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ELECTRON SPIN RESONANCE INVESTIGATIONS OF MEMBRANE PROTEINS IN ERYTHROCYTES IN MUSCLE DISEASES

DUCHENNE AND MYOTONIC MUSCULAR DYSTROPHY AND CONGENITAL MYOTONIA

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Summary

Comparison of electron spin resonance spectra of spin labeled erythrocyte membranes from patients with the dystrophic conditions Duchenne and myotonic muscular dystrophy with those of normal controls suggests that alterations in membrane protein conformation and/or organization are present in these disease states. These protein alterations are not apparent in the non-dystrophic disease congenital myotonia. The results suggest a correlation between changes in the physical state of proteins in membranes with the presence of dystrophy. In addition, the present results from erythrocytes lend support for the concept of a generalized membrane defect in these diseases.

Introduction

Myotonic muscular dystrophy has been considered a defect of muscle surface-membrane based upon the persistance of curare-resistant repetitive membrane depolarization [1]. A similar locus has been postulated for the membrane defect in congenital myotonia [1]. Duchenne muscular dystrophy has no myotonia (prolonged muscle contracton upon stimulation due to repetitive membrane depolarization) and its chief clinical manifestation is an inherited, progressive muscle weakness and degeneration (dystrophy) [2]. The primary biochemical defects for all of these diseases are unknown.

Biophysical and biochemical studies of erythrocyte membranes in myotonic and Duchenne muscular dystrophy and congenital myotonia have suggested these inherited diseases may have more widespread membrane involvement

Abbreviations: SDS, sodium dodecyl sulphate; MAL-6 2,2,6,6-tetramethylpiperidin-1-oxyl-4-maleimide.

[3—9]. Electron spin resonance (ESR) investigations utilizing spin labeled derivatives of the methyl esters of stearic acid demonstrated erythrocyte membranes in myotonic dystrophy to be more fluid near the membrane surface than those of normal controls [3,4]. Studies involving the same surface-sensitive lipid spin label have led to a suggested correlation of increased erythrocyte membrane surface fluidity with the presence of myotonia [5]. ESR studies using a protein specific spin probe also suggested a generalized membrane alteration in this disease [6]. The enzymatic activity of endogenous membrane-bound protein kinase in erythrocytes was found to be altered in myotonic [7] and Duchenne muscular dystrophy [8] when compared to controls. In addition, a non-specific increased number of stomatocytes were noted in red blood cells in these two dystrophic states and congenital myotonia by scanning electron microscopy [9].

In contrast to the noncovalently bound, lipid-specific spin labels which are intercalated into the lipid region of the membrane, the protein specific spin probe 2,2,6,6-tetramethylpiperidin-1-oxy-4-maleimide (MAL-6) is covalently bound to sulfhydryl (SH) groups almost exclusively, although a small amount of amino group binding may also occur [10]. At least two classes of spin labeled SH groups are detected in erythrocyte membrane proteins: those that are strongly immobilized and those that are only weakly immobilized [11]. ESR studies have been performed on erythrocyte membranes labeled with MAL-6 to assess the effect of pH [10], pharmacological agents [12], membrane protein solubilization procedures [13] and protein denaturants [14,15] on membrane protein organization. Changes in this organization affected the strongly and weakly immobilized SH groups and were monitored by changes in the ESR spectrum of MAL-6.

Recently, alterations in the physical state of membrane proteins in erythrocytes in myotonic muscular dystrophy were demonstrated by the spin labeling method [6]. The present experiments were performed to evaluate the specificity of these membrane organization alterations detected by MAL-6. In addition to erythrocyte membranes from patients with myotonic dystrophy (which has both myotonia and dystrophy), red blood cell ghost membranes from patients which Duchenne muscular dystrophy who demonstrate dystrophy with no myotonia and congenital myotonia who present myotonia with no dystrophy were utilized. The alterations in the erythrocyte membrane protein conformation and/or organization in Duchenne as well as myotonic muscular dystrophy and two other non-myotonic muscular dystrophies suggested by these studies and their possible correlation to the presence of dystrophy represent the principal subjects of this report.

