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ENZYMATIC ACTIVITY OF DYSTROPHIC CHICKEN SARCOPLASMIC RETICULUM

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Summary

We have isolated sarcoplasmic reticulum from normal and dystrophic chicken muscle, using an improved isolation procedure. Dystrophic sarcoplasmic reticulum has a reduced level of calcium-sensitive ATPase activity, phosphoenzyme formation, and steady-state calcium transport. Anion-stimulated calcium transport by dystrophic sarcoplasmic reticulum is also reduced when measured under the proper conditions, and dystrophic sarcoplasmic reticulum shows no alteration in calcium efflux rate. Active calcium phosphate loading of the normal and dystrophic sarcoplasmic reticulum preparations indicates that a reduced percentage of the dystrophic vesicles are capable of active calcium transport. The loaded dystrophic sarcoplasmic reticulum vesicles exhibit the same relative reductions in enzymatic activity as the starting sarcoplasmic reticulum preparations. However, the enzyme activities of normal and dystrophic sarcoplasmic reticulum are similar in the presence of detergent and exogenous phospholipid. On the basis of these results, we suggest that the lipid microenvironment of the dystrophic enzyme is altered.

Introduction

Previous studies from this and other laboratories have demonstrated the existence of major biochemical alterations in isolated dystrophic chicken sarcoplasmic reticulum. Reductions of calcium-sensitive ATPase activity, phosphoenzyme level, sarcoplasmic reticulum yield, and steady-state level of calcium

Abbreviations: SDS, sodium dodecyl sulfate; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

transport have been reported [1—3]. In contrast, the rate of oxalate-potentiated calcium transport is reduced only in adult dystrophic sarcoplasmic reticulum [4], while this rate of transport is either increased [1,4,5] or unchanged in chickens 8 weeks of age or younger.

On a structural level, dystrophic chicken sarcoplasmic reticulum exhibits both a reduced particle density on freeze-fracture faces and a reduced protein to lipid ratio [3,6]. The protein composition of dystrophic sarcoplasmic reticulum is similar to that of normal sarcoplasmic reticulum as indicated by SDS-polyacrylamide gel electrophoresis [1,3,7]. Furthermore, the ATPase enzyme of dystrophic chicken sarcoplasmic reticulum has been examined by total amino acid analysis, isoelectric focusing, and petide-mapping methods, and appears identical to the normal ATPase protein [7].

Several important questions about the impairment of dystrophic chicken sarcoplasmic reticulum remain unanswered. First, the increased or unaltered calcium transport rate in the presence of oxalate is at odds with the observed reduction in calcium-sensitive ATPase activity, phosphoenzyme formation and steady-state calcium transport. Second, since it has been demonstrated that the protein composition of dystrophic chicken sarcoplasmic reticulum is not altered, the primary defect may be either an amount of inactive dystrophic protein or an alteration in the dystrophic protein-lipid interaction. Third, the biochemical alterations in dystrophic chicken sarcoplasmic reticulum could be due to either a reduced function in all of the isolated dystrophic sarcoplasmic reticulum vesicles or the presence of two discrete vesicle populations, one active and one inactive. In the latter case, the inactive vesicles might come from muscle fibers affected by the dystrophic process, while the active vesicles could come from fibers as yet unaffected by the dystrophic process.

The present work is an effort to further elucidate the mechanism(s) of alteration of dystrophic chicken sarcoplasmic reticulum. Using an improved sarcoplasmic reticulum isolation procedure, we have firmly established the biochemical differences that exist in dystrophic sarcoplasmic reticulum. These differences are not eliminated after the purification of 'active' vesicles by a calcium phosphate-loading procedure, but any differences in calcium-dependent ATPase activity are eliminated by high detergent or detergent plus phospholipid concentration. This implicates the lipid portion of the membrane in the dystrophic process.

Materials and Methods

Isolation of sarcoplasmic reticulum vesicles

Crude pellet. Pectoralis major and supracoracoideus muscles from normal chicken (line 412) and dystrophic chicken (line 413) ranging in age from 6 to 8 weeks were excised and used for the isolation of the microsomal fraction. Muscle tissues from each chicken were minced and placed in ice-cold buffer (50 mM Tes/0.25 M sucrose, pH 7.0). Volume of each muscle mass was determined and the volume of buffer was adjusted to equal 6-times the tissue volume. Homogenization was accomplished using a Waring blender set at high speed for 15 s at 5-min intervals for a total homogenization time of 60 s. After each 15 s

step, pH value of the homogenate was checked and adjusted back to neutral by the addition of 6 M KOH.

