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Erythrocyte membrane fluidity in malignant hyperthermia

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Malignant hyperthermia (MH) is a rare but serious complication of general anesthesia that potentially carries a high mortality and morbidity. It is associated with excessive release of calcium into skeletal muscle following exposure to certain drugs, including the volatile general anesthetics. Since these are recognized membrane fluidizing agents it has been speculated that this condition might represent a generalized defect in membrane physical properties either at rest or inducible by fluidizing agents. If this hypothesis were found to be correct, malignant hyperthermia might conveniently be detected by examining membrane physical properties of easily accessible cells rather than the cumbersome method of muscle biopsy currently employed. To test this hypothesis we identified patients proven to be susceptible to MH by muscle biopsy and a cohort of patients not susceptible to MH as defined by negative muscle biopsy testing. Erythrocytes were isolated from both groups and membrane physical properties examined using conventional, widely available, steady-state fluorescence polarization techniques. Erythrocyte membranes were evaluated with multiple probes both in the basal condition and following fluidization with either increasing temperature or two concentrations of a fluidizing alcohol. We report, contrary to previous publications, that no discernable differences were detectable between MH-positive or negative patients. Thus, we find no evidence for a generalized membrane defect in MH and conclude that the determination of erythrocyte membrane physical properties, by these techniques, are of no use in the preoperative screening for this disorder.

Introduction

Malignant hyperthermia (MH) is a rare, inherited condition, in which sufferers react to volatile anaesthetic agents and depolarizing muscle relaxants by developing muscle rigidity, high fever (greater than 41° C), and a metabolic acidosis [1]. Unless recognized and promptly treated death may rapidly ensue making the prevention of this condition a priority amongst anesthetists. In general, MH susceptible individuals are recognized by investigating patients who have either survived an episode or are the relatives of sufferers. Currently the only reliable way to preoperatively diagnose MH is by measuring the in vitro contracture of muscle obtained during an open muscle biopsy [2].

While the human disease is fortunately rare, great strides have been made in elucidating the pathogenesis of MH as a similar illness afflicts a specific strain of pig. With the help of this model and investigations in man several important points have emerged. Excessive calcium release from skeletal muscle sarcoplasmic reticulum appears to be important in the pathogenesis of MH. Furthermore, since exposure to membrane fluidizing agents, such as halogenated anaesthetic agents, plays an important role in triggering MH it has been suggested that changes in membrane fluidity might be involved in the development of MH. If the postulated membrane defect in muscle were reflected elsewhere in the body there exists the possibility of diagnosing MH preoperatively by examining membrane structure in organs more amenable to investigation. In the porcine model it has recently been demonstrated that the sarcoplasmic reticulum from afflicted animals is more fluid than seen in controls [3]. Furthermore, investigation of erythrocytes from pigs susceptible to MH has demonstrated that the plasma membrane is more fragile than seen in controls [4]. Thus,there is a precedent suggesting that not only does a membrane defect exist but it may be generalized. Finally, recent work has suggested that patients susceptible to MH may have an abnormal increase in erthrocyte plasma

Abbreviation: MH, malignant hyperthermia.

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membrane fluidity following exposure to halothane [5]. Therefore, we have examined a group of patients with proven MH susceptibility and evaluated red blood cell membrane physical properties with and without the addition of a membrane fluidizer attempting to confirm these observations. Since fluorescent methodology is widely available now we used fluorescent rather than spin labelled probes.

Materials and Methods

Patient identification

Patients were categorized as MH-positive or MH-negative according to the results of in vitro testing of isolated muscle biopsy [2]. Muscle was obtained by open biopsy from the vastus lateralis muscle under appropriate regional anaesthesia. A strip 3 mm in diameter was suspended in Krebs solution, and bubbled with 5% CO_2 in oxygen. Either increasing amounts of halothane (up to 2%) were added to the gas passing through the muscle or caffeine was added directly in concentrations ranging from 0.25–8 mM. Patients were defined as MH-positive if the muscle contracted (> 1 g tension) in response to halothane or less than 4 mM caffeine. Negative muscle biopsies developed no contracture following stimulation. All tests were performed in the laboratory of Dr. K. Brownell at the University of Calgary, Alberta from the years 1980 to 1990 using the North American protocol. Over this period of time there has been an evolution in the methods used to make the diagnosis of MH-susceptibility. However, all patients labelled as MH-positive had clear muscle contraction to whichever agonist was in use in the laboratory at the time of their testing. Since this diagnosis requires an open muscle biopsy we did not feel it ethical to repeat this procedure in 1990 solely for the purpose of this investigation.