Experimental

MAL-6 was obtained from Syva. ESR spectra were recorded on a Ventron-Magnion MVR-9X spectrometer utilizing a quartz aqueous sample cell. Modulation and power broadening of the spectral lines were carefully avoided by employing low microwave powers (<20 mW) incident on the rectangular ESR cavity and by working with small modulation amplitudes (0.2 *G*).

Erythrocyte membranes were obtained from fresh, heparinized blood in each

of three separate sets of experiments. In the myotonic dystrophy set of experiments, 12 different patients and 12 control subjects were utilized. In the experiments involving Duchenne muscular dystrophy and congenital myotonia samples, 11 control and Duchenne's patients and 6 normal and congenital myotonia patients, respectively were employed. In each set of experiments, intact erythrocytes were prepared by immediately centrifuging blood at 1570 × *g* for 10 min at 4°C and washing the cells 3 times with 5 mM sodium phosphate/150 mM NaCl buffer, pH 8.0. The buffy coat was carefully removed. Permeable erythrocyte ghost membranes were prepared by hypotonic lysis of these freshly washed intact cells in 5 mM sodium phosphate buffer, pH 8.0, according to Fairbanks et al. [16] and membrane protein content was estimated by the method of Lowry et al. [17]. SDS-polyacrylamide gel-electrophoresis of erthrocyte membranes was performed in the usual manner [16].

Membrane ghosts were spin labeled with MAL-6 as previously described [6] except a 1 : 25 weight ratio of spin label to total membrane protein was employed. Erythrocyte membranes from patients with myotonic muscular dystrophy were spin labeled overnight at 4°C 2 or 3 days after being prepared as described above, while erythrocyte membranes from patients with Duchenne muscular dystrophy and congenital myotonia were spin labeled with MAL-6 overnight at 4°C the same day on which they were prepared. In each respective set of experiments, control and disease state membranes were treated identically.

Results

The spin label used in the present study is covalently bonded principally to sulfhydryl groups although fewer than 5% of the labeled sites may be amino groups [10]. A typical ESR spectrum of MAL-6 incorporated into control erythrocyte membranes is shown in Fig. 1. Spectra similar to that in Fig. 1 have been described previously [6,10—15] as having ESR parameters reflecting at least two different classes of SH group sites in the red blood cell membrane. One type of site is indicated by the powder-like spectrum whose amplitude

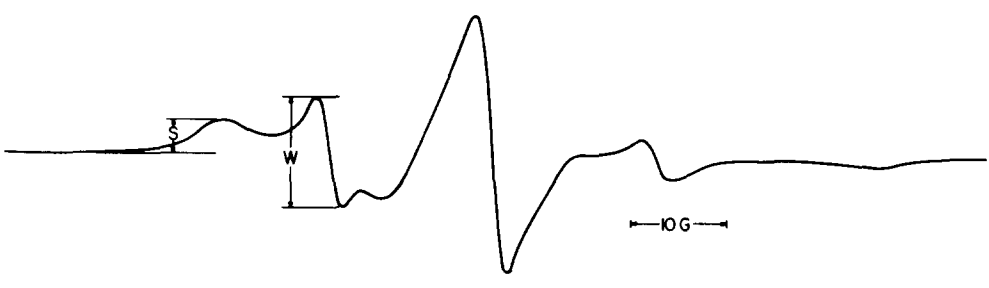

Fig. 1. A typical ESR spectrum of normal human erythrocyte ghosts with which MAL-6 has been reacted overnight as described in Experimental. The amplitudes of the strongly and weakly immobilized lines are indicated by *S* and *W*, respectively.

of the low-field line is indicated by *S* (for strongly immobilized). Sulfhydryl groups in the *S* sites are essentially completely immobilized as evidenced by comparison of one-half the splitting of the outer hyperfine extrema to the T_{zz} principal values of nitroxides doped in appropriate host single cyrstals [18,19]. The second SH group environment reported by MAL-6 is one in which the spin probe is only weakly immobilized; the low field peak-to-peak amplitude of this weakly immobilized spectrum is indicated by *W* (for weakly immobilized). Radical decay studies employing membrane impermeable ascorbate have suggested these *W* sites to be exposed to the polar aqueous medium and not buried in the lipid milieu of the membrane [6].

