Biochimica et Biophysica Acta, 1146 (1993) 236–242

BBAMEM 75901

Abnormal fatty acid composition in sarcolemma and sarcoplasmic reticulum from myotonic ADR mouse muscle

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(Received 29 May 1992)
(Revised manuscript received 10 December 1992)

Key words: Muscle; Myotonia; ADR; Fatty acid; Sarcolemma; Sarcoplasmic reticulum

The fatty acid composition of membrane lipids from sarcolemma and sarcoplasmic reticulum isolated from biceps and gastrocnemius muscle has been compared in normal (wildtype, $+/adr^{mto}$ or $+/+$) and affected (adr^{mto}/adr^{mto}) myotonic mice. The adr^{mto} mouse exhibits an arrested development of the righting response, and arose spontaneously from the SWR/J strain. These mice exhibit classical myotonia similar to the human disease, Becker's myotonia [1]. Significant alterations, characterized by a decrease in the saturated fatty acid, palmitic acid (16:0), and the polyunsaturated fatty acid, arachidonic acid (20:4), and an increase in stearic (18:0) and linoleic (18:2) acids, were observed between sarcolemma and sarcoplasmic reticulum from normal and affected mice. These changes in fatty acid composition of muscle membrane from ADR mice may be adequate to cause an alteration in membrane fluidity and affect the function of ion channels. The fatty acid composition of erythrocytes ghosts was also examined, as a potential marker for alterations in muscle membranes. In erythrocyte ghosts isolated from affected mice, the only alteration observed was a decrease in the proportion of oleic acid (18:1), an effect completely different from those observed in muscle membranes. Therefore, erythrocyte ghosts do not serve as an adequate indicator of changes in fatty acid composition of muscle membranes in this model of myotonia.

Introduction

Myotonia is a skeletal muscle symptom characterized by delayed relaxation following muscle contraction. Electromyography in affected muscle demonstrates the presence of waxing and waning high-frequency membrane discharges that occur after trains of voluntarily evoked action potentials [2]. In certain types of myotonia there is a defect of unknown origin in the voltage-dependent Cl^- channel, resulting in decreased Cl^- conductance [2]. Because Cl^- conductance accounts for over 60% of the total membrane conductance in normal resting vertebrate skeletal muscle, a reduction in Cl^- conductance adversely affects the electrical stability of the membrane [3].

Several hereditary myotonias have been identified in humans [4], goats [5] and mice [1], including a mutant strain of mice (adr/adr and adr^{mto}) exhibiting a syndrome similar to Becker's myotonia (recessive generalized myotonia) [6–8]. In the ADR strain, the myotonia is inherited recessively, and is characterized by decreased Cl^- conductance [9]. The adr/adr mutation, which arose from the A2G strain, and the adr^{mto} mutation, which arose from the SWR/J strain, are allelic [7,8]. Transplantation studies have shown that the defect is intrinsic to the ADR muscle tissue [10]. The density of Cl^- channels in the sarcolemmal fraction, as measured by the binding of indanyl oxyacetic acid, is reduced by 75% in ADR mice [11]. A recent report demonstrated that the defect in Cl^- conductance in myotonia is due to an insertion mutation in the gene that codes for the Cl^- channel, resulting in a loss of coding potential for several membrane spanning domains [12]. Myotonia is evident at about 2 weeks postnatal age, and manifests as an arrested develop-

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Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 20:4, arachidonic acid; 22:6, docosahexaenoic acid; ADR, arrested development of righting reflex; GLC, gas liquid chromatography; mto, myotonic; DBI, double bond index; S:U, saturated to unsaturated fatty acid ratio.

ment of the righting reflex. By 30 days, affected mice are about 10% smaller, and are about 40% smaller by adulthood [1]. There are no signs of muscle fiber degeneration [13], atrophy or inflammatory infiltrates [1].

In this study we investigated the fatty acid composition of muscle membrane to determine whether there are any alterations that may result from or occur in conjunction with a defect in Cl^- channel conductance. Our results demonstrate a change in the fatty acid composition of lipids of sarcolemma and sarcoplasmic reticulum isolated from adr^{mto}/adr^{mto} (affected, ADR) mice, as compared to wild-type ($+/adr^{mto}$ or $+/+$) mice. It is possible that the altered fatty acid composition of muscle membrane from ADR mice is adequate to cause an alteration in membrane fluidity and effect the function of ion channels. We have also compared muscle membrane with erythrocyte plasma membrane (ghosts) to determine whether the changes in fatty acid composition were generalized, and if erythrocyte ghosts could serve as a marker for changes in the muscle. While similar alterations in fatty acid composition were observed in both sarcolemma and sarcoplasmic reticulum from ADR mice, these changes were not reflected in erythrocyte ghosts.