The homogenate was centrifuged at 8000 × *g* for 15 min. The supernatant was collected and placed on ice while the pellet was combined with buffer equalling 3-times the original tissue volume. This suspension was homogenized again for 15 s and the pH adjusted. The homogenate was centrifuged as before and the resulting supernatant was combined with the first supernatant fraction. The suspension was filtered through glass wool and centrifuged at 8000 × *g* for 30 min. The supernatant was collected and centrifuged at 43 000 × *g* for 45 min. The resulting pellet was suspended in buffer (10.0 mM Tes/0.6 M KCl, pH 7.0) and homogenized in a glass homogenizer. The homogenate was then centrifuged a final time at 43 000 × *g* for 45 min. The crude sarcoplasmic reticulum pellet recovered was homogenized in a small volume of buffer (10.0 mM Tes/ 1.0 mM KCl).

Purification of the crude sarcoplasmic reticulum. 5 mg of sarcoplasmic reticulum (by protein) was loaded with $Ca_3(PO_4)_2$ in the following manner. A reaction mixture totaling 5 ml containing 5 mM ATP, 20.0 mM potassium phosphate and the appropriate amount of buffer (50.0 mM Tes/4.0 mM $MgCl_2$/ 100.0 mM KCl/0.1 mM EDTA, pH 7.0) was used. 50 μl of 100 mM $CaCl_2$ was added 5-times at 2-min intervals. After the second addition of Ca^{2+}, an additional 250 μl of 100 mM ATP and 100 μl of potassium phosphate was added. After the fourth addition of Ca^{2+}, 250 μl of ATP was again added. The resulting mixture was placed on a discontinuous sucrose gradient (30%, 40%, 50%) and centrifuged in a Beckman L5-75 ultracentrifuge for 1 h at 210 000 × *g*. Four distinct fractions were recovered, the supernatant/30% interface, the 30% layer and 30/40% interface, the 40% layer and 40/50% interface, and the 50% layer and pellet.

Each fraction was kept on ice and allowed to unload Ca^{2+} overnight in 2 ml of buffer (10.0 mM Tes, pH 7.0) containing 2.0 mM EGTA. After the unloading step, the fractions obtained from the 30/40% and 40/50% interfaces were each placed on another sucrose gradient (30%, 40%, 50%). These were centrifuged at 210 000 × *g* for 60 min. Each sample resulted in two fractions. The lighter fractions were collected and combined with the pellet originally collected in the 50% sucrose fraction. This was regarded as the collection of vesicles capable of accumulating Ca^{2+} and is referred to as 'loaded sarcoplasmic reticulum'. The heavier fraction was also collected and combined with the supernatant/30% sucrose interface fraction previously recovered. This collection of vesicles was considered to be not capable of sequestering Ca^{2+} and is referred to as 'not-loaded sarcoplasmic reticulum'. The resulting four fractions, dystrophic 'loaded' and 'not-loaded' and normal 'loaded' and 'not-loaded' were placed on a 60% sucrose shelf and centrifuged at 210 000 × *g* for 30 min to concentrate the vesicles. Pellets recovered from the 60% shelf were resuspended by shear forces generated by passage through a pipette tip. Protein determination was by a modification of methods described by Bradford [8] using a Bio-rad protein dye.

Phosphoenzyme

Phosphoenzyme was determined by Millipore filtration. Reaction mixture

consisted of 0.04 mg sarcoplasmic reticulum and buffer (50 mM Tes/4 mM $MgCl_2$/100 mM KCl/0.1 mM EDTA, pH 7.0) to bring the volume to 0.19 ml. Each sample was assayed with and without 0.5 mM $CaCl_2$. The reaction was started by adding 0.1 mM [^{32}P]ATP. Each mixture was immediately agitated and placed in an ice-water bath. 30 s following the addition of ATP, 300 μl of quenching solution (5% trichloroacetic acid/1 mM KH_2PO_4) was added to terminate the reaction. A 400 μl sample from each mixture was filtered through a 0.45 μm Millipore filter and chased with 3 ml of 5% trichloroacetic acid. The filters were dissolved overnight in 10 ml of Aquasol scintillation cocktail and counted. Specific activity of the ATP was determined by counting three samples of 5 μl each from the stock solution of 2 mM ATP and [^{32}P]ATP (ICN). Phosphoenzyme values were determined by calculating the net counts per sample (counts with Ca^{2+} minus counts without Ca^{2+}) and expressed in terms of nmol/mg sarcoplasmic reticulum.

Calcium transport

Calcium transport was assayed by Millipore filtration. Transport in the presence of 5 mM potassium oxalate or 20 mM potassium phosphate was performed using a sarcoplasmic reticulum concentration of 0.03 mg/ml, and transport in the absence of precipitant anions (steady state) was performed using a sarcoplasmic reticulum concentration of 0.1 mg/ml. The transport assay mix contained 50 mM Tes, 0.2 mM $^{45}CaCl_2$, 5 mM ATP, 0.4 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA at pH 7.0. At the appropriate times, 3 ml were filtered through a 0.45 μm Millipore filter. Filters were dissolved in 15 ml Aquasol overnight and counted.

