Arrhenius plots, were similar for MHS and normal membranes derived from a given cellular location. However, the E_a of probe motion for TT membranes (2.3 ± 0.1 and 2.4 ± 0.1 kcal/mol/degree for MHS and normal, respectively) was significantly less than for SL (3.4 ± 0.4 and 2.9 ± 0.1 kcal/mol/degree for MHS and normal, respectively) which, in turn, was significantly less than the E_a for HSR (3.7 ± 0.1 and 3.7 ± 0.1 kcal/mol/degree for MHS and normal, respectively). Since 16-SASL motion was similar in MHS and normal membranes, we conclude that there is no evidence for a generalized membrane defect affecting lipid mobility in these MHS muscle membranes.

Introduction

Malignant hyperthermia (MH) is an inherited metabolic disorder, generally believed to result from a defect in skeletal muscle calcium regulation [1]. Although the primary defect responsible for MH has not yet been defined, an abnormality in calcium release by the sarcoplasmic reticulum (SR) is thought to play a major role in this syndrome [2–8]. This conclusion is supported by reports of abnormal calcium release [4–7] and ryanodine binding [8] activities of heavy SR (HSR) isolated from MH susceptible (MHS) pig muscle. A number of studies, however, have indicated that there may be a generalized membrane defect in all tissues of MHS individuals. We have recently reported altered [^{3}H]nitrendipine and (−)[^{3}H]desmethoxyverapamil binding to the dihydropyridine receptor of isolated MHS transverse tubules (TT) [9], as well as diminished calcium-accumulating activity in both isolated MHS TT [10] and sarcolemma (SL) [11]. Furthermore, red blood cells (RBC) from MHS pigs have been shown to have increased osmotic fragility relative to normal pig RBC [12], while both MHS RBC ghosts [13] and isolated MHS SR [14] have been reported to exhibit lower than normal lipid mobility. A possible explanation for these

Abbreviations: MH, malignant hyperthermia; MHS, malignant hyperthermia susceptible; SR, sarcoplasmic reticulum; HSR, heavy sarcoplasmic reticulum; SL, sarcolemma; TT, transverse tubule; RBC, red blood cell(s); SASL, stearic acid spin label(s); S, order parameter; τ_r, effective correlation time; E_a, apparent activation energy.

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various alterations is a defect in a lipid component or the lipid environment of the MHS membranes.

To investigate the possibility of altered membrane function in MH, the rotational dynamics of lipid hydrocarbon chain motion in SL, TT and HSR membranes isolated from MHS and normal pig muscle was examined by electron paramagnetic resonance (EPR) spectroscopy. In contrast to previous findings utilizing RBC ghosts [13] and isolated SR [14], no difference in membrane fluidity was detected over a wide range of temperatures between MHS and normal SL, TT or HSR membranes. Thus, we find no evidence for altered MHS muscle membrane mobility and conclude that a generalized membrane defect does not appear to be the cause of the defects exhibited by MHS muscle.

Materials and Methods

Membrane isolation and purification

Skeletal muscle membranes were isolated from the longissimus dorsi muscle of MHS and normal pigs, which had been tested for susceptibility to MH by a halothane-challenge test [7,11] at least 3 weeks prior to use. During deep anesthesia, the longissimus dorsi muscle was removed, and immediately placed on ice. MHS and normal SL membranes were isolated by a LiBr-extraction method adapted specifically for porcine muscle [11]. TT membranes were prepared by a modification of the method of Rosemblatt et al. [15]. Briefly, crude microsomes were prepared according to Fernandez et al. [16], loaded on tubes containing 34 ml of 25% (w/v) sucrose and centrifuged at $85\,000 \times g$ for 15 h in a swinging bucket rotor. The light membrane fraction collected from the top of the 25% sucrose layer was used for this study and has been extensively characterized [9]. HSR membranes were isolated from MHS and normal pig muscle by the procedure of Meissner [17] as described by Mickelson et al. [7].

Spin-labeling

The fatty acid spin labels, 4′,4′-dimethyl-oxazolidine-*N*-oxyl derivatives of stearic acid, which were labeled at carbons 5 or 16, designated 5- and 16-SASL, respectively, were obtained from Aldrich Chemical Co. Spin labels were diluted from a stock solution (10 mM) in dimethyl formamide to 1 mM with absolute ethanol [18]. The spin label was added to membranes at a ratio of 1 mol spin label per 200 mol of phospholipid with the final ethanol concentration less than 1%.

EPR spectroscopy

Conventional EPR spectra were obtained with a Bruker ER200 spectrometer using 100 kHz field modulation, with a peak-to-peak amplitude of 2 G and a microwave field amplitude of 0.14 G. All spectra were obtained from samples containing either 10 mg protein per ml (SL and TT) or 40 mg protein per ml (HSR) in 0.3 M sucrose, 20 mM Tris-maleate buffer, pH 7.0 [19]. Samples were placed in a capillary tube made from the gas permeable plastic TPX [18] and degassed with N_2 for 15 min prior to scanning. Sample temperature was controlled with a Bruker VT-100 variable temperature controller and monitored during data acquisition with a Bailey Digital thermometer (model BAT-12), using a thermocouple probe (IT-21) positioned outside the sample cell in the center of the cavity.

