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Electron Spin Resonance Studies of an Animal Model of Human Congenital Myotonia: Increased Erythrocyte Membrane Fluidity in Rats with 20,25-Diazacholesterol-Induced Myotonia

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Summary. Electron spin resonance experiments have been performed on erythrocyte membranes from rats with myotonia induced by treatment with 20,25-diazacholesterol. The results suggest that erythrocyte membranes in this animal model of human congenital myotonia possess a highly significantly increased surface membrane fluidity compared to that of controls. Alterations in the physical state of membrane proteins were not apparent. These findings, also present in human congenital myotonia [Butterfield, Chesnut, Roses & Appel, 1976, *Nature* (London) **263**:159; Butterfield, 1977 (*Submitted for publication*)], strengthen the concepts that increased membrane fluidity is associated with the presence of myotonia and that congenital myotonia may be a diffuse membrane disease.

Myotonia, defined as a prolonged muscle contraction upon stimulation, is a consequence of repetitive depolarizations [27] and occurs congenitally in the goat [4, 26], horse [42], mouse [13], and man [2]. The genetically determined myotonias affecting man include myotonic muscular dystrophy (MyD), hyperkalemic periodic paralysis with paramyotonia, and congenital myotonia (CM).

The defect responsible for myotonia in CM and MyD has been localized to muscle sarcolemma based upon repetitive depolarizations in the presence of neuromuscular blockade [27]. Recent biophysical studies have suggested that these diseases may possess widespread membrane involvement [7–10]. Electron spin resonance (ESR) studies of erythrocyte membranes have demonstrated increased membrane surface fluidity in MyD [7, 8, 10]. Additional ESR investigations of erythrocyte membranes from patients with MyD [which has both myotonia and muscle degeneration (dystrophy)], Duchenne muscular dystrophy (DMD) as a model of dystrophy with no myotonia, and CM as a model of myotonia with no

dystrophy have been performed [8, 9][1]. These studies suggested increased erythrocyte membrane fluidity may be correlated with the presence of myotonia [8], while alterations in the membrane protein conformation and/or organization may be correlated with the presence of dystrophy (*see* footnote 1). That is, erythrocyte membranes in CM appear to have increased membrane surface fluidity whereas the physical state of the membrane proteins as assessed by the protein-specific spin label employed is apparently not altered compared to normal controls. These ESR results on tissue outside the central nervous and neuromuscular systems suggested that MyD, DMD, and CM may be diffuse membrane diseases.

20,25-diazacholesterol (20,25-DC) is a chemical agent capable of producing myotonia in laboratory animals [25] and man [41]. 20,25-DC reversibly inhibits Δ^{24}-reductase, the enzyme which reduces 24-dehydrocholesterol (desmosterol) to cholesterol and results in an accumulation of desmosterol as the principal plasma sterol [32]. Muscle membrane physiology [35] and phosphatide content [25] and erythrocyte membrane $(Na^+ + K^+)$-ATPase [31] have been studied in rats with 20,25-DC-induced myotonia.

The physiological explanation of myotonia in both rats treated with 20,25-DC and in human CM may be similar. In both cases, a decreased chloride conductance and increased membrane resistance is observed [27, 36]. In addition, a curare-resistant repetitive depolarization occurs in myotonic rats [3] suggesting that the defect responsible for myotonia in these animals is also located in the muscle surface membrane.

The present experiments on erythrocyte membranes from this cogent experimental model of CM were carried out utilizing the spin labeling method in order to gain additional insight into the physical basis of myotonia and to assess whether widespread membrane involvement may also be suggested in this apparent pathological condition of muscle. The increased fluidity near the membrane surface of erythrocytes, and the possible relationship of an altered membrane fluidity to the development of myotonia in muscle represent the subject of the present report.

Materials and Methods

The spin labels used in the current experiments, 2,2,6,6-tetramethylpiperidin-1-oxyl-4-maleimide (MAL-6) and 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolindinyloxyl (5-

1 *Also* Butterfield, D.A. 1977. A spin label investigation of membrane proteins in human erythrocytes in Duchenne and myotonic muscular dystrophy and congenital myotonia: Evidence for a correlation between changes in the physical state of membrane proteins and the presence of dystrophy. (*Submitted for publication.*)

nitroxide stearic acid or, 5-NS) were obtained from Syva. 20,25-DC was provided by Searle. All other reagents were of the highest purity available.

Spectra were recorded on a Magnion-Ventron MVR-9X ESR spectrometer employing a quartz aqueous sample cell. Modulation and power broadening of the spectral lines were carefully avoided.

