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CALCIUM TRANSPORT, ATPase ACTIVITY AND LIPID COMPOSITION IN SARCOPLASMIC RETICULUM ISOLATED FROM ISOGENIC LINES OF NORMAL AND DYSTROPHIC CHICKENS

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Two new lines of chickens with near identical genotypes (greater than 90% isogeneity), one demonstrating avian dystrophy, were used for isolation of sarcopalsmic reticulum vesicles. Vesicles from line 433 (dystrophic) displayed reduced Ca^{2+}-ATPase activity, phosphoenzyme formation and steady-state calcium transport capabilities in comparison with vesicles from line 03 (normal). Lipid analyses show that dystrophic vesicles have greater amounts of cholesterol and lesser amounts of phosphatidylcholine. The results support the use of isogenic chickens in further studies of avian dystrophy. However, the results also suggest that current sarcoplasmic reticulum vesicle purification procedures dependent on differential calcium accumulation may not fully achieve the intended purpose.

Introduction

Chickens afflicted with genetic muscular dystrophy have provided a useful model in attempting to understand similar conditions in humans. Although the exact causes of dystrophy have not been determined, it was first reported by Sreter et al. [1] that the sarcoplasmic reticulum from dystrophic chicken pectoralis muscle is less capable of calcium accumulation than is normal sarcoplasmic reticulum. Later investigations have reported a reduced Ca^{2+}-sensitive ATPase activity, phosphoenzyme formation and steady-state calcium transport in dystrophic chicken sarcoplasmic reticulum preparations [2–4]. Subsequently, it has been demonstrated that the amino-acid composition of the (Ca^{2+} + Mg^{2+})-ATPase from normal and dystrophic sarcoplasmic reticulum shows no differences [5] and when the (Ca^{2+} + Mg^{2+})-ATPase is solubilized in Triton, the ATPase activities are similar [6]. Consequently, recent investigations have focused on the lipid component of the sarcoplasmic reticulum membranes to explain the functional differences.

It has been stated that the genetic manifestation of dystrophy may be affected by background genes [7]. In recent years, efforts have been made to use dystrophic birds with closely related normal controls. In such investigations, chicken lines 412 (normal) and 413 (dystrophic) have been selected in which breeding has resulted in some genetic similarities [8]. However, Rushbrook et al. [9] have shown that in comparisons of myosin light chains obtained from 412 and 413 lines, the normal bird is genetically heterozygous for this protein, suggesting that line 412 birds may not necessarily represent the ideal control for line 413 birds.

Information on the lipid profiles of normal and

Abbreviation: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

dystrophic sarcoplasmic reticulum from chickens is limited and the available data is conflicting. Because cholesterol is present in large amounts in dystrophic muscle [10], it has been of interest to link dysfunction of lipid metabolism to avian dystrophy. Hsu and Kaldor [11] reported that dystrophic sarcoplasmic reticulum preparations contained much higher cholesterol and lower phosphatidylcholine than normal. Recently. Sumnicht and Sabbadini [12] reported somewhat different results, in which the dystrophic sarcoplasmic reticulum contained a much smaller increase in cholesetrol content, and phospholipid differences involved sphingomyelin rather than phosphatidylcholine. Interestingly, Mrak and Fleischer [13] found no major abnormalities in genetically dystrophic mice. If differences in normal and dystrophic chicken sarcoplasmic reticulum are represented in the lipid compositions, background genes may provide ambiguous results without appropriate control animals.

Recently, we have been able to obtain lines of chickens, line 03 (normal) and 433 (dystrophic), which through inbreeding have resulted in near identical genotypes differing by the genes for dystrophy. The birds from these two lines are considered to be greater than 90% isogenic [8]. We believe the use of these birds may minimize possible confusion derived from background gene contributions. The availability of nearly isogenic animals in studies of hereditary disorders represents a unique and significant situation. Dissimilarities between dystrophic and normal animals can be interpreted in terms of genes for dystrophy without what would otherwise be uncertainty based on whether or not observed differences might be attributable to background gene variations.

In this investigation, we have applied methods previously reported [6] to isolate sarcoplasmic reticulum from breast muscle of 03 and 433 birds. Biochemical analyses of these preparations are compared with those obtained from birds of lines 412 and 413 to establish the value of isogenic lines of chickens for further studies on avian dystrophy.