ATPase assay

Calcium-sensitive ATPase activity was determined by an enzyme coupled assay described by Warren et al. [9]; the assay was performed in 1 ml cuvettes with a sarcoplasmic reticulum protein concentration of 0.01 mg/ml.

Succinate dehydrogenase and (Na^+ + K^+)-ATPase activities

Degree of contamination by mitochondria and sarcolemma was checked by assaying for succinate dehydrogenase activity and (Na^+ + K^+)-ATPase activity. Succinate dehydrogenase activity was determined by the method of Mircheff and Wright [10], and (Na^+ + K^+)-ATPase activity was determined by the method of Garaham et al. [11], which is based on the potassium-stimulated hydrolysis of the artificial substrate *p*-nitrophenyl phosphate.

Calcium efflux

Calcium efflux experiments were performed by incubating 0.5 mg sarcoplasmic reticulum in 0.2 mM $^{45}CaCl_2$, 5 mM acetyl phosphate, 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EGTA, 20 mM potassium phosphate, and 50 mM Tes, pH 7.0, in a total volume of 0.5 ml. After 15—20 min, 19.5 ml of 1 mM EGTA/100 mM KCl/50 mM Tes, pH 7.0, were added to the samples. Samples of 3 ml were then filtered through 0.45 μm Millipore filters at 0, 5, 10 and 15 min. The filters were dissolved in 15 ml of Aquasol overnight and counted.

Polyacrylamide gel electrophoresis

Polyacrylamide gels were run using the system of Laemmli [12] as described by Weber and Osborn [13].

ESR measurements

ESR measurements were made according to the method of Eckstein et al. [14]. Samples of normal and dystrophic sarcoplasmic reticulum from three different preparations were run in triplicate and the results were averaged.

Chemicals

Unless otherwise specified, all chemicals used were of the highest grade available from Sigma (St. Louis, MO).

Results

Biochemical characterization

The biochemistry of the isolated normal and dystrophic sarcoplasmic reticulum, as described in Table I, demonstrates significant alterations in dystrophic sarcoplasmic reticulum. It should be noted that most of the activities measured are higher than we measured using another muscle homogenization method [1]. We attribute this improvement in sarcoplasmic reticulum activities to the careful maintenance of neutral pH during homogenization, as suggested by Scales et al. [3]. Using the present isolation method, we find that dystrophic sarcoplasmic reticulum has a yield comparable to that of normal sarcoplasmic reticulum. Dystrophic sarcoplasmic reticulum also exhibits reduced calcium-sensitive ATPase activity, phosphoenzyme level, and steady-state calcium transport level. The basal ATPase of dystrophic sarcoplasmic reticulum is not significantly different from that of normal sarcoplasmic reticulum. The calcium-sensitive ATPase rate in the presence of 50 mM Triton X-100 is also unchanged in dystrophic sarcoplasmic reticulum, suggesting that the dystrophic ATPase enzyme may be released from a relatively restrictive membrane environment by detergent solubilization.

TABLE I

BIOCHEMICAL CHARACTERIZATION OF NORMAL AND DYSTROPHIC SARCOPLASMIC RETICULUM

Results are mean ± S.E. n.s., not significant.

Activity	Normal	Dystrophic	*t*-test
Yield (mg/g muscle)	0.80 ± 0.04	0.87 ± 0.06	n.s.
ATPase (μmol/mg per min)			
Basal	2.66 ± 0.20	3.38 ± 0.33	n.s.
Ca^{2+} sensitive	1.02 ± 0.10	0.44 ± 0.06	$P < 0.001$
Ca^{2+} sensitive + 50 mM Triton	1.79 ± 0.22	1.34 ± 0.17	n.s.
Phosphoenzyme (nmol/mg)	3.29 ± 0.47	2.00 ± 0.23	$P < 0.02$
Steady-state transport (μmol/mg)	0.132 ± 0.015	0.070 ± 0.004	$P < 0.005$
Potassium *p*-nitrophenylphosphatase (μmol/mg per h)	0.79 ± 0.09	2.11 ± 0.38	$P < 0.01$
Succinate dehydrogenase (μmol/mg per h)	0.73 ± 0.11	0.74 ± 0.09	n.s.

The differences in steady-state calcium transport levels are probably even greater than indicated by our results. We measured calcium transport in terms of μmol of calcium transported/mg of protein, not in terms of the calcium gradient across the membrane. As dystrophic sarcoplasmic reticulum has been shown to have an increased amount of phospholipid/mg of protein, dystrophic sarcoplasmic reticulum vesicles should have a greater membrane surface area and intravesicular volume/mg of protein than normal sarcoplasmic reticulum. Each μmol of calcium transported/mg of dystrophic protein would therefore be transported into a greater intravesicular volume than in normal sarcoplasmic reticulum, generating less of a calcium gradient in the process. As a result, the differences we find in steady-state levels of calcium transport are less than the differences in magnitude of calcium gradient that are developed by normal and dystrophic sarcoplasmic reticulum.