Because of the small amplitudes of the high field lines and the overlap of the central lines, analyses of the spectra were confined to the low field lines. The linewidths of the strongly and weakly immobilized signals are respectively equal in controls and each disease state indicating the respective labeled erythrocyte membrane protein SH groups have essentially the same mobility in each case. The ratio of the ESR spectral amplitudes of MAL-6 bonded to weakly immobilized sulfhydral groups (*W*) to that of MAL-6 attached to strongly immobilized SH groups (*S*) is a convenient monitor of protein conformational and/or organization changes in membranes [6,10—15].

Because the myotonic dystrophy set of experiments involved labeling ghost membranes 2 or 3 days after they were prepared, the effect of aging of the membranes on the *W/S* ratio of control membranes was studied. *W/S* was observed to increase from 3.7 for ghosts labeled on the day they were prepared to 5.2 for ghosts labeled 2 days after preparation. This latter value is essentially that of the mean value of the control samples in the myotonic dystrophy set of experiments (Table I). "2-day old" ghosts were found by SDS-polyacrylamide gel electrophoresis to lose some of the approx. 200 000 dalton protein "spectrin" from the membrane.

The current results of the spin labeling studies of membrane proteins in erythrocytes in myotonic and Duchenne muscular dystrophy and congenital myotonia were obtained in three separate sets of experiments. In each respective set of experiments, control and disease state membranes were treated identically. The mean *W/S* values for controls and each disease state performed in these three separate sets of experiments were compared by a two-way

TABLE I

COMPARISON OF THE ESR SPECTRAL AMPLITUDE RATIO (*W/S*) * FOR MAL-6 IN ERYTHROCYTE MEMBRANES IN NORMAL (N), MYOTONIC DYSTROPHY (MyD), DUCHENNE MUSCULAR DYSTROPHY (DMD) AND CONGENITAL MYOTONIA (CM) **

	$(W/S)_N$	$(W/S)_{MyD}$	$(W/S)_N$	$(W/S)_{DMD}$	$(W/S)_N$	$(W/S)_{CM}$
Mean	5.37	5.81	3.65	4.25	3.87	3.81
SEM	0.12	0.14	0.13	0.21	0.12	0.10
n	12	12	11	11	6	6
$P^{\dagger}$	$P < 0.001$		$P < 0.01$		$P < 0.5$	

* Defined in text.

** The results from three separate sets of experiments are presented.

† *P*-value calculated by a two-way analysis of variance.

analysis of variance [21] (Table I). This method of statistical analysis is a two-tailed test which minimizes the effects of possible flucuations from day to day that may occur with biological samples. P is the significance of the difference of the mean values of $(W/S)_{\text{control}}$ and $(W/S)_{\text{disease state}}$ calculated from the two-way analysis of variance in each set of experiments. The null hypothesis was that W/S values in myotonic and Duchenne muscular dystrophy and congenital myotonia are, respectively, not different from that of their corresponding controls. The W/S ratio in myotonic dystrophy or Duchenne muscular dystrophy is significantly increased compared to the respective controls ($P < 0.001$ and $P < 0.01$, respectively, Table I) suggesting alterations in the physical state of membrane proteins in erythrocytes in these dystrophic conditions. As opposed to these results, (W/S) in congenital myotonia is not different from that of controls ($P > 0.5$). Some experiments in the this non-dystrophic case tended to show this parameter to be less than $(W/S)_{\text{control}}$ but statistical significance of this observation could not be demonstrated.

A few experiments were conducted with the non-myotonic muscular dystrophies, hyperkalemic periodic paralysis (without para-myotonia) and oculopharyngeal muscular dystrophy with results similar to the myotonic and Duchenne muscular dystrophy experiments.