Materials and Methods

Isolation of sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum vesicles were isolated according to previously described methods employing calcium phosphate loading [6].

ATPase activity. Basal and Ca^{2+}-sensitive ATPase activities were determined by an enzyme-coupled assay described by Warren et al. [16].

Calcium transport and phosphoenzyme determination. Calcium transport and phosphoenzyme formation were determined by Millipore filtration methods previously described [6].

Lipid determination. Lipids were extracted in 2-times the sample volume with chloroform/methanol (2:1). The mixture was sonicated for 15 s with a Bronwill Biosonik sonicator at a setting of 30 with a small probe. The chloroform phase was recovered after centrifugation in a clinical centrifuge set to full speed for 5 min. The lipids were analyzed in an Iatroscan TH-10 Analyser in which thin-layer chromatography was carried out on silica sintered glass rods (chromarods-II) and analyzed by flame ionization detection. Chromarods were developed in hexane/diethyl ether (9:1) to resolve neutral lipids. The same rods were subsequently developed in $CHCl_3/CH_3OH/H_2O$ (65:25:4) to separate phospholipids. Major lipid species of cholesterol, phosphatidylcholine and phosphatidylethanolamine were investigated. Results are expressed as weight percent of total lipids.

SDS-polyacrylamide gel. Polyacrylamide gels were run using the system of Laemmli [14] as described by Weber and Osborn [15].

Results

Muscle tissue

The appearance of the excised muscle is similar in 03/433 and 412/413 chickens. In each case, the dystrophic breast muscle is characteristically rubbery and pale. However, there is a major difference in the overall muscle tissue yields (Table I). Dystrophic birds of line 413 undergo muscle hypertrophy during ex ovo development. During the ages of interest (6–8 weeks), dystrophic chickens have larger muscle mass than normal. The difference appears to be insignificant at 8 weeks, but this is probably the result of large variations in muscle size and small sample size. The situation is different in the isogenic lines. First, when compared to 412 and 413, in all cases, the overall tissue mass is far less for the isogenic chickens.

TABLE I

PECTORAL MUSCLE MASS OF NORMAL (412, 03) AND DYSTROPHIC (413, 433) BIRD LINES

Results are quested in g wet wt. Values are means ± S.E.; P values are based on Student's t-test.

Age (weeks)	Line 412	Line 413	t-test	Line 03	Line 433	t-test
6	23.3 ± 1.94	31.1 ± 2.92	$P < 0.05$	16.8 ± 0.6	12.1 ± 0.6	$P < 0.001$
7	43.5 ± 1.6	55.6 ± 2.7	$P < 0.001$	22.1 ± 0.6	15.8 ± 0.5	$P < 0.001$
8	61.4 ± 3.0	71.2 ± 4.8	n.s.	23.3 ± 0.3	14.3 ± 0.5	$P < 0.001$

Second, it is apparent that both normal and dystrophic birds of 412 and 413 lines show muscle growth through the 8th week. Yet in the isogenic lines, the muscle growth is much less and appears to cease between 7–8 weeks. Finally, the dystrophic chickens (line 433) show muscle atrophy rather than the hypertrophy observed in line 413 [8].

Crude microsomes

Table II shows the biochemical characterizations of the crude sarcoplasmic pellets prior to calcium phosphate loading. There is a slightly greater yield of microsomes per gram of muscle tissue from the normal than from the dystrophic chicken. Such a difference was not apparent in the 412 and 413 strain preparation, in which both yielded microsomes in quantities similar to the 03 line. As indicated, the dystrophic (433) bird is smaller than normal animals of equivalent age; however, the lesser yield is still sufficient for extensive biochemical determinations.

The basal ATPase activities are comparable between 412/03 and 413/433 lines. In each case, the dystrophic animals (413 and 433) exhibit significantly higher activities. Comparisons of the calcium-sensitive ATPase activities show the opposite difference in that both normal animals (412 and 03) demonstrate greater activities than dystrophic birds. However, the absolute values for both 03 and 433 animals are nearly twice as great as the values for their equivalent counterparts.