Recent investigations of the basal ATPase activity in chicken and rabbit sarcoplasmic reticulum have indicated that this enzyme activity is primarily associated with the sarcolemma and/or mitochondria [15,16]. The presence of this enzyme activity in our preparation may therefore be a result of contamination by other organelles.

Dystrophic sarcoplasmic reticulum contains a higher specific activity of $(Na^+ + K^+)$-ATPase than the normal sarcoplasmic reticulum. This may indicate the presence of a greater amount of sarcolemmal contamination in the dystrophic preparation or a higher specific activity of the $(Na^+ + K^+)$-ATPase found in dystrophic sarcolemma. The degree of contamination of both preparations by mitochondria, based on succinate dehydrogenase activity, appears similar.

ESR results

The alterations in the biochemical activities that have been demonstrated in dystrophic sarcoplasmic reticulum could be readily explained by an overall reduction in the fluidity of the dystrophic sarcoplasmic reticulum membrane. A reduction in the fluidity of the dystrophic sarcoplasmic reticulum membrane would presumably cause a reduction in the enzyme activity of the $(Ca^{2+} + Mg^{2+})$-ATPase, which would then explain the biochemical alterations observed in dystrophic sarcoplasmic reticulum.

We have measured the fluidity of normal and dystrophic sarcoplasmic reticulum membranes by ESR, using the fatty acid probe 5-doxylstearic acid. We then calculated three different parameters from the ESR spectra. These were the order parameters, S, the isotropic coupling constant, a, and the half-width at half-height for the low-field line, $W_{1/2}$, as suggested by Eckstein et al. [14].

TABLE II

ESR RESULTS

Results are mean ± S.E.

	Normal	Dystrophic
S	0.666 ± 0.001	0.669 ± 0.003
a	29.32 ± 0.03	29.39 ± 0.10
$W_{1/2}$	4.00 ± 0.03	4.11 ± 0.23

A difference in any one of these parameters would have indicated an alteration in membrane fluidity of dystrophic sarcoplasmic reticulum. As indicated in Table II, we find no differences in fluidity between normal and dystrophic sarcoplasmic reticulum. Therefore, using the 5-doxylstearic acid label, we cannot demonstrate any reduction in fluidity of the dystrophic sarcoplasmic reticulum membrane. However, ESR measurements using this particular probe have recently been shown to be relatively insensitive to the condensing effect of cholesterol [22]. Our ESR results are consistent with those of Eckstein et al. [14], which showed no fluidity differences between normal and dystrophic chicken erythrocytes. In view of the possible lack of sensitivity with the fatty acid probe used and the fact that we did not obtain a positive difference in ESR spectra, the ESR results must be viewed as inconclusive.

Polyacrylamide gel electrophoresis

SDS-polyacrylamide gels of normal and dystrophic sarcoplasmic reticulum are presented in Fig. 1. Three discrete bands appear in a molecular weight range typical of the $(Ca^{2+} + Mg^{2+})$-ATPase, 103 000—112 000. These bands may correspond to the three $(Ca^{2+} + Mg^{2+})$-ATPase found by isoelectric focusing [7], although isoelectric focusing of sarcoplasmic reticulum proteins tends to produce multiple banding artifacts [17]. We do not know if these multiple bands all represent active units or subunits of the $(Ca^{2+} + Mg^{2+})$-ATPase, or if one or two of the bands are due to contamination. The relative amounts of the

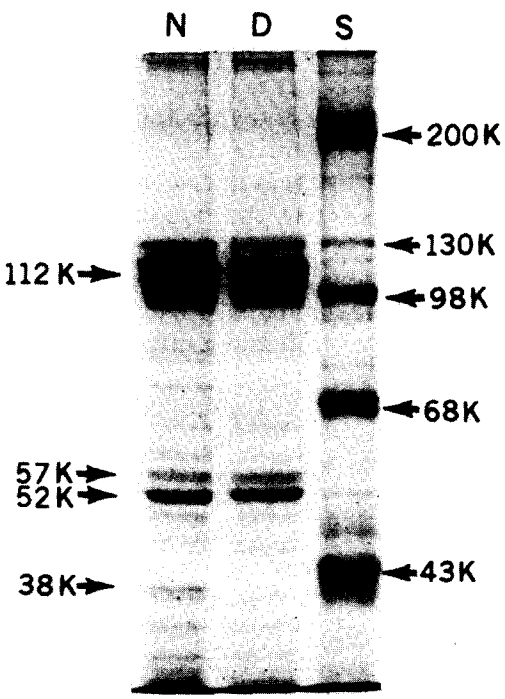

Fig. 1. Polyacrylamide gel electrophoresis. Samples of 10 μg were run on a 12-slot 7.5% acrylamide slab gel. N, normal sarcoplasmic reticulum; D, dystrophic sarcoplasmic reticulum, and S, standards.