Materials and Methods

Animals

Tested breeding pairs of $+/adr^{mto}$ mice on BALB/C background were obtained from Jackson Labs (Bar Harbour, Maine), and maintained in the animal quarters at West Virginia University. Two pairs were purchased, and six carrier females were subsequently identified from interbreeding within the WVU colony. For the experiments described, a total of 12 affected (adr^{mto}/adr^{mto}) mice and 12 unaffected (wild-type, $+/adr^{mto}$ or $+/+$) mice were used in four separate experiments. These mice were obtained from six different litters. Three of the litters were obtained from the two original breeding pairs. The remaining three litters were obtained from backcrossing carrier daughters with their carrier fathers. Affected (ADR) mice were identified by testing the righting reflex at 3 weeks of age. The affected mice exhibited arrested righting reflex, with hindlimb extension, and were about 50% smaller than their unaffected littermates. Mice were killed between 6 and 9 weeks of age. All animal procedures were conducted following protocols approved by the WVU Institutional Animal Care and Use Committee.

Isolation of erythrocyte ghosts [14]

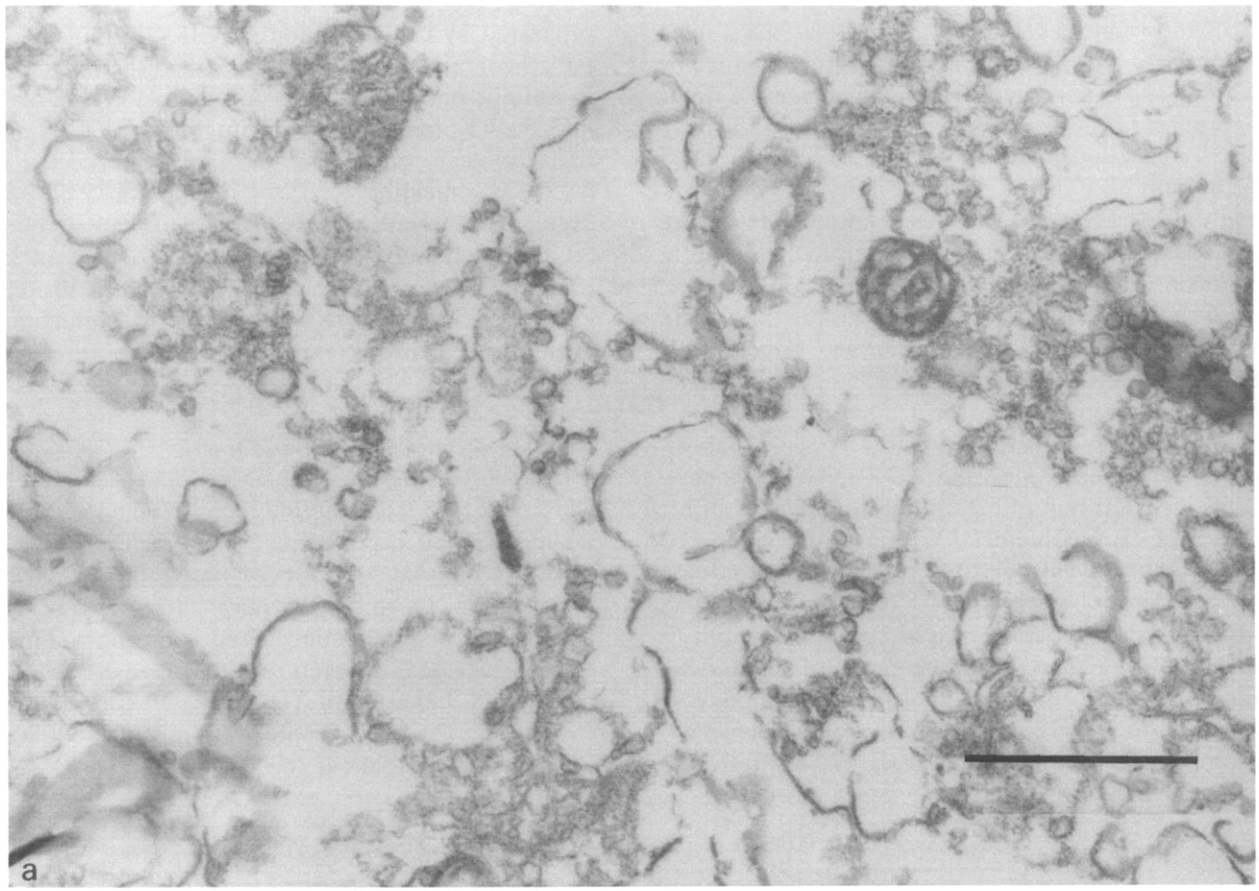
Blood (300–500 μl) was drawn into heparinized tubes from the left ventricle of mice anesthetized with pentobarbital (40 mg/kg, i.p.). Plasma and cells were separated by centrifugation at 150 g for 10 min. The pellet, containing erythrocytes, was washed three times in isotonic phosphate-buffered saline (150 mM NaCl, 5 mM $NaPO_4$, pH 8.0), by centrifuging at $2000 \times g$ for 10 min for each wash. The purified erythrocytes were lysed by sonicating for 30 s in hypotonic phosphate buffer (5 mM $NaPO_4$, pH 8.0). The plasma membranes (ghosts) were recovered by washing 3 times in hypotonic buffer, centrifuging at $22000 \times g$ for 10 min for each wash. Pellets were resuspended in 2 ml hypotonic buffer and transferred to glass tubes for lipid extraction.