Phosphoenzyme and steady-state calcium transport values are both greater in the normal (03) chickens. These relationships are also consistent with the findings from 412- and 413-line birds.

Calcium phosphate-loaded vesicles

SDS-gels of the vesicle fractions are shown in Fig. 1. Following $CaSO_4$ loading, purification is reflected in the enrichment of the $(Ca^{2+} + Mg^{2+})$-ATPase (100 kDa) protein and calsequestrin (51 kDa) protein.

Table III shows the biochemical characterization of sarcoplasmic reticulum vesicles following calcium phosphate loading. Of the material re-

TABLE II

BIOCHEMICAL CHARACTERIZATION OF NORMAL AND DYSTROPHIC SARCOPLASMIC RETICULUM FROM ISOGENIC CHICKENS

Values are mean ± S.E. Number of animals tested is shown in parentheses.

Activity	Normal (03)	Dystrophic (433)	t-test
Yield (mg/g muscle)	0.75 ±0.11 (9)	0.54 ±0.05 (10)	$P < 0.05$
ATPase (μmol/mg per min)			
Basal	1.50 ±0.17 (9)	2.94 ±0.17 (9)	$P < 0.001$
Ca^{2+}-senstive	1.83 ±0.13 (9)	0.76 ±0.08 (9)	$P < 0.001$
Phosphoenzyme (nmol/mg)	3.46 ±0.39 (9)	1.24 ±0.17 (10)	$P < 0.001$
Steady-state Ca^{2+} transport (μmol/mg)	0.094 ± 0.012 (10)	0.050 ± 0.006 (10)	$P < 0.01$

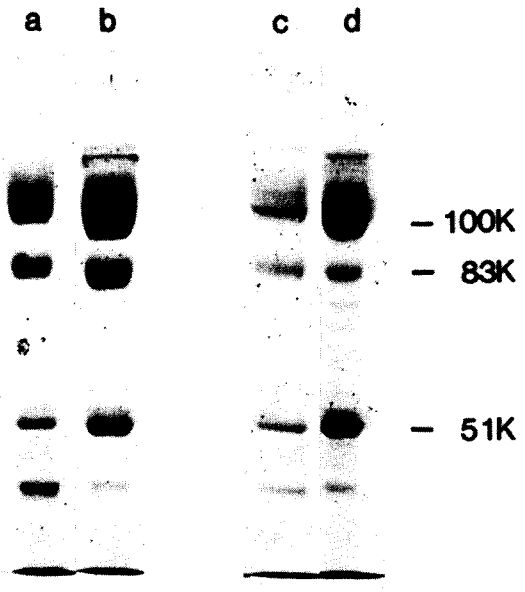

Fig. 1. $7\frac{1}{2}$ SDS gels of vesicle fractions (15 μg per lane). (a) Normal crude microsomes. (b) Normal 'loaded' sarcoplasmic reticulum. (c) Dystrophic crude microsomes. (d) Dystrophic 'loaded' sarcoplasmic reticulum.

covered, a greater percentage of vesicles in normal animals is found to accumulate calcium. Similar results were reported for 412 and 413 chickens. Whether the initial dystrophic microsomal pellet contains a proportionally larger amount of non-sarcoplasmic reticulum membranes or whether the dystrophic sarcoplasmic reticulum fraction contains populations which cannot accumulate calcium is not clear.

As reported for 412 and 413 birds, the 03 (normal) chickens show greater calcium-sensitive ATPase, phosphoenzyme and steady-state transport values than do the 433 (dystrophic) chickens. In both the normal and dystrophic preparations, the loaded vesicles exhibit much reduced basal ATPase activities. Conversely, the calcium-sensitive ATPase activities and the steady-state transport value (for normal) are enhanced. Furthermore, when the activity values are expressed as percent of normal, generally, the 03/433 lines express much greater differences than shown in 412/413 comparisons (Table III).