three bands in the (Ca^{2+} + Mg^{2+})-ATPase region of the gels are similar in normal and dystrophic sarcoplasmic reticulum. Two other major bands are present on both gels, with molecular weights of 57 000 and 52 000. In addition, normal sarcoplasmic reticulum contains a minor band at 38 000 daltons which appears absent from dystrophic sarcoplasmic reticulum. The 57 000 and 52 000 dalton bands may correspond to the high-affinity calcium-binding protein and calsequestrin, respectively, as described in rabbit sarcoplasmic reticulum [18,19]. However, we cannot be certain of the identity of these two lower molecular weight bands. Calsequestrin has been isolated from normal and dystrophic sarcoplasmic reticulum, and the two calsequestrins have identical molecular weights of 44 000 (Yap, J.L. and MacLennan, D.H., unpublished results). We did not find a protein band with a molecular weight of 44 000, but this could be attributed to differences in gel systems and acrylamide percentages. The 38 000 dalton band may be one of the acidic proteins [19] and we do not know its function.

Anion-stimulated calcium transport

Previous studies from this and other laboratories have shown that the rate of oxalate-stimulated calcium transport in dystrophic chicken sarcoplasmic reticulum is either unchanged or increased relative to normal sarcoplasmic reticulum [1,3—5]. This observation is inconsistent with the observed reductions in other biochemical activities of dystrophic sarcoplasmic reticulum. In particular, the increased or unchanged rate of oxalate-stimulated calcium transport by dystrophic sarcoplasmic reticulum is directly at odds with the observed reduction in the rate of calcium transport by dystrophic sarcoplasmic reticulum in the absence of divalent anions, as measured by rapid kinetic methods [2,3]. The measurement of anion-stimulated calcium transport is a non-physiological phenomenon that permits estimates of calcium transport by precipitating intravesicular calcium concentration at low levels. Comparative differences in calcium transport are assumed to represent actual differences in calcium transport rates, assuming free movement of divalent anions across the sarcoplasmic reticulum membrane. Under these conditions, differences in anion permeability might give misleading calcium transport rates, and we have therefore attempted to ascertain whether or not the anomalous anion-stimulated calcium transport rates in normal and dystrophic chicken sarcoplasmic reticulum might be due to differences in anion permeability of the membranes.

We initially attempted to measure the permeability of sarcoplasmic reticulum membranes to [^{14}C]oxalate using the Millipore filtration procedure, but high levels of background [^{14}C]oxalate binding to the filters precluded the use of this method. As a result, we have investigated the anion permeability of chicken sarcoplasmic reticulum membranes by a more indirect method. We have measured the calcium transport rates of normal and dystrophic sarcoplasmic reticulum under three sets of conditions: (1) calcium transport in the presence of ATP and 5 mM oxalate; (2) calcium transport in the presence of ATP and 20 mM phosphate, and (3) calcium transport in the presence of acetyl phosphate and 20 mM phosphate. Calcium transport rates are highest under the conditions in (1) and lowest under the conditions in (3). If the anion permeabilities of the two sarcoplasmic reticulum preparations are the same, then