Erythrocyte preparation

Following an overnight fast, blood samples were collected from MH susceptible volunteers into vacutainer tubes containing acid-citrate-dextrose as a preservative (Becton-Dickinson, Rutherford, NJ), and stored at 4 °C for 1–3 h. The blood was washed with phosphate-buffered saline (mM), NaCl (145); KCl (5); Na_2PO_4 (5); pH 7.4 and filtered through cotton wool to remove platelets and leucocytes followed by gentle centrifugation for 10 min [6]. The sample was washed and centrifuged twice more and any remaining buffy coat aspirated with the supernatant. Erythrocytes were then suspended to a hematocrit of 1% and fluorescent probe added. All probes were purchased from Molecular Probes (Junction City, OR) and used as supplied. All other reagents were purchased from Sigma and were of the highest purity available. The fluorescent probes used were diphenylhexatriene (DPH) and a series of *n*-(9-anthroyloxy) stearic or palmitic acids. For these experiments *n* took the values of 3, 6, 9, 12 or 16. All probes were stored, protected from light at −20 °C, in ethanol and were added to the erythrocyte preparation to give a final probe concentration of $12.5 \cdot 10^{-6}$ M (AS and AP probes) or $5 \cdot 10^{-6}$ M (DPH), with a final ethanol concentration of 0.5%, according to the method of Schacter et al. [7]. After incubation for 10–15 min at 37 °C, the erythrocytes were gently pelleted and the supernatant discarded. The pellet was washed twice more with approx. 25 vol. of PBS. Labelled erythrocytes were resuspended to a hematocrit of 0.025% in PBS. Steady-state fluorescence polarization was measured at an excitation wavelength of 385 nm (AS and AP probes) or 360 nm (DPH) in an SPF-500C fluorometer specifically adapted for polarization work (SLM Aminco). Emission maxima were observed at 450 nm and a bandpass of 10 nm was used for both the excitation and emission monochromaters.

Under these conditions the samples were slightly turbid, however, no increase in anisotropy parameter was observed with a 2–5-fold dilution suggesting that light scattered from cells was not contributing to the loss of polarization observed. Thus, anisotropy values obtained at this hematocrit are reported. Steady-state anisotropy values for DPH were used to estimate the

TABLE I

Steady-state anisotropy values for erythrocytes at 25 °C

Probe	MH negative (n = 10)			MH-positive (n = 7)		
	steady-state anisotropy (r_s)	total fluorescence	order parameter S_{DPH}	steady-state anisotropy (r_s)	total fluorescence	order parameter S_{DPH}
3-AS	0.111 ± 0.001	6.80 ± 0.13	–	0.118 ± 0.002	5.37 ± 0.27	–
6-AS	0.117 ± 0.001	8.43 ± 0.24	–	0.115 ± 0.001	6.93 ± 0.22	–
9-AS	0.107 ± 0.001	9.02 ± 0.32	–	0.105 ± 0.001	7.56 ± 0.25*	–
12-AS	0.086 ± 0.001	8.11 ± 0.30	–	0.086 ± 0.001	7.12 ± 0.17*	–
16-AP	0.073 ± 0.001	3.73 ± 0.92	–	0.077 ± 0.002	3.37 ± 0.12	–
DPH	0.246 ± 0.001	4.46 ± 0.14	0.794 ± 0.002	0.239 ± 0.003	4.23 ± 0.19	0.777 ± 0.007

* $P < 0.05$ vs. cells from MH negative individuals.

membrane order parameter by a previously observed empiric relationship [8]. In all experiments total fluorescence was determined in order to evaluate the possibility that fluorescent lifetime was altered. This parameter cannot be directly measured with steady-state methodology, however, a constant total fluorescence for a constant amount of probe and membrane is evidence that this parameter did not change since total fluorescence is directly proportional to fluorescent lifetime.

Results

Table I illustrates the observed steady-state anisotropy parameters for all probes in both groups of patients. MH-positive denotes susceptibility for MH, while MH-negative represents the absence of this condition as defined by the criteria outlined in the methods section. Data in Table I were obtained at 25 °C while Table II shows similar data obtained at 37 °C. The fluorescent intensity of the blanks were usually 8–12% of total sample fluorescence and invariably less than 18%.

The two classes of probes reflect different types of molecular motion within the bilayer. DPH is felt to localize near the core of the bilayer and because of both its rodlike shape and the orientation of excitation and emission dipoles this probe appears to wobble within the bilayer. Thus, it is sensitive to what is termed the static component of membrane fluidity. As illustrated in Table I no detectable difference existed between the physical environment sampled by this probe in erythrocytes obtained from either MH-positive or -negative individuals. Furthermore, with an increase in temperature to 37 °C, a fluidizing event (Table II), the environment examined by DPH became more fluid but still no difference was evident between the MH-positive or -negative groups.