Isolation of muscle membrane fractions [15,16]

Muscles of the hindlegs (biceps and gastrocnemius) were dissected from the mice (muscle and blood were matched in all experiments), yielding 0.5 g of tissue from ADR mice and 1 g of tissue from unaffected mice. Nerve, fat and connective tissue were removed. Muscle was weighed, minced and homogenized in 9 vol 0.25 M sucrose (prepared in 1 mM Tris-1 mM EDTA buffer, pH 7.3) using a Technika tissuemizer (30 s at full speed). The homogenate was centrifuged at $800 \times g$ for 10 min to sediment the debris fraction (P1), containing unbroken cells, nuclei, contractile proteins and connective tissue. The P1 fraction was washed once by resuspension in 0.25 M sucrose. The two S1 supernatants were combined and centrifuged at $100000 \times g$ for 1 h to obtain P2, containing sarcoplasmic reticulum, sarcolemma and mitochondria. P2 was resuspended in 0.25 M sucrose and layered on a discontinuous sucrose gradient of 1.5, 1.38, 1.22, and 1.1 M sucrose. The gradients were centrifuged at $111000 \times g$ for 90 min. Sarcolemma was recovered from the uppermost interface, and sarcoplasmic reticulum was collected from the second and third interfaces. Mitochondria were well-separated from the other fractions and found at the fourth interface, and the cell debris formed a pellet. The collected fractions were diluted at least 5-fold with 1 mM Tris-EDTA buffer and centrifuged at $100000 \times g$ for 30 min to form a pellet. Pellets were resuspended in 2 ml Tris-EDTA buffer and transferred to glass tubes for lipid extraction. Subcellular fractions were examined by electron microscopy (Fig. 1) and characterized by assaying the marker enzymes, 5′-nucleotidase for sarcolemma, Ca^{2+}/Mg^{2+}-ATPase for sarcoplasmic reticulum, and succinate dehydrogenase for mitochondria [17]. Insignificant cross contamination of the fractions was observed, and no differences in these parameters were noted between normal and ADR muscle (Table I).

Preparation of lipid extracts [18]