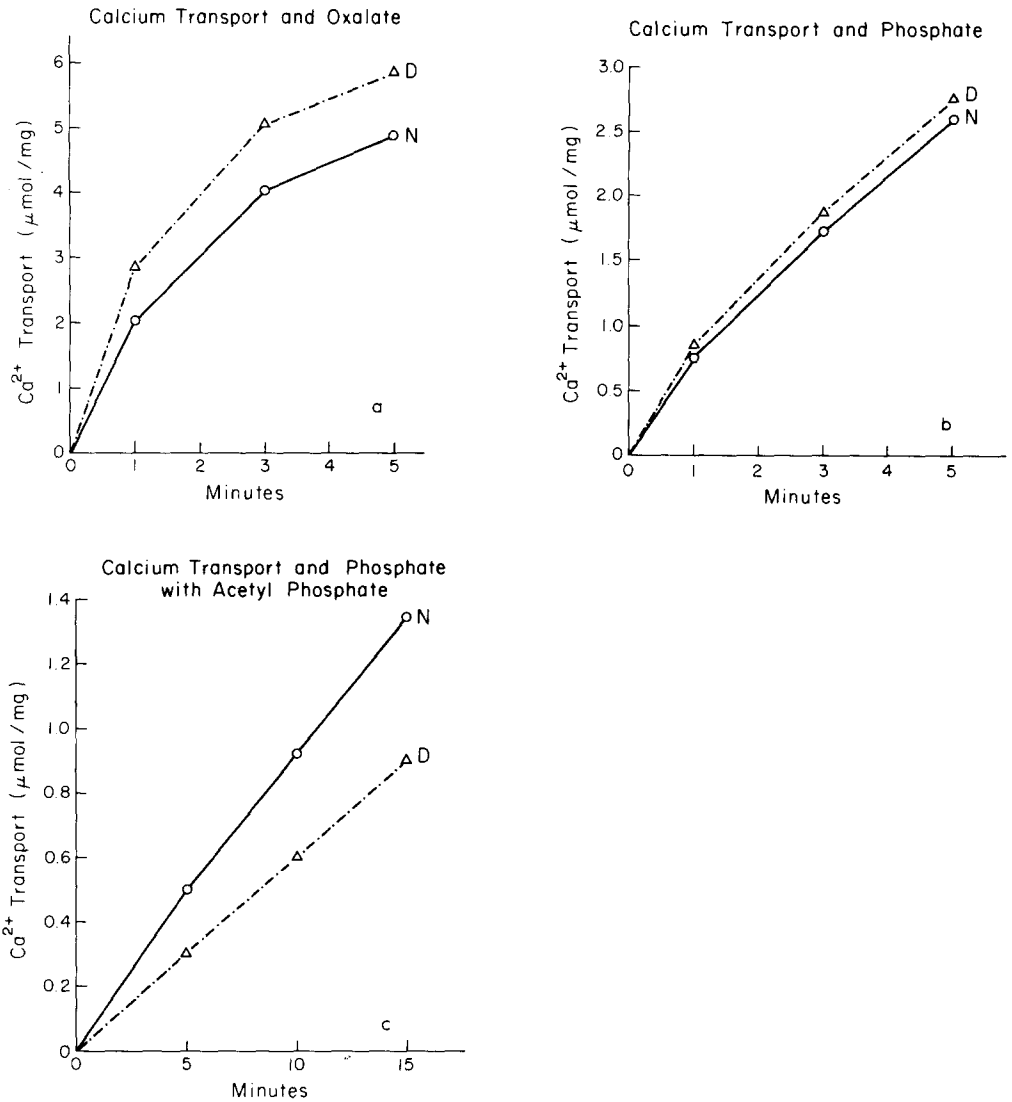

Fig. 2. Anion-stimulated calcium transport. Note the differences in the scales. Transport measurements were performed as described in Materials and Methods. N, normal sarcoplasmic reticulum; D, dystrophic sarcoplasmic reticulum. (a) Calcium transport in the presence of 5 mM potassium oxalate and 5 mM ATP. (b) Calcium transport in the presence of 20 mM potassium phosphate and 5 mM ATP. (c) Calcium transport in the presence of 20 mM potassium phosphate and 5 mM acetyl phosphate.

the relative differences in calcium transport rates between the two membranes should be the same under all three sets of conditions. However, if the normal sarcoplasmic reticulum membrane is less permeable to divalent anions, its rate of calcium transport relative to the dystrophic sarcoplasmic reticulum membrane should increase with decreasing rate of calcium transport.

The results are shown in Fig. 2a—c. As expected, the calcium transport rates of both membranes are highest in the presence of oxalate (Fig. 2a) and lowest

in the presence of acetyl phosphate and phosphate (Fig. 2c). Under the fastest calcium transport conditions (Fig. 2a), the dystrophic sarcoplasmic reticulum is slightly faster than the normal sarcoplasmic reticulum. Under the intermediate calcium transport conditions, the dystrophic and normal sarcoplasmic reticulum have virtually identical calcium transport rates (Fig. 2b). However, under the slowest calcium transport conditions the dystrophic sarcoplasmic reticulum shows the expected reduction in calcium transport rates relative to normal sarcoplasmic reticulum (Fig. 2c). This implies that a difference exists in the anion permeabilities of the two membranes, and that the calcium transport rates measured in the presence of oxalate represent a condition where anion permeability, not calcium transport, is the rate-limiting factor in normal sarcoplasmic reticulum.

Calcium efflux

Sabbadini and Inesi [20] have shown no alteration in the rate of calcium efflux from dystrophic sarcoplasmic reticulum, although they did observe a faster efflux rate than in mammalian sarcoplasmic reticulum. Using a slightly different experimental procedure, we also find no alteration in the rate of calcium efflux from dystrophic sarcoplasmic reticulum (Fig. 3), and we find a slower efflux rate than in lobster sarcoplasmic reticulum. Lobster sarcoplasmic reticulum is an exceptionally active preparation, so any conclusions about chicken sarcoplasmic reticulum function should not be based upon the assumption of an increased rate of calcium efflux relative to the sarcoplasmic reticulum of other species.