The AS and AP series of membrane probes sense a distinctly different type of motion within the bilayer. These probes are based upon a long chain fatty acid and localize within a membrane with the carboxyl end at the membrane water interface and the terminal methyl group approaching the core of the bilayer. The fluorescent recorder anthroyloxy group may be attached to any of the base carbons of the fatty acid and is sensitive to rotational movements of the molecule. Thus, the 3-AS probe has the AS group located on carbon number 3 from the carboxyl end and senses rotational motion near the surface of the bilayer. The remaining probes examine similar motions but at progressively deeper levels within the membrane, 16-AP sensing events that take place very close to the core of the bilayer. It is apparent from Table I that rotational freedom is relatively restricted close to the surface of the erythrocyte membrane (3-AS, 6-AS and 9-AS) but at deeper levels a greater degree of motional freedom became evident (decreasing values of r_s). However, in a fashion similar to that observed for DPH, absolutely no differences were apparent between erythrocytes from MH-positive or -negative individuals.

Increasing temperature (Table II vs. Table I) produced some interesting results. Examination of the data obtained with DPH suggested that increasing temperature produced a dramatic increase in motional freedom for this probe in both sets of erythrocytes that was not accompanied by any alteration in the total fluorescence of the sample. This would suggest that the environment sampled by this probe was strongly influenced by ambient temperature, a finding not found with the probes assessing superficial regions of the bilayer. No significant increases in motional freedom of the AS (n = 3,6, 9 or 12) probes were observed when temperature was increased from 25 °C to 37 °C as assessed by the steady-state anisotropy parameter. However, a significant reduction in total fluorescence was observed in many of the samples suggesting that the fluorescent lifetime of these probes may have decreased, thus obscuring a reduction in the steady-state anisotropy. Despite this the 15–20% reduction in r_s

TABLE II

Steady-state anisotropy values for erythrocytes at 37 °C

Probe	MH negative (n = 10)			MH positive (n = 7)		
	steady-state anisotropy (r_s)	total fluorescence	order parameter S_{DPH}	steady-state anisotropy (r_s)	total fluorescence	order parameter S_{DPH}
3-AS	0.107 ± 0.002	5.20 ± 0.26 **	–	0.099 ± 0.003 **	4.04 ± 0.36 **	–
6-AS	0.111 ± 0.004	6.10 ± 0.26 **	–	0.105 ± 0.002	5.32 ± 0.42 **	–
9-AS	0.100 ± 0.001	6.48 ± 0.28 **	–	0.094 ± 0.002	5.73 ± 0.39 **	–
12-AS	0.081 ± 0.003	6.24 ± 0.29 **	–	0.086 ± 0.005	5.14 ± 0.37 **	
16-AP	0.061 ± 0.001 **	3.33 ± 0.11	–	0.061 ± 0.003 **	2.98 ± 0.21	–
DPH	0.201 ± 0.002 **	3.92 ± 0.12	0.681 ± 0.005 **	0.204 ± 0.004 **	3.65 ± 0.16	0.689 ± 0.010 **

** $P < 0.05$ vs. data obtained at 25 °C (Table I).

TABLE III

Fluidized erythrocyte membrane physical properties at 25 °C

Control cells were suspended in either PBS or 50 mM methanol and aliquots were fluidized with either 25 or 50 mM benzyl alcohol. Data obtained with three membrane probes (3-AS, 16-AP and DPH) are reported. Parameters include both the steady-state anisotropy parameter and the total fluorescence (in arbitrary fluorescence units) for a constant amount of probe and erythrocytes. Data are reported as the mean ± S.E.