Spectral analysis

The parameters used to characterize EPR spectra were chosen to provide maximum precision over the entire temperature range to be studied [18,20]. EPR spectra obtained with 16-SASL were evaluated by two methods. An empirical motion parameter, τ_c (effective correlation time) was calculated from the formula [21]:

$$\tau_c = (6.5 \times 10^{-10}) W_0 \left[(h_0/h_{-1})^{1/2} - 1 \right],$$

where W_0 is the peak-to-peak line width of the midfield line, and h_0 and h_{-1} are the peak-to-peak line heights in the midfield and high-field regions of the spectrum, respectively. In addition, the effective order parameter, S was calculated by the relationship [22]:

$$S = \frac{T_0 - T'_\perp}{T_0 - T_\perp}$$

T_0 (14.0 G), or one-half of the separation between the low-field and high-field zero-crossing points, was the mean value measured from the spectra recorded at 40 °C. The value used for $T_\perp$ was 6.0 Gauss [20].

EPR spectra obtained with 5-SASL were evaluated using the standard formula relating S to both inner and outer extrema [22,23]:

$$S = \frac{T'_\parallel - (T'_\perp + C)}{T'_\parallel + (2T'_\perp + C)} \times 1.66$$

where $C = 1.4 - 0.053\,(T'_\parallel - T'_\perp)$. $2T'_\parallel$ and $2T'_\perp$ are the measured inner and outer extrema resolved in the EPR spectrum.

Arrhenius analysis

Lines in the Arrhenius plots for a given membrane type were fit by linear regression least-squares analyses. Apparent activation energies (E_a) were calculated from the slopes of the plots obtained from each membrane preparation.

Results

The 16-SASL spin probe has been successfully used to study the structural transitions in a variety of mem-

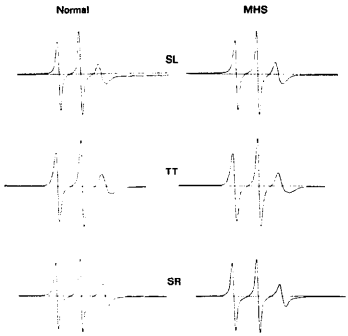

Fig. 1. EPR spectra of 16-SASL incorporated into normal and MHS muscle membranes. The membranes were suspended in 0.3 M sucrose, 20 mM Tris-maleate buffer (pH 7.0) and spectra were obtained at 37°C. All spectra were recorded with a 100 G scan range.

branes [24] including that of the TT [19] and SR [18,19,25,26]. To examine the motion of this probe in MHS and normal pig muscle SL, TT, and HSR, EPR spectra were recorded at every 2-3°C over the temperature range of 2–40°C. Representative spectra for this probe incorporated into normal and MHS SL, TT, and HSR (Fig. 1) appear as narrow three-line spectra due to motional averaging, indicating little anisotropy (little orientational order). When S was calculated and plotted as a function of temperature (Fig. 2), it was observed that for any given temperature, S values obtained from

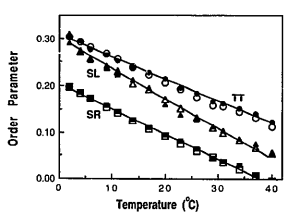

Fig. 2. Order parameter (S) as a function of temperature for 16-SASL incorporated into MHS (solid symbols) and normal (open symbols) TT (●, ○), SL (▲, △) and SR (■, □). S was calculated as described in Materials and Methods and the symbols represent the mean for three or more different MHS and normal preparations. The lines drawn through the data were obtained from linear regression least-squares analysis, with correlation coefficients of −0.999, −0.997 and −0.999 for TT ($n = 3$), SL ($n = 5$) and SR ($n = 5$), respectively.

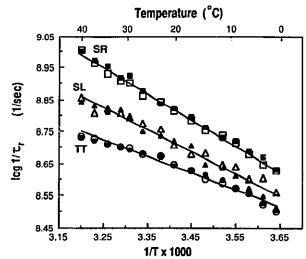

Fig. 3. Arrhenius plot of lipid hydrocarbon chain mobility in MHS (solid symbols) and normal (open symbols) SR (■, □), SL (▲, △) and TT (●, ○). Rates of motion were plotted as $1/\tau_r$ for 16-SASL. When data were fit to a single line, Mean correlation coefficients for MHS and normal SR, SL and TT were −0.986, −0.993, −0.991, −0.991, −0.985 and −0.988, respectively. If MHS and normal TT lipid were fit to two lines, the best fits were for a break point at 23°C in each case with mean correlation coefficients of −0.971 and −0.985, respectively, indicating that the single-line fit was superior in each case.

TT membranes were significantly greater than those of SL ($P < 0.005$ for all temperatures). In addition, S values of SL membranes were significantly greater than those of SR membranes ($P < 0.005$ for all temperatures). It is also apparent from Fig. 2 that for each membrane type studied (ie., SL, TT and HSR), normal and MHS membranes were similar with respect to the calculated S values over the temperature range studied.