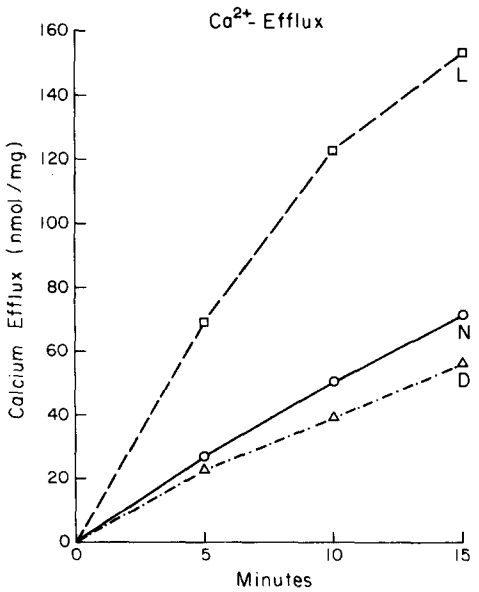

Fig. 3. Calcium efflux. Calcium efflux was measured as described in Materials and Methods. N, normal sarcoplasmic reticulum; D, dystrophic sarcoplasmic reticulum, and L, lobster sarcoplasmic reticulum.

Detergent-lipid interactions with sarcoplasmic reticulum

It has been previously postulated that the major site of alteration in dystrophic sarcoplasmic reticulum could be the lipid environment of the membrane [1,3,7]. We have examined this possibility by placing normal and dystrophic sarcoplasmic reticulum in detergent environments with a large excess of exogenous dioleoylphosphatidylcholine relative to the amount of endogenous lipid present. As shown in Fig. 4, the response of dystrophic sarcoplasmic reticulum to dioleoylphosphatidylcholine is similar to that of normal sarcoplasmic reticulum. When the results are presented relative to the ATPase activity present without added dioleoylphosphatidylcholine, the dystrophic and the normal response are identical (Fig. 5). This indicates that dystrophic sarcoplasmic reticulum interacts with phospholipid in a manner similar to normal sarcoplasmic reticulum and that dystrophic sarcoplasmic reticulum exhibits an ATPase activity similar to that of normal sarcoplasmic reticulum when the two are placed into identical lipid environments.

Calcium phosphate loading

One problem with interpreting results from isolated dystrophic preparation is that the individual fibers of dystrophic muscle may not all be affected to the same extent by the dystrophic process. A dystrophic preparation could conceivably consist of 'bad' membranes, isolated from fibers affected by the disease process, and 'good' membranes, isolated from fibers relatively unaffected by the disease process. The observed reductions in dystrophic sarcoplas-

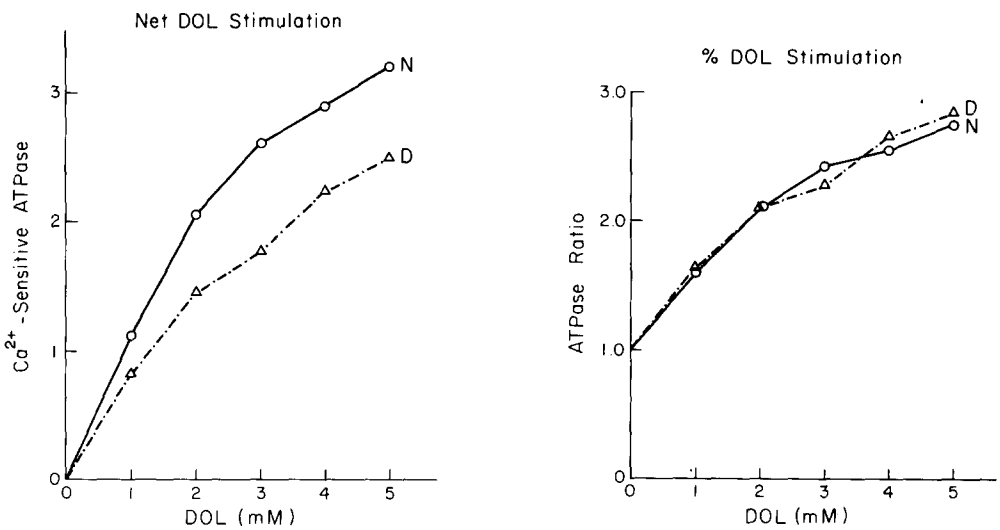

Fig. 4. Phospholipid stimulation of calcium-sensitive ATPase activity. ATPase measurements were performed in the presence of 50 mM Triton X-100 and varying amounts of dioleoylphosphatidylcholine (DOC). The activity in 50 mM Triton and no dioleoylphosphatidylcholine was used as a zero reference and, as such, was subtracted from the ATPase activities determined with dioleoylphosphatidylcholine present. N, normal sarcoplasmic reticulum, and D, dystrophic sarcoplasmic reticulum.

Fig. 5. Relative phospholipid stimulation of calcium-sensitive ATPase activity. ATPase determinations were as in Fig. 4. Calcium-sensitive ATPase activities were divided by the activity without dioleoylphosphatidylcholine (DOL) to give the relative stimulation of activity by dioleoylphosphatidylcholine.

TABLE III

BIOCHEMISTRY OF CALCIUM PHOSPHATE-LOADED SAMPLES

n.s., not significant.