Male Wistar rats weighing 250 g were fed on a normal diet of Purina laboratory chow and divided into two groups. The subject group was dosed daily by esophageal cannula with 30 mg 20,25-DC per kg weight in 1 ml of distilled water, while controls received daily dosages of 1 ml of distilled water. All animals were weighed weekly.

Two rats from each group were studied by electromyography (EMG) at 0, 1, 2, 3, and 4 weeks after the initiation of the 20,25-DC treatment. The rats were sacrificed the following day as described below. At the end of eight weeks, all remaining rats were studied by EMG and sacrificed.

EMG data were obtained employing a coaxial needle electrode inserted into the right thigh muscles of the rats with a ground electrode attached to the tail of the animal. The EMG responses were evaluated by two independent investigators in a blind manner and graded on a scale of 1–4 as indicated in Table 1.

Rats were sacrificed by decapitation and the blood collected into a heparinized syringe. The intact erythrocytes were washed three times with 5 mM sodium phosphate buffer (pH 8.0) 150 mM NaCl buffer (PBS) in the cold by centrifugation at $1570 \times g$. The buffy coat was carefully removed. Erythrocyte ghost membranes were obtained by hypotonic lysis in 5 mM sodium phosphate buffer, pH 8.0 (5P8) according to Fairbanks *et al.* [15]. Membrane protein content was estimated by the method of Lowry *et al.* [28].

The erythrocyte membrane ghosts were spin labeled with MAL-6 by reacting one volume of packed permeable ghosts at 4 °C overnight with 10 volumes of 5P8 buffer containing the spin probe in a 1:25 weight ratio to total membrane protein. The spin labeled ghosts were centrifuged at $27{,}000 \times g$ for 10 min and washed five times in the cold with 5P8 to remove all unbound MAL-6.

Intact erythrocytes were spin labeled by 5-NS as follows: 0.25 ml of the spin label in chloroform was deposited in a vial and the chloroform evaporated by a stream of dry nitrogen followed by vacuum. 0.5 ml of a 50% hematocrit solution of washed intact cells in PBS was then added to the vial and subsequently shaken at room temperature for 30 min. The ratio of spin label molecules to membrane lipid molecules was kept at 1:40 in order to avoid spin exchange and morphological effects [11].

Biopsy of the left gastrocnemius muscle was immediately obtained after the blood was collected from the sacrificed rat. A specimen was placed in 0.9% normal saline, rapidly frozen in a mixture of dry ice-acetone, and sectioned in a −30 °C cryostat at 6–8 μ. Sections were stained with the modified Gomori trichrome method, hematoxylin

Table 1. Rating scheme for EMG data

Scale	Definition
1	Very brief insertional activity decaying to complete inactivity
2	Limited insertional activity decaying to isolated bursts of brief activity
3	Enduring insertional activity with steady bursts of activity and brief dive-bomber effects
4	Intense insertional activity with steady bursts of activity and intense dive-bomber effects

and eosin, adenosine triphosphatase and nicotine adenine dinucleotide methods according to Dubowitz and Brook [14]. Another specimen was fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. A third specimen was immediately immersed in 3.5% glutaraldehyde for 4 hr. It was then postfixed in 2% osmium tetroxide and embedded in Epon 812. One-micron sections were stained with toluidine blue while thin sections were stained with uranyl acetate and lead citrate.

Results

Myotonia was demonstrated in rats given 20,25-diazacholesterol by electromyography. Rats sustained on this daily regimen appeared myotonic even after two weeks (Fig. 1).

No alterations in the gastrocnemius muscle of myotonic rats were detected by histological or histochemical reactions either as a function of time or at the end of the experiment.

In an effort to determine if the red blood cell membrane was in a different physical state compared to controls, the electron spin resonance technique of spin labeling was employed. MAL-6 is a nitroxide group derivative of N-ethyl maleimide and is covalently attached principally to sulfhydryl groups of membrane proteins (a very small amount of amino group binding may also occur). A typical spectrum of MAL-6 in erythrocyte ghost membranes from the rat control group is shown in Fig. 2. Spectra similar to that in Fig. 2 have been described previously