Erythrocyte ghosts (in 2 ml hypotonic buffer) or muscle membranes (in 2 ml Tris buffer) were suspended in 12 ml chloroform/methanol (2:1). The ex-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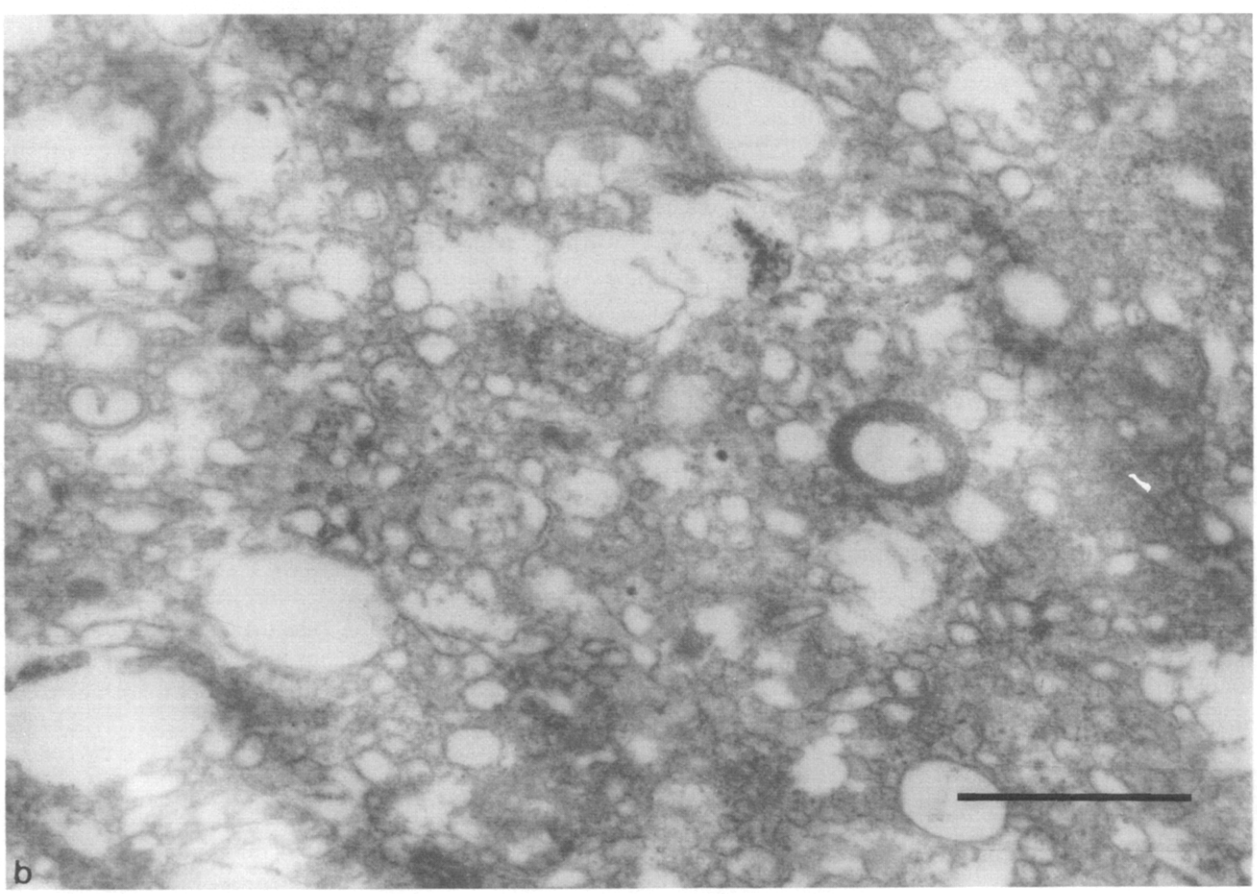
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TABLE I

Characterization of subcellular fractions from mouse muscle

Marker enzymes were assayed in crude homogenates (CH), sarcolemma (SL), sarcoplasmic reticulum (SR) and mitochondria (MT) fractions isolated by density graident centrifugation as described in Materials and Methods. Values are means ± S.E. of three determinations and are expressed as specific activity (nmol/mg protein per min). No differences were observed between normal and ADR muscle fractions.

	CH	SL	SR	MT
Protein (mg/g tissue)				
Normal	154 ±16	0.55±0.02	1.17± 0.11	2.88±0.14
ADR	146 ± 9	0.53±0.04	1.23± 0.14	3.40±0.31
5′-Nucleotidase				
Normal	3.0± 0.01	129 ±2.6	5.4 ± 0.04	1.15±0.13
ADR	3.0± 0.00	131 ±2.0	5.5 ± 0.02	1.20±0.08
Ca^{2+}/Mg^{2+}-ATPase				
Normal	121 ± 1.96	39 ±0.7	1584 ±16	60 ±0.9
ADR	120 ± 1.70	40 ±0.7	1615 ±24	60 ±1.4
Succinate dehydrogenase				
Normal	3.0± 0.02	1.0 ±0.02	1.16± 0.14	30 ±0.4
ADR	2.9± 0.01	0.99±0.02	1.27± 0.03	30 ±0.7

tracts were centrifuged (1000 × *g* for 10 min) to separate the aqueous and organic layers. The small amount of protein was trapped at the interface. The upper (aqueous) phase was removed and discarded, and the lower phase was evaporated to dryness under nitrogen.