Additive (mM)	Probe	MH negative ($n = 10$)		MH positive ($n = 7$)	
		steady-state anisotropy (r_s)	total fluorescence	steady-state anisotropy (r_s)	total fluorescence
PBS	3-AS	0.119 ± 0.002	6.56 ± 0.24	0.111 ± 0.001	7.88 ± 0.28
	16-AP	0.075 ± 0.001	4.51 ± 0.07	0.079 ± 0.001	4.52 ± 0.08
	DPH	0.242 ± 0.001	5.34 ± 0.16	0.239 ± 0.003	5.47 ± 0.11
Methanol (50)	3-AS	0.112 ± 0.001	8.45 ± 0.11	0.113 ± 0.001	8.20 ± 0.03
	16-AP	0.072 ± 0.001	5.20 ± 0.05	0.070 ± 0.001	5.38 ± 0.14
	DPH	0.244 ± 0.001	6.47 ± 0.17	0.246 ± 0.003	6.82 ± 0.09
Benzyl (25)	3-AS	0.113 ± 0.001	7.29 ± 0.22	0.111 ± 0.001	7.75 ± 0.19
	16-AP	0.066 ± 0.002 *	5.18 ± 0.06	0.072 ± 0.001 *	5.11 ± 0.06
	DPH	0.233 ± 0.002 *	6.32 ± 0.12	0.235 ± 0.002	6.43 ± 0.15
Benzyl (50)	3-AS	0.114 ± 0.003	7.54 ± 0.36	0.111 ± 0.001	8.47 ± 0.24
	16-AP	0.058 ± 0.001 *	6.77 ± 0.16	0.060 ± 0.001 *	6.88 ± 0.36
	DPH	0.204 ± 0.003 *	7.82 ± 0.22	0.200 ± 0.002 *	6.88 ± 0.17

* $P < 0.05$ vs. membrane suspended in PBS.

seen for DPH is significantly greater than that observed for 3-AS (4%) in control erythrocytes. Finally, the anisotropy parameter for 16-AP, which also senses a central membrane environment, also decreased significantly with increased temperature. Thus, increasing temperature may produce a selective increase in motional freedom close to the core of the bilayer.

It has been suggested that erythrocytes from MH-positive individuals may respond differently to pharmacological fluidization, as occurs during general anes-

TABLE IV

Fluidized erythrocyte membrane physical properties at 37 °C

Control cells were suspended in either PBS or 50 mM methanol and aliquots were fluidized with either 25 or 50 mM benzyl alcohol. Data obtained with three membrane probes (3-AS, 16-AP and DPH) are reported. Parameters include both the steady-state anisotropy parameter and the total fluorescence (in arbitrary fluorescent units) for a constant amount of probe and erythrocytes. Data are reported as the mean ± S.E.

Additive (mM)	Probe	MH negative ($n = 8$)		MH positive ($n = 7$)	
		steady-state anisotropy (r_s)	total fluorescence	steady-state anisotropy (r_s)	total fluorescence
PBS	3-AS	0.111 ± 0.001	5.15 ± 0.23	0.107 ± 0.001	6.31 ± 0.19
	16-AP	0.065 ± 0.001	3.66 ± 0.07	0.070 ± 0.001	3.89 ± 0.04
	DPH	0.208 ± 0.002	4.34 ± 0.10	0.214 ± 0.004	4.46 ± 0.08
Methanol (50)	3-AS	0.107 ± 0.001	7.17 ± 0.10	0.109 ± 0.001	6.97 ± 0.24
	16-AP	0.065 ± 0.002	4.47 ± 0.02	0.059 ± 0.002 *	4.69 ± 0.05
	DPH	0.206 ± 0.002	5.59 ± 0.13	0.201 ± 0.002	5.60 ± 0.15
Benzyl (25)	3-AS	0.107 ± 0.001	5.98 ± 0.22	0.094 ± 0.004	6.20 ± 0.29
	16-AP	0.060 ± 0.001	4.30 ± 0.07	0.056 ± 0.001 *	4.25 ± 0.07
	DPH	0.182 ± 0.002 *	5.50 ± 0.12	0.182 ± 0.003 *	5.20 ± 0.09
Benzyl (50)	3-AS	0.111 ± 0.001	6.01 ± 0.22	0.105 ± 0.002	6.38 ± 0.33
	16-AP	0.046 ± 0.001 *	5.58 ± 0.19	0.047 ± 0.002 *	5.85 ± 0.19
	DPH	0.157 ± 0.005 *	7.18 ± 0.44	0.164 ± 0.003 *	6.00 ± 0.20

* $P < 0.05$ vs. membranes suspended in PBS.

thesia. In order to investigate this, samples from all patients were prepared and allowed to incubate for 30 min in isosmotic PBS containing either 25 or 50 mM benzyl alcohol. Measurements were obtained at both 25 °C or 37 °C. Ideally, a volatile anesthetic agent such as halothane would have been a preferable fluidizer but we experienced great difficulty in using this compound owing to its extreme volatility which limited our ability to control the concentration of this agent in the sample. Furthermore, halothane appeared to quench the fluorescence of our probes. Hence, we elected to utilize benzyl alcohol, a compound commonly used for studies examining the role of increased membrane fluidity. In order to control for the osmotic effects of the exogenous alcohol equimolar concentrations of methanol were added to control preparations. The results obtained with methanol did not differ from those observed with PBS alone. The results of these experiments are shown in Tables III and IV, representing experiments performed at 25 °C and 37 °C, respectively.