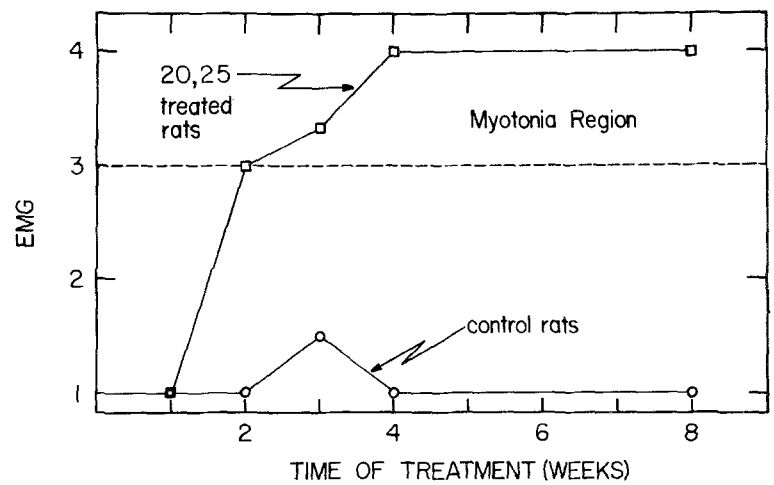

Fig. 1. Electromyographic responses of normal (○) and myotonic (□) rats as a function of time

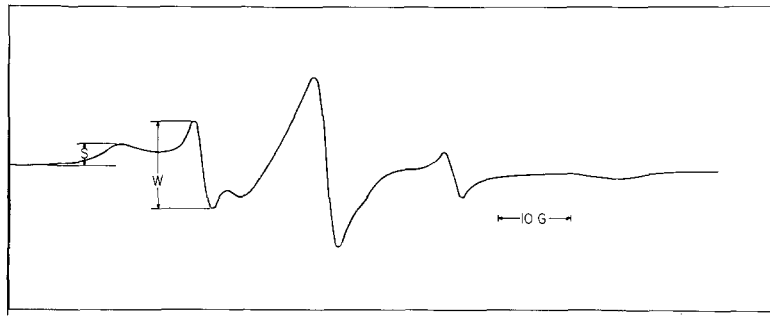

Fig. 2. A typical ESR spectrum of normal rat erythrocyte membranes spin labeled with MAL-6. The spectral amplitudes of the $M_I = +1$ strongly and weakly immobilized lines are indicated by S and W, respectively. The modulation amplitude, microwave power, and the relative spectral gain were 0.2 G, 1 mW, and an attenuation of 19, respectively

[9, 12, 18, 23, 24, 37, 38] as having ESR parameters reflecting at least two kinds of sulfhydryl groups in the erythrocyte membrane: those that are strongly immobilized and those that are only weakly immobilized. Due to the overlap of the strongly and weakly immobilized central lines and their small amplitudes in the high field lines, analyses were concentrated on the well-resolved low field lines. The ratio of the ESR spectral amplitude of MAL-6 attached to weakly immobilized SH groups (W) to that of MAL-6 bonded to strongly immobilized groups (S) is a measure of the relative population of each class of SH sites [38] and is a sensitive monitor of conformational changes of membrane proteins in erythrocytes [9, 12, 18, 23, 24, 37, 38].

The mean values of the W/S ratio of MAL-6 attached to membrane proteins in erythrocytes from control and myotonic rats is presented in Table 2. These means were compared by a two-way analysis of variance [6], a two-tailed test which minimizes the effects of possible daily variations common to experiments on biological samples. The membrane protein conformation and/or organization in erythrocytes from myotonic rats is apparently not altered compared to normal controls ($p > 0.1$). This result suggests that as in the case of human congenital myotonia (*see* footnote 1), myotonia in this animal model of CM may not be associated with alterations in the physical state of proteins in erythrocyte membranes.

An increased membrane surface fluidity was, however, demonstrated in this myotonic state by the lipid specific spin label 5-NS. 5-NS is thought to orient in the membrane with its polar acid group in close proximity to the polar head groups of the phospholipids and with its

Table 2. Ratio of spectral amplitude of MAL-6 attached to weakly immobilized SH groups (W) to that of MAL-6 attached to strongly immobilized SH groups (S) of membrane proteins in erythrocyte ghosts from myotonic and control rats[a]

	$(W/S)_{control}$	$(W/S)_{myotonic}$
Mean	5.44	6.09
SEM	0.42	0.77
n	8	8
	$p^b > 0.1$	

[a] Results presented are for rats sacrificed eight weeks after the initiation of the experiment.
[b] p value calculated by a two-way analysis of variance using the null hypothesis that W/S is the same in normal and myotonic membranes (to be expected if the membrane proteins are identical in every way) and an alternative hypothesis that the values are different.