The calculation of S assumes that the only variable is the angular amplitude of probe motion with the rotational correlation time (τ_r) short enough to be in the rapid-motion limit ($\leq 10^{-10}$ s). Since the S values of 16-SASL are low, indicating that the spectral anisotropy is almost completely averaged, the calculation of S in the case of 16-SASL is not rigorously valid. Thus, such spectra are more commonly characterized by a correlation time (τ_r), with the assumption that the probe motion is isotropic. Arrhenius plots (Fig. 3) for τ_r of 16-SASL motion in MHS or normal HSR, SL or TT membranes demonstrated no temperature breaks. Over the temperature range studied (2–40°C), HSR membranes exhibited significantly lower τ_r (note that rates of motion were plotted as $1/\tau_r$ in Fig. 3) than SL and TT ($P < 0.005$). Although SL and TT τ_r values were not significantly different from 2 to 14°C ($P > 0.100$), from 17 to 40°C SL membranes exhibited significantly lower τ_r than TT membranes ($P < 0.025$, Fig. 3).

From the slopes of the Arrhenius plots (Fig. 3) E_a values were calculated. The mean E_a of MHS and normal TT (2.3 ± 0.1 and 2.4 ± 0.1 kcal/mol per degree, respectively) were significantly lower than those of MHS and normal SL (3.4 ± 0.4 and 2.9 ± 0.1 kcal/mol

per degree, respectively) which were significantly less than the mean E_a of MHS and normal HSR membranes (3.7 ± 0.1 and 3.7 ± 0.1 kcal/mol per degree, respectively). However, in comparing MHS and normal membranes from the same cellular location, there was no significant difference in E_a (compare MHS and normal SL, TT and HSR).

To determine whether a defect in lipid hydrocarbon chain mobility is localized nearer the hydrocarbon core-water interface of MHS HSR membranes, 5-SASL was incorporated into 5 MHS and 5 normal HSR preparations and the EPR spectra obtained at 25°C. The mean S value for 5-SASL motion in MHS HSR (0.609 ± 0.002) was not significantly different from the mean S value obtained from 5-SASL in normal SR (0.608 ± 0.002).

Discussion

In the present study, EPR spectroscopy was utilized to obtain measurements of lipid chain mobility in SL, TT and HSR membranes isolated from MHS and normal pig skeletal muscle. Over a wide range of temperatures, no difference in S, τ_r or E_a were detected between MHS and normal membranes regardless of cellular location (Figs. 2, 3). The biochemical characterization of these preparations has been previously reported [7,9–11]. MHS and normal SL membranes exhibited similar lipid contents, protein distribution, enzymatic activities and vesicle size and sidedness [11]. MHS and normal TT were similar in protein distribution, lipid contents, vesicle sidedness, Ca^{2+}-ATPase activity and saxitoxin binding activity [9]. MHS and normal HSR membranes were also similar with regard to many of these properties [7]. Thus, our finding that these MHS membrane preparations exhibit no difference in lipid mobility when compared with their normal counterparts further indicates that the preparations likely suffered no differential damage during the isolation procedure.

It has been previously reported that SR membranes from MHS muscle exhibit greater than normal S values for both 5-SASL and 12-SASL motion at 25°C [14]. In the present study, the mean S values calculated from 5-SASL spectra obtained at 25°C for MHS and normal SR were not significantly different. However, the previous study reported a difference in S between normal and MHS SR of only 2% [14]. Furthermore, the SR preparative method used did not include fractionation on sucrose density gradients, and the cholesterol contents were not determined for the preparations used. It is thus not possible to distinguish whether the increased S reported [14] was the result of increased surface membrane contamination of MHS SR relative to normal SR, or due to a defect related to MH.

It has been previously reported that, when compared to normal, MHS pig RBC ghosts exhibit a decreased lipid mobility [13]. In addition to diminished lipid fluidity, the MHS pig RBC ghosts exhibited a significantly higher cholesterol content than did normal RBC ghosts [13]. Why normal and MHS RBC ghosts differ in these two parameters, while the normal and MHS muscle membranes do not, is not immediately clear. That membrane fluidity and cholesterol content do not appear to be altered in MHS muscle, where the primary defect of MH has been localized [2–8], suggests, however, that an altered membrane fluidity is not the primary defect in MH.

Our results cannot eliminate the possibility that a minor lipid component which could modulate the activities of proteins involved in excitation-contraction coupling, i.e., the dihydropyridine and ryanodine receptors, as well as surface membrane calcium pumps, could be altered in MHS muscle membranes. Previous studies have reported altered lipid profiles in microsomes isolated from MHS muscle [27,28]. However, more recently it has been demonstrated that MHS and normal longissimus dorsi muscle were not different with respect to lipid profiles, including phospholipid and fatty acid composition [29]. Furthermore, these authors suggest that the previously reported differences in lipid profile between MHS and normal muscle were probably artifacts of the homogenization and/or extraction protocols used [29]. We conclude that our own and previous data indicate that the lipid environment(s) of MHS muscle membrane systems are probably not perturbed.

Acknowledgements

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