Analysis of the major lipid species found in crude microsomes and calcium phosphate-loaded vesicles is shown in Table IV. Based on weight percent of total lipid, phosphatidylcholine represents the major lipid class in normal crude sarcoplasmic reticulum. However, in dystrophic crude sarcoplasmic reticulum preparations, phosphatidylcholine and cholesterol are found to be the dominant lipid species. Also, the data show a significantly greater weight percentage of cholesterol and a lesser percentage of phosphatidylcholine in dystrophic animals. However, the extremely high cholesetrol content in dystrophic microsomes may not be solely attributable to membrane lipids. The generally greater cholesterol content in dystrophic muscle suggests the possibility of non-membrane lipids including cellular and serum cholesterol. Similar differences are also detected in calcium phosphate-loaded vesicles. However, in the loaded dystrophic fraction, the weight percentage of cholesterol has dropped significantly

TABLE III

BIOCHEMICAL CHARACTERIZATION OF CALCIUM PHOSPHATE-LOADED VESICLES

Values are mean ± S.E. Number of animals tested is shown in parentheses.

Activity	Normal (03)	Dystrophic (433)	*t*-test	% of Normal	
				$\frac{433}{03}$	$\frac{413}{412}$
Percent loaded (of recovered protein)	50.0 ±4,2 (10)	35.0 ±4.7 (10)	$P < 0.001$		
ATPase (μmol/mg per min)					
Basal	0.50 ±0.05 (9)	2.25 ±0.18 (9)	$P < 0.001$	450	163
Ca^{2+}-sensitive	2.29 ±0.25 (9)	0.99 ±0.09 (9)	$P < 0.001$	43	76
Phosphoenzyme (nmol/mg)	2.71 ±0.010 (10)	0.055 ±0.007 (9)	$P < 0.001$	39	65
Steady-state Ca^{2+} transport (μmol/mg)	0.140 ±0.010 (10)	0.055 ±0.007 (9)	$P < 0.001$	39	65

TABLE IV

LIPID ANALYSIS OF NORMAL AND DYSTROPHIC SARCOPLASMIC RETICULUM FROM ISOGENIC CHICKENS

Values are mean ± S.E. of the weight percentage of total lipid. Number of animals tested is shown in parentheses. n.s., not significant.

Microsomes	Lipid	Normal (03)	Dystrophic (433)	*t*-test
Crude microsomes	cholesterol	15.9 ± 2.7 (4)	34.9 ± 5.5 (4)	$P < 0.001$
	phosphatidylcholine	54.7 ± 3.8 (4)	35.2 ± 7.1 (4)	$P < 0.01$
	phosphatidylethanolamine	18.9 ± 3.3 (4)	17.6 ± 8.6 (4)	n.s.
Calcium phosphate-loaded vesicles	cholesterol	7.6 ± 0.6 (4)	22.0 ± 3.6 (4)	$P < 0.01$
	phosphatidylcholine	66.7 ± 3.9 (4)	49.3 ± 3.9 (4)	$P < 0.02$
	phosphatidylethanolamine	14.0 ± 1.4 (4)	15.9 ± 0.7 (4)	n.s.

with a simultaneous rise in the phosphatidylcholine content. The level of phosphatidylethanolamine was shown not to differ between animals or treatments.

Discussion

The biochemical characterization of sarcoplasmic reticulum vesicles isolated from normal (03) and dystrophic (433) chickens correlate well with the results previously reported for chickens of lines 412 and 413 [6]. Following Ca^{2+}-loading purification, both normal and dystrophic preparations show a significant decrease in the basal ATPase activity but, surprisingly, only minor increases in calcium stimulated values. Hydrostatic pressures generated during ultracentrifugation have been found to inactivate the $(Ca^{2+} + Mg^{2+})$-ATPase [17]. However, 0.3 M sucrose was found to prevent the inactivation. In our gradient centrifugation, sucrose concentrations exceed 0.3 M; consequently, we believe ultracentrifugation is not responsible for the lack of specific activity enhancement. We have found (data not shown) that when assays of the crude sarcoplasmic reticulum from either animal are repeated after a 24 h interval, the Ca^{2+}-sensitive ATPase value is much reduced in the second measurement, suggesting inactivation may occur spontaneously over time. Consequently, in these preparations, prolonged purification procedures may not necessarily be paralleled by enhanced activity.