In a fashion similar to increasing temperature benzyl alcohol selectively increases motional freedom deep within the bilayer, seen as alterations in the anisotropy of probes that localize to this region, DPH and 16-AP. However, once again no differences were observed between erythrocytes from MH-positive or -negative patients. This pattern was evident at 25 °C as well as 37 °C. Once again it can be observed, by comparing Tables III and IV, that the effect of either increasing temperature or the addition of a fluidizing alcohol produces a fluidization that is relatively specific for the core of the bilayer. Furthermore, the quantitative effect of 50 mM benzyl alcohol is similar to that obtained by increasing temperature from 25 °C to 37 °C.

Discussion

MH is felt to be initiated by increased calcium release from skeletal muscle sarcoplasmic reticulum following exposure to volatile anaesthetic agents in susceptible individuals. Presently, the only reliable method to diagnose this potentially fatal condition involves a muscle biopsy and a difficult, time consuming in vitro assay. If the abnormality present in muscle sarcoplasmic reticulum were reflected in other organs it might be possible to detect the propensity to develop MH without the need for muscle biopsy. Such a test would make the prospect of surgery in patients with either a family or personal history of adverse anesthetic reactions much less daunting.

In order to investigate these matters we examined membrane physical properties of intact erythrocytes from biopsy confirmed MH-positive and MH-negative individuals utilizing steady-state fluorescent techniques which are becoming widely available. Both the static and dynamic components of membrane fluidity were examined, the latter at multiple depths in the bilayer. Furthermore, since the underlying defect of MH has been postulated to be an abnormal sensitivity to the fluidizing effects of the volatile anesthetics we examined erythrocyte membrane physical properties following conditions known to increase membrane fluidity, increasing temperature and incubation with a fluidizing alcohol. The results of our study are unambiguous. In the native state no differences were apparent in erythrocyte membrane physical properties between MH-postive or -negative patients. Furthermore, as expected both increasing temperature and the addition of benzyl alcohol increased motional freedom of the erythrocyte plasma membrane. However, the observed changes in membrane physical properties were not significantly different in MH-positive patients. Thus, we conclude that utilizing these techniques MH-positive individuals cannot be distinguished from normal volunteers. This would imply that either the underlying defect in MH does not involve alterations in membrane physical properties, it is not expressed in the erythrocyte plasma membrane or finally, that techniques employing fluorescent probes are not sensitive to these alterations.

Ohnishi et al. [5], using EPR methodology, were able to distinguish between MH-positive and -negative individuals by measuring the change in fluidity induced by halothane in both porcine and human erythrocytes. We have attempted to reproduce these data utilizing fluorescent membrane probes since this technique may be more widely available. Unfortunately, we have been unable to confirm the findings of Ohnishi et al. and find no evidence that differences exist between the physical properties of human erythrocytes isolated from control patients or those susceptible to MH. Clearly, this discrepancy may be the result of differences in the methodology utilized to quantitate membrane physical properties, the fluidizing agent employed or both. In order to minimize these differences we attempted to incubate erythrocytes with halothane but were unable to obtain useful data since this agent significantly quenched the fluorescence of the probes utilized and we were unable to rigidly control the halothane concentration.

Research into the pathogenesis of MH has benefited greatly from the existence of an animal model for the human disease. Utilizing the porcine model Harrison and Verburg [12] has demonstrated that erythrocytes from susceptible animals have a greater osmotic fragility than controls and, furthermore, Thatte et al. [4] has reported differences in membrane physical properties utilizing EPR techniques. These latter measurements were apparent without the addition of membrane fluidizers and were present over a wide temperature range. However, differences between disease expression in pigs and man are clearly apparent. Erythro-

cytes from human MH-positive individuals do not appear to display the increased osmotic fragility characteristic of the porcine model [10,11]. Furthermore, recent work by Ervasti et al. [9] utilizing EPR methodology has failed to demonstrate differences in physical properties of various skeletal muscle membrane preparations in the porcine model. This work, in conjunction with the present study, casts some doubt upon the concept that susceptibility to MH is associated with a generalized defect in membrane physical properties. Finally, it is apparent that measurement of erythrocyte membrane physical properties utilizing steady-state fluorescence polarization methodology is simply not a tenable method for the detection of the human disease. Membrane physical properties are not only identical between control and MH-positive individuals,at rest, they are also similar when the cell membrane is fluidized either by thermal means or the addition of exogenous fluidizing alcohols.

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