Activity	Normal	Dystrophic	*t*-test
%loaded (of recovered protein)	38.6 ± 3.8	28.0 ± 2.1	$P < 0.05$
ATPase (μmol/mg per min)			
Basal	1.32 ± 0.15	2.15 ± 0.16	$P < 0.001$
Ca^{2+} sensitive	1.86 ± 0.15	1.42 ± 0.12	$P < 0.005$
Phosphoenzyme (nmol/mg)	2.27 ± 0.37	0.96 ± 0.25	$P < 0.01$
Steady-state transport (μmol/mg)	0.223 ± 0.023	0.145 ± 0.025	$P < 0.05$
Potassium *p*-nitrophenylphosphatase (μmol/mg per h)	0.55 ± 0.08	1.17 ± 0.20	$P < 0.05$
Succinate dehydrogenase (μmol/mg per h)	0.38 ± 0.02	0.70 ± 0.18	n.s.

mic reticulum activities would then be due to an average of the activities of the 'good' and 'bad' vesicle populations. To test this hypothesis, we have isolated vesicles capable of active calcium transport by a calcium phosphate-loading procedure. The calcium phosphate-loading procedure involves the incubation with ATP, calcium, and phosphate under conditions which allow the accumulation of appreciable amounts (1—5 μmol/mg) of calcium phosphate crystals in the interior of those vesicles capable of active calcium transport. The loaded vesicles are then separated from those vesicles not loaded by centrifugation on a sucrose density gradient. All fractions are then unloaded overnight by the addition of EGTA and then collected by centrifugation.

Dystrophic sarcoplasmic reticulum shows a significant reduction in the amount of recovered vesicular protein that can be calcium phosphate loaded (Table III), indicating that dystrophic sarcoplasmic reticulum does not contain as many active vesicles as normal. The loaded dystrophic vesicles show a reduction in basal ATPase activity, calcium-sensitive ATPase activity, phosphoenzyme level, and steady-state calcium transport level. This demonstrates that we are unable to separate a population of vesicles from dystrophic sarcoplasmic reticulum that is as active as normal sarcoplasmic reticulum vesicles. The effectiveness of the loading procedure is indicated by the increased calcium-sensitive ATPase and steady-state calcium transport level in the loaded sarcoplasmic reticulum as compared to the non-loaded sarcoplasmic reticulum. Significant levels of potassium *p*-nitrophenylphosphatase and succinate dehydrogenase are still present in the loaded vesicles, however, indicating that the loading procedure was not 100% effective.

Discussion

This investigation has established the following major points:

(1) Biochemical alterations. Dystrophic chicken sarcoplasmic reticulum has a reduced level of calcium-sensitive ATPase activity, phosphoenzyme formation, and steady-state calcium transport. These alterations indicate a reduced level of function of the dystrophic (Ca^{2+} + Mg^{2+})-ATPase. These alterations are probably not due to an alteration in the primary structure of the enzyme, as we have previously demonstrated [7].

(2) Anion-stimulated calcium transport. The lack of a reduction in the oxalate-stimulated calcium transport rate of dystrophic sarcoplasmic reticulum appears to be an artifact caused by the rate-limiting movement of oxalate across the normal membrane. This is shown by the reduction in dystrophic phosphate-stimulated calcium transport rate using acetyl phosphate. Normal and dystrophic vesicles have similar calcium efflux rates, indicating that differences in anion-stimulated calcium transport rates are not due to different calcium efflux rates.

(3) Calcium phosphate loading. The calcium phosphate-loading experiments indicate that more of the normal vesicular protein is present in active vesicles than in the dystrophic preparation. The loaded dystrophic vesicles show the same alterations in enzyme activity as the crude dystrophic sarcoplasmic reticulum, indicating that all of the vesicles are affected by the disease process.

(4) Lipid-protein interactions. In the presence of high concentrations of Triton X-100 and exogenous phospholipid, the calcium-sensitive ATPase activities of normal and dystrophic membranes are similar. This implies that the lipid environment is altered. However, a recent investigation has shown no differences in the relative percentages of the phospholipid classes and cholesterol present in the dystrophic membrane (Tovar, A.M. and Verjovski-Almeida, S., unpublished results).

The $(Ca^{2+} + Mg^{2+})$-ATPase has been shown to be very sensitive to its microenvironment, particularly to the lipid composition of its surrounding annular lipid layers [9,21]. This lipid annulus appears to interact strongly with the $(Ca^{2+} + Mg^{2+})$-ATPase, and cholesterol is normally excluded from the annular lipid layer. Our results are consistent with the hypothesis that the lipid microenvironment of the dystrophic enzyme is altered. This alteration could be due to an alteration in the overall lipid composition of the dystrophic membrane or to an alteration only in the lipid microenvironment of the dystrophic $(Ca^{2+} + Mg^{2+})$-ATPase. The occurrence of the latter situation is more difficult to explain, but could occur during membrane biosynthesis.

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