Discussion

Alterations in the membrane protein conformation and/or organization in erythrocytes in the dystrophic conditions myotonic and Duchenne muscular dystrophy have been suggested by the present studies employing the protein specific spin label MAL-6, while these alterations are apparently not present in the non-dystrophic disease state congenital myotonia.

The present results probably cannot be attributed to a decreased amount of protein present in the erythrocyte membranes since the total protein content and SDS-polyacrylamide gel electrophoretic patterns are not altered in ghosts from the various disease states compared to controls [22].

The difference between the mean values of the control parameters in the myotonic dystrophy experiments compared to those of the congenital myotonia and Duchenne muscular dystrophy experiments probably reflects the different methods of labeling used. Erythrocyte membrane ghosts which stand in contact with 5 mM sodium phosphate bufer, pH 8.0, will gradually lose some membrane bound "spectrin" and such ghosts tend to give an increased value of the W/S ratio. This last result, suggesting more mobile membrane protein SH groups, is consistent with the postulated "anchoring"role of spectrin on erythrocyte membrane proteins [23]. Separate experiments on myotonic dystrophic erythrocyte membranes labeled without the 2—3 day delay gave increased W/S values compared to controls, consistent with the present experiments; however, the W/S ratios obtained were similar to those of the Duchenne muscular dystrophy set of experiments reported in Table I (D.A.B., unpublished observations).

Although membrane protein alterations are present in erythrocyte membranes from each of the dystrophic states studied, the biophysical and biochemical defect peculiar to each disease which gives rise to these changes may

well be different. ESR studies of erythrocyte membranes detected increased surface membrane fluidity in myotonic muscular dystrophy [3,4] with no apparent change in Duchenne muscular dystrophy compared to normal controls [5]. Decreased phosphorylation of Component a in red blood cell membranes has been shown in myotonic dystrophy [7] while increased phosphorylation of "spectrin" in erythrocyte ghosts occurs in the Duchenne type of dystrophy [8]. In addition, the genetic and the clinical expression of these diseases are different. Duchenne muscular dystrophy, inherited as a sex-linked recessive trait, initially involves dystrophy of proximal muscles, while this symptom in myotonic dystrophy, inherited as an autosomal dominant trait, is initially expressed in the distal musculature [2].

Studies involving the same three disease states as in the current experiments suggested increased erythrocyte surface membrane fluidity was correlated with the presence of myotonia [5]. The present results suggest membrane protein conformational and/or organizational changes are correlated with the presence of dystrophy. These findings may provide a means by which the separate symptoms of myotonia and dystrophy may be studied.

That no apparent protein alteractions were observed in the current studies with congenital myotonia suggests that the previously demonstrated increased erythrocyte membrane surface fluidity in this disease [5] may not involve boundary lipids immediately in contact with membrane proteins. Differential assemblage of such lipids might be expected to affect the conformation of the integral proteins to which they are bound. That such a phenomenon exists is exemplified by the requirement for phosphatidylserine for the recovery of enzymatic activity of isolated $(Na^+ + K^+)$-ATPase [24].

A possible alternative explanation for the apparent lack of differences in erythrocyte membrane proteins in congenital myotonia as assessed by MAL-6 may be the sensitivity of the spin label. As was observed in earlier spin probe studies of the effect of oxygenation on the conformation of hemoglobin [25], several alternate spin labels may be necessary in the study of congenital myotonia before a spectrum which is sufficiently sensitive may be found. Recent results with an animal model of congenital myotonia however, suggest that in rat erythrocyte membranes, membrane protein alterations also may not be present [26].

The alterations in the physical state of membrane proteins in erythrocytes in myotonic and Duchenne muscular dystrophy may possibly explain the alterations in protein kinase activity [7,8] and response to fixation [9] observed in these dystrophic conditions and differences in oubain-sensitive sodium efflux in myotonic dystrophic membranes [27]. While the relationship of the ESR results presented here to enzymatic and clinical properties of Duchenne and myotonic muscular dystrophy and congenial myotonia is still unclear, the current studies suggest a correlation between membrane protein conformational and/or organizational alterations and the presence of dystrophy. Moreover, these findings strongly suggest these respective diseases are associated with general membrane abnomalities.

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