alkyl carbon chain on the average parallel to the alkyl chains of the membrane phospholipids [20]. Although the exact location of the spin probe in the membrane is unknown, 5-NS is probably distributed on both halves of the membrane lipid bilayer and its paramagnetic center reports on an average environment near the cytoplasmic and outside membrane surfaces. Rapid anisotropic rotation is thought to occur about the long axis of the spin label giving rise to new effective T-tensor parameters (Fig. 3). The order parameter S and the nitrogen isotropic coupling constant a_N are calculated from these experimentally determined, motionally averaged nitrogen hyperfine coupling values. Regardless of whether the random walk model of Jost *et al.* [22] or the model of Mason *et al.* [29] (which involves an inverse relationship between

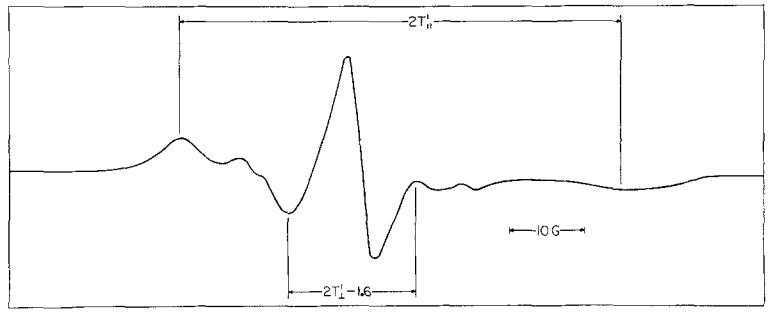

Fig. 3. A typical ESR spectrum of 5-NS incorporated into normal rat erythrocyte membranes. The measurements of the motionally averaged T-tensor parameters are indicated. The same instrumental settings as those noted in Fig. 2 were employed

Table 3. Magnetic resonance parameters[a] of 5-NS in intact erythrocyte membranes from control and myotonic rats[b]

	Control	Myotonic	p[c]
$T'_{\parallel}$	29.25 ±0.16	28.46 ±0.12	<0.025
$T'_{\perp}$	9.55 ±0.096	9.72 ±0.081	<0.05
TrT' ($\equiv 3a_N$)	48.36 ±0.16	47.91 ±0.20	<0.25
S	0.661±0.008	0.634±0.05	<0.005

[a] S is calculated by $\frac{T'_{\parallel}-T'_{\perp}}{T_{\parallel}-T_{\perp}}\frac{a_{x_1}}{a'}$ where the primed values are obtained experimentally (Fig. 3) and $a'=(T'_{\parallel}+2T'_{\perp})/3$. The unprimed values are obtained from the results of Jost *et al.* [22] on doxyl propane.

[b] Means ± SEM are presented for ($n=9$) rats sacrified eight weeks after the initiation of the experiment.

[c] p value calculated by a two-way analysis of variance utilizing the null hypothesis that the respective parameters are identical (expected if the physical state of the membrane is the same in each case) and an alternative hypothesis that these parameters are different.

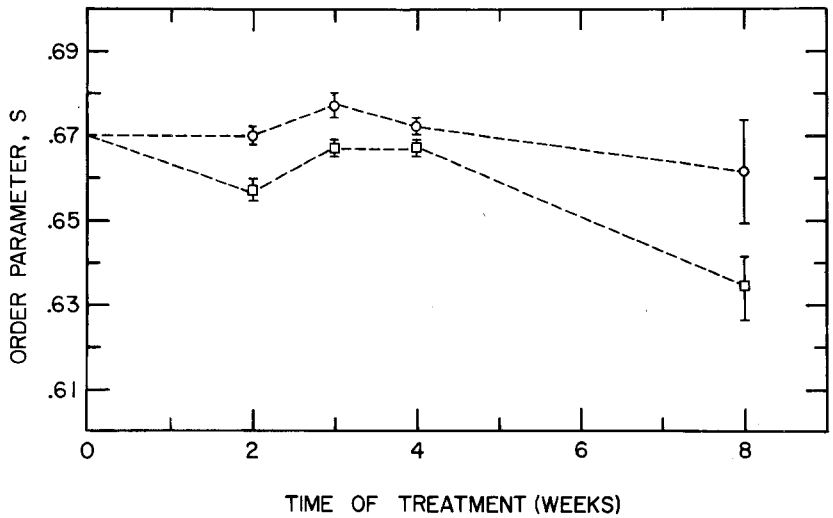

Fig. 4. Variation of the order parameter S of 5-NS in normal (○) and myotonic (□) erythrocyte membranes as a function of time. The means and standard deviations are presented. Two rats from each group were utilized through four weeks while nine rats from each group were employed at eight weeks

the rate of anisotropic rotation of the symmetry axis of the probe and the apparent value of S) is employed to explain spectra like that in Fig. 3, the interpretation of S as a measure of membrane fluidity is valid. The smaller the values of S and a_N, the more fluid and less polar, respectively, is the local environment in which the paramagnetic center

of the label is found. The polarity correction factor used by Hubbell and McConnell [20] and the crystal T-tensor values for doxyl propane as reported by Jost *et al.* [22] were employed in the calculation of S.