Quantitative analysis of fatty acid composition [19]

Aliquots of lipid extracts equivalent to 10–30% of the total lipid extract were transesterified to form fatty acid methyl esters by reaction with boron trifluoride-methanol. Fatty acid methyl esters were analyzed by

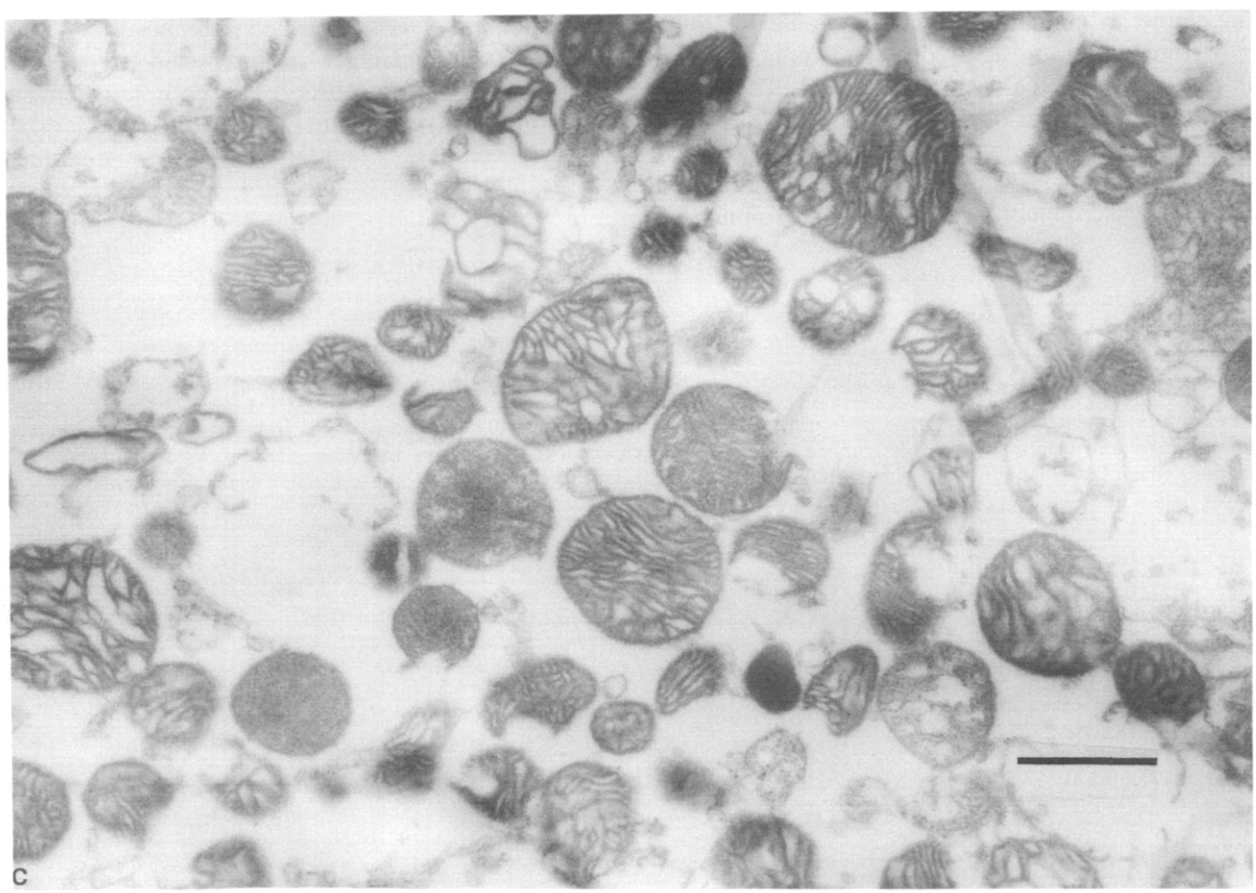

Fig. 1. Electron micrographs of subcellular fractions isolated from normal mouse muscle. Panel a, sarcolemma. Panel b, sarcoplasmic reticulum. Triads (arrows) and tubular structures (boxes) in the sarcoplasmic reticulum fractions are indicated. Panel c, mitochondria. Scale bar = 1 μm.