Some degree of purification is evident as a slight increase in calcium-sensitive ATPase activity and enrichment in the $(Ca^{2+} + Mg^{2+})$-ATPase protein is observed. The decrease in basal ATPase activity, coupled with a shift in lipid composition is also suggestive of purification. The slight decrease in the phosphoenzyme level in normal sarcoplasmic reticulum despite modest increases in Ca^{2+}-ATPase and transport values suggests that some uncoupling may have also occurred during purification. Vesicles of transverse tubule (T-tubule) origin from rabbit skeletal muscle have been demonstrated to have a high Mg^{2+}-ATPase (basal) activity [18] as well as higher cholesterol and lower phosphatidylcholine content than sarcoplasmic reticulum membranes [19]. Similar results exist in chicken microsomes following calcium phosphate loading. This observation strongly suggests that contaminating T-tubules were selectively removed from our sarcoplasmic reticulum preparations.

As indicated earlier, the published data on lipid in normal and dystrophic sarcoplasmic reticulum membranes is conflicting, although there seems to be agreement on the increased levels of cholesterol in dystrophic birds [11,12]. Possible differences in phospholipid content are still questionable. Our analysis of cholesterol levels in sarcoplasmic reticulum vesicles agrees with published results. In particular, the cholesterol/phospholipid ratio in normal is in good agreement with others [12]. The decreased level of phosphatidylcholine in dystrophic sarciplasmic reticulum in our preparations is in agreement with the findings of Hsu and Kaldor [11], though not with Sumnicht and Sabbadini [12]. However, any assessment of lipid composition is tenuous as each study employed differ-

ent lines of chickens. Consequently, at this time it would be difficult to establish those lipid differences that are truly attributable to dystrophy.

Chicken T-tubules have been shown to contain a greater amount of cholesterol than does the sarcoplasmic reticulum [12]. As such, it is reasonable to believe that because loaded vesicles show a lower cholesterol content, sarcoplasmic reticulum and T-tubule vesicles are being segregated by this procedure. Loaded dystrophic vesicles maintain elevated cholesterol, and it is possible to interpret this as a condition of dystrophy. However, reported comparisons of lipids in rabbit T-tubules and sarcoplasmic reticulum suggest that it may also be an indication of incomplete removal of surface membrane contamination.

Sarcoplasmic reticulum vesicle purification based on calcium loading (either with phosphate or oxalate as the precipitant anion), unfortunately, does not preclude the possibility of T-tubule contamination since they also accumulate calcium (data not shown). Furthermore, T-tubules of rabbit have been shown to have Ca^{2+}-sensitive ATPase activity [18,20]. The procedure also fails to exclude the likelihood of aggregation causing co-migration of T-tubule and sarcoplasmic reticulum vesicles to regions of a sucrose density gradient assumed to be available only to sarcoplasmic reticulum membranes. Consequently, it is feasible that lipid differences between normal and dystrophic sarcoplasmic reticulum vesicles can be explained by the degree to which surface membranes are a contaminant. Stereologic analysis of muscle fibers from dystrophic chicken has shown significantly greater amounts of T-system membrane than normal [21]. Therefore, at least in the case of dystrophic preparations, T-tubules must be considered as a serious source of contamination. A prerequisite to resolving lipid disparities between normal and dystrophic sarcoplasmic reticulum is the categorical elimination of non-sarcoplasmic reticulum membrane contamination.

The biochemical characterization which we have presented here for normal and dystrophic sarcoplasmic reticulum vesicles of chickens from lines 03 and 433, respectively, is consistent with previously reported results for chickens of lines 412 and 413 with one major exception. The activity differences represent a much greater differential between normal and dystrophic animals than previously reported. This suggests that other differences attributable to dystrophy might also be more pronounced in isogenic chickens. Thus, we believe the use of isogenic chickens is supported in further investigations of avian dystrophy. It would seem that the isogenic quality of these birds is a major asset, as possible perturbations resulting from background genes is minimized and symptoms characteristic of dystrophy maximized.

The high cholesterol values observed in dystrophic sarcoplasmic reticulum implies that a purification procedure based on preferential calcium loading may be inadequate to remove contaminating surface membranes. Consequently, we are pursuing alternate isolation protocols in a systematic effort to obtain the purest possible sarcoplasmic reticulum membrane preparations from isogenic chickens.

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