No significant difference in a_N of 5-NS in erythrocytes from normal and myotonic rats could be demonstrated (Table 3), suggesting that the polarity of the local milieu reported by the probe is the same in normal and myotonic samples.

Increased erythrocyte surface membrane fluidity that parallel the time course for the development of myotonia is suggested in rats made myotonic by 20,25-diazacholesterol (Fig. 4 and Fig. 1). Erythrocyte membranes from myotonic rats sacrificed at the conclusion of the experiment were significantly more fluid near the membrane surface than those of normal controls ($p < 0.005$, Table 3).

Discussion

Increased erythrocyte membrane surface fluidity has been suggested by the present spin labeling experiments on rats made myotonic by treatment with 20,25-diazacholesterol. No apparent alterations in the conformation and/or organization of membrane proteins were present in these erythrocytes as assessed by MAL-6. These findings previously observed in erythrocyte membranes in human congenital myotonia [8] (*see also* footnote 1) together with the similar physiological explanations of myotonia in the animal and human conditions [27, 36] re-enforce the validity of this experimental model of CM. The present results also suggest that the apparent correlation of increased membrane fluidity with the presence of myotonia observed in human systems [8] may be found in myotonic conditions affecting some other species as well.

No structural lesions were demonstrable by histological methods in these myotonic rats, an observation also reported in goat myotonia [1] and in rats made myotonic by treatment with 2,4-dichlorophenoxyacetic acid [16].

The increased surface membrane fluidity observed in myotonic rats appeared to parallel the development of myotonia suggesting that these two phenomena may be related. The mechanism by which myotonia occurs in this model is not known. It has been shown that a nonspecific action of the diazacholesterol is not responsible for myotonia in rats since animals dosed with this drug but maintained on a high cholesterol diet did not develop myotonia [33]. The plasma sterol content is com-

prised of approximately 85% desmosterol in 20,25-DC-treated rats after 24 days [33]. This accumulation of desmosterol has been suggested as being responsible for the decreased chloride conductance and the increased membrane resistance in these myotonic animals [32]. However, it has been reported by Winer *et al.* [43] that myotonia is not developed by administration of triparanol which also inhibits the conversion of desmosterol to cholesterol.

Although the data in Table 2 suggest myotonia may not be associated with the membrane protein conformational and/or organizational alterations, it is conceivable that MAL-6 could be insensitive to very small changes in protein organization and these may play a role in myotonia. Other spin labels with different functional group specificities may help elucidate this point.

Increased membrane fluidity may be the common factor in the myotonia process. Erythrocyte membranes in MyD and CM are more fluid than those of normal controls [8, 10] while no significant difference in membrane fluidity was observed in the nonmyotonic disease state DMD [8]. In addition, diphenylhydantion, a drug which clinically relieves myotonia [30], caused the membrane surface of MyD erythrocytes to become as rigid as that of normal controls [34]. Also in support of the suggestion that increased membrane fluidity may be intimately involved in myotonia are the data of Kuhn *et al.* [25] which show that 20,25-DC-treated rats have an increased amount of the unsaturated fatty acids $C_{18:2}$, $C_{20:4}$, and $C_{22:6}$ in skeletal muscle. Such an alteration in fatty acid content would be expected to increase membrane fluidity as was observed in the present study.

The rigidizing effects of Ca^{2+} [21], and cholesterol [19] on membrane lipids is well documented. The beneficial action of these chemical agents in the treatment of myotonia [3, 33] may result from their ability to increase membrane rigidity. Experiments designed to test this hypothesis are currently in progress.

The requirement of the correct membrane fluidity for maximal enzymatic activity has been amply demonstrated [40]. The increased surface membrane fluidity observed in the present study and in human CM [8] may explain the reported alterations in ouabain inhibition of $(Na^+ + K^+)$-stimulated ATPase [5], the decrease in ability of the sarcoplasmic reticulum to concentrate Ca^{2+} [39], and the increase in hexokinase activity [17] observed in these myotonic conditions.

While the relationships of the present results to the physiological explanation of myotonia are still uncertain, the current data suggest

that increased membrane surface fluidity is correlated with the presence of myotonia and that human congenital myotonia and myotonia induced by diazacholesterol are conditions associated with general membrane abnormalities.

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