capillary gas-liquid chromatography, with flame ionization detection, using an SP-2330 (cyanopropyl, 30 m × 0.32 mm i.d.) column (Supelco), operated in a temperature gradient mode from 70°C to 200°C. The carrier gas was helium, the injector temperature was 220°C, and the detector temperature was 250°C. Fatty acids were identified by comparing retention times to known standards, and quantified by comparing the peak areas of fatty acid methyl esters with the peak area of an internal standard (heptadecanoate methyl ester). Blanks were samples prepared with no tissue present, and were subtracted from all samples.

Data analysis

Data were collected in four experiments, each using three affected and three unaffected mice for a total of 12 animals per group. Data are expressed as mol% for each fatty acid, calculated as the percent of the total mass of fatty acids. Multiple comparisons were done by analysis of variance and Tukey's protected *t*-tests. Differences were considered significant at $P < 0.05$.

Results

Comparison of membrane fatty acid composition from normal muscle revealed substantial differences in profiles between membrane fractions (Table II). Over 30% of the total fatty acids of sarcolemma was the saturated fatty acid, palmitic acid (16:0). The major unsaturated fatty acids present in sarcolemma were oleic acid (18:1), linoleic acid (18:2) and docosahexaenoic acid (22:6). As has been previously reported [15,20,21], muscle membranes contain high amounts of 22:6 compared to membranes from other organs. The ratio of saturated to unsaturated fatty acids (S:U ratio, calculated by dividing the sum of 16:0 plus stearic acid (18:0) by the sum of the unsaturated fatty acids), an index of membrane fluidity [22,23], was 0.96. Another indicator of membrane fluidity [22–24], the double bond index (DBI, calculated as the average number of double bonds per fatty acid) was 1.61.

Sarcoplasmic reticulum was also rich in 16:0, although it contained lesser amounts than sarcolemma (27.06 mol% vs. 34.47 mol%, $P < 0.05$). Over 18% of the total fatty acids in sarcoplasmic reticulum was 22:6, significantly higher than sarcolemma. This resulted in a lower S:U ratio of 0.76, and a higher DBI of 2.03, possibly indicating a more fluid membrane.

Erythrocyte ghosts contained levels of 16:0 similar to sarcoplasmic reticulum, and significantly lower than sarcolemma, an interesting observation since both sarcolemma and erythrocyte ghosts are plasma membranes. This membrane fraction was also not particularly enriched in 22:6, but instead contained significantly higher amounts of 18:1 compared to sarcoplasmic reticulum and arachidonic acid (20:4) compared to both sarcoplasmic reticulum and sarcolemma. The S:U ratio of erythocyte membrane was 0.86. The DBI was 1.47, which was significantly lower than either sarcolemma or sarcoplasmic reticulum.

Substantial changes in fatty acid composition in ADR muscle were observed in both sarcolemma and sarcoplasmic reticulum (Fig. 2). Both sarcolemma and sarcoplasmic reticulum from ADR mice contained significantly less 16:0 and 20:4, and significantly more 18:0 and 18:2. The decline in 16:0 was offset by the

TABLE II

Fatty acid composition of membrane fractions from normal and ADR mouse muscle and erythrocyte ghosts

Total lipids were extracted from membrane fractions isolated from gastrocnemius muscle and erythocytes from heterozygous and homozygous ADR mice. Fatty acid composition was determined by capillary gas chromatography. Values are means ± S.E. of 9–12 samples. S:U ratio is calculated as (nmol saturated fatty acids/nmol unsaturated fatty acids). DBI (double bond index) is calculated as the average number of double bonds per fatty acid.

Membrane fraction	Fatty acid (mol%)							
	16:0	18:0	18:1	18:2	20:4	22:6	S:U ratio	DBI
Sarcolemma								
Normal	34.47 ± 3.27	13.84 ± 1.53	15.01 ± 1.69	13.40 ± 1.36	9.80 ± 0.78	13.48 ± 1.09	0.96 ± 0.08	1.61
ADR	26.82 ± 1.57 **	18.71 ± 0.46 *	17.76 ± 0.77	17.72 ± 0.36 *	7.21 ± 0.25 *	11.78 ± 0.68	0.86 ± 0.06	1.52
Sarcoplasmic reticulum								
Normal	27.06 ± 2.61	15.78 ± 1.53	10.32 ± 0.68	16.42 ± 1.05	11.45 ± 0.54	18.97 ± 1.60	0.76 ± 0.05	2.03
ADR	21.49 ± 2.23 **	20.18 ± 0.91 **	13.11 ± 0.96	22.12 ± 1.27 **	7.68 ± 0.47 *	15.43 ± 1.16	0.80 ± 0.14	1.81
Erythrocytes								
Normal	29.62 ± 3.21	15.84 ± 0.78	19.07 ± 1.08	13.22 ± 0.45	15.77 ± 0.71	6.48 ± 0.65	0.86 ± 0.09	1.47
ADR	33.86 ± 4.22	16.14 ± 1.13	14.42 ± 0.89 *	14.78 ± 1.71	13.71 ± 1.65	7.08 ± 1.10	1.29 ± 0.38	1.41

* $P < 0.05$; ** $P < 0.01$, comparing normal vs. ADR by analysis of variance and Tukey's protected *t*-test.

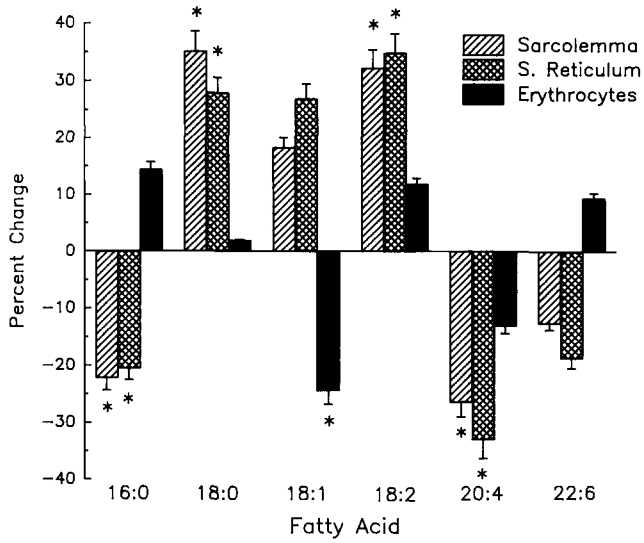

Fig. 2. Comparison of the level and direction of changes in fatty acid composition in sarcolemma, sarcoplasmic reticulum and erythrocyte plasma membranes from normal and ADR mice. Values were calculated from the data in Table I as ((ADR-normal)/normal), and are means ± S.E. of 9–12 samples. * $P < 0.05$, comparing normal vs. ADR by analysis of variance and Tukey's protected t-test.

increase in 18:0, therefore the S:U ratios did not differ from normal values. However, the DBI in both sarcolemma and sarcoplasmic reticulum fractions were lower, as compared to normal muscle, possibly indicating a more rigid membrane structure in the ADR membranes. These results suggest that 18-carbon saturated fatty acids (18:0) may replace palmitic acid (16:0), while 18-carbon unsaturated fatty acids (18:2) may replace arachidonic acid (20:4).

In contrast to the muscle membranes, erythrocyte ghosts from ADR mice contained 24% less 18:1. The other fatty acids were not significantly changed. There was an increased S:U ratio in ADR erythrocyte ghosts, due to the decline in 18:1, but the difference was not statistically significant. There was no change in the DBI. These alterations in fatty acid composition are completely different from the changes observed in muscle membranes.

Discussion

The major changes in fatty acid composition in sarcolemma and sarcoplasmic reticulum from ADR mouse muscle were a decrease in 16:0 and 20:4, and an increase in both 18:0 and 18:2. These alterations may indicate a defect in fatty acid acylation in muscle membranes, the biochemical process important to the retailoring of membrane fatty acid composition. This retailoring consists largely of replacing saturated fatty acids with more unsaturated species [25]. The observed alterations in fatty acid composition could also reflect a change in phospholipid composition, e.g., increased proportions of phosphatidylinositol (which is enriched in 18:0) or decreased proportions of phosphatidylcholine or sphingomyelin (which are enriched in 16:0). Indeed, Rubello and Watts [21] have reported that the proportion of phosphatidylcholine was decreased in whole ADR muscle. In their studies, they detected no change in the total phospholipid per g muscle, and no change in the cholesterol/phospholipid ratio, therefore the amount of lipid in whole muscle was not altered. Rather, the relative proportions of phospholipids were changed. Furthermore, there were alterations in the fatty acid composition of particular phospholipids from whole ADR muscle, specifically a decrease in the mol% of 16:0 in phosphatidylethanolamine and phosphatidylcholine, and an increase in 18:1 and 18:2 in phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. They also detected a decrease in the mol% of 20:4 in phosphatidylcholine and phosphatidylserine and a slight increase in the mol% of 18:0 in phosphatidylcholine. In so far as whole muscle lipid composition can be compared with the composition of subcellular fractions, their results agree with the present findings.

In the ADR mouse, several secondary biochemical defects arise due to abnormal Cl^- conductance [26]. These include increased numbers of mitochondria [27], reduced parvalbumin and mRNA for parvalbumin (a calcium binding protein found in fast-twitch muscle fibers), increased mRNA for slow-muscle specific protein, and decreased phosphorylation of myosin light chain [28]. All of these effects are reversed by preventing myotonic episodes with the fast sodium channel blocker, tocainide [26]. The changes in fatty acid composition observed in these experiments may be secondary to alterations in Cl^- conductance, and could alter the environment of other proteins in the membranes, particularly if changes are confined to specific microdomains in the membrane. Based on calculations done with artificial membranes, a small decrease in mol% of 16:0 reduces the cholesterol-ordering coefficient, so that cholesterol is less effective in ordering the membrane lipids, thus resulting in a more fluid membrane [29]. Increasing the mol% of 20:4 or other unsaturated fatty acids has a similar effect. In detergent/lipid micelle systems, increasing the chain length and level of unsaturation of fatty acids in phosphatidylcholine increases the thermal stability of the membrane bound protein, rhodopsin [30]. Catalytic hydrogenation of plasma membrane fractions or whole cells, which reduces the DBI by 40% causes a proportional decrease in the fluidity of the membrane, and changes the density of cell-surface proteins [24]. In the present studies, we observed decreases in the DBI on the order of 10% to 20%.

Although most myotonias do not manifest as a generalized defect affecting other tissues, the fatty acid composition of erythrocytes has been studied as a potential, easily obtainable marker for changes in muscle membranes. For example, alterations of erythrocyte membrane fatty acid composition in paramyotonia congenita have been reported [6,31]. That study demonstrated small decreases in the proportions of unsaturated fatty acids, resulting in a elevation of the S:U ratio. Although no measurements of the fatty acid composition of muscle membranes were done, the authors suggested that changes in erythrocytes may be indicative of similar changes in muscle plasma membrane. Our results directly demonstrate that erythrocyte ghosts do not serve as an indicator of changes in muscle membrane fatty acid composition in the ADR mouse. The only change we observed in the fatty acid composition of erythrocyte ghosts was a decrease in 18:1, which in fact did not occur in muscle. This decrease in 18:1 was offset by small changes in other fatty acids so that neither the S:U ratio nor the DBI were altered. Furthermore, in a study comparing the fatty acid composition of sarcolemma, sarcoplasmic reticulum and erythrocyte ghosts from patients with facioscapulohumeral muscular dystrophy or myotonia congenita, we found that the muscle membrane fractions from the myotonia congenita patient showed substantial increases (172% in sarcolemma and 30% in sarcoplasmic reticulum) in the S:U ratios compared to muscle membrane fractions from the patient with facioscapulohumeral muscular dystrophy [32]. However, the S:U ratio of erythrocyte ghosts from the myotonic patient was actually 29% lower than the S:U ratio of erythrocyte ghosts from the muscular dystrophy patient or from normal subjects. Therefore, we failed to observe any similarities between changes in muscle membrane fractions and changes in erythrocyte ghosts in myotonia congenita. In light of these results, we suggest that the membrane of erythrocytes is not an appropriate model system for investigating alterations fatty acid composition of phospholipids in myotonic muscle.

Acknowledgements

This work was supported in part by the WVU Medical Corporation and NIH-BMRS grant No. 2SO RR05433-28 awarded to J Riggs. J. Lucci was a Van Liere Summer Fellow. The excellent technical assistance of Barbara J. Victor, M.S. is gratefully appreciated.

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