EVIDENCE FOR SARCOLEMMA ABNORMALITIES IN THE DYSTROPHIC MOUSE SKELETAL MUSCLE

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ABSTRACT

Sarcolemma-enriched fractions were isolated from skeletal muscles of normal and dystrophic mice. Comparison between the two muscles was possible because almost equivalent subcellular fractions were obtained with respect to the distribution of marker enzymes. Unusual findings in the dystrophic sarcolemma include a marked decrease in the specific activity of 5'-nucleotidase, and an alteration in immunoreactivity of NaK-ATPase. Also, quantitative changes in some glycoproteins were seen in the dystrophic sarcolemma; disappearance of a glycoprotein with an apparent molecular mass of 30 kDa was the most noteworthy finding.

Since the first demonstration of an elevated level of serum aldolase which implied an abnormal leakiness of the muscle membrane in Duchenne muscular dystrophy (41), the *membrane hypothesis* has stimulated a considerable number of researches on the structure and function of the dystrophic muscle. Ultrastructural studies on the dystrophic muscle have shown some defects in the sarcolemma (9, 34), which may be relevant to an abnormal leakiness of the dystrophic muscle membrane. Recent electron microscopic studies on biopsied muscles (12, 46) have revealed abnormalities of the skeletal sarcolemma which may involve cell-surface glycoproteins (31, 32).

5'-Nucleotidase, acetylcholinesterase (AChE) and NaK-ATPase (β -subunit) have been described as glycoproteins of the plasma membrane (8, 11, 38, 39). The latter two have been implicated in a variety of biological processes and play important roles in the neuromuscular function (5, 35, 36, 47).

The physiological role of 5'-nucleotidase is not yet fully understood, but a number of functions have been postulated for this enzyme. It may allow nucleosides to cross the plasma membrane (22, 23), be involved in the regulation of blood flow in cardiac and skeletal muscles (6, 52), play a role in the neurotransmission process (10, 42), regulate the adenosine level during cell prolifera-

tion (43), and have some relevance to the myelination process (3).

The present study deals with the activity of 5'-nucleotidase, NaK-ATPase and AChE in the sarcolemma isolated from normal and dystrophic skeletal muscles. Also, the distribution of glycoproteins in the normal and dystrophic sarcolemma was studied. The dystrophic sarcolemma showed some unusual features.

MATERIALS AND METHODS

Highly Purified Sarcolemmal Fraction

Three-month-old dystrophic mice (C57 BL dy/dy) and their controls (C57 BL dy/+) were used. Animals were anesthetized with ether, and gastrocnemius muscles were dissected out. Tissues were subjected to the procedure originally used for the isolation of the cardiac sarcolemma (30), with modifications (Fig. 1).

Enzyme Assays

All enzyme activity was expressed in micromoles of the product per hour per mg of protein. 5'-Nucleotidase was determined by measuring the release of Pi from 5'-AMP (30), and checked by the method of Arkesteijn (2). Glucose 6-phosphatase activity was determined at 37°C by meas-

Fig. 1 Scheme for the isolation of sarcolemma, sarcoplasmic reticulum and mitochondria from the mouse skeletal muscle. *Buffer I: 30 mM imidazole-HCl pH 6.8, 120 mM KCl, 30 mM NaCl, 20 mM sodium pyrophosphate, 0.1 mM PMSF (Phenylmethylsulfonyl-fluoride), 250 mM sucrose. **Buffer II: 5 mM Tris HCl pH 7.4, 50 mM sodium pyrophosphate, 5 mM $MgCl_2$ and 135 mM NaCl

uring Pi release from glucose 6-phosphate. NADPH cytochrome *c* reductase activity was measured by the method of Sottocasa *et al.* (48), and AChE activity according to Ellman *et al.* (19). NaK-ATPase activity was measured according to Mansier *et al.* (30), and cytochrome *c* oxidase according to Cooperstein *et al.* (15). Protein was determined by the method of Lowry *et al.* (28).

Electrophoresis and Immuno-blotting

The sarcolemmal fraction was solubilized in 9 mM Tris-glycine buffer, pH 8.3, containing 1% sodium dodecyl sulfate (SDS), and 4% 2-mercaptoethanol, and heated for 3 min at 90°C, except for the case of NaK-ATPase whose sample was incubated at 37°C for 1 h (49). Solubilized protein samples (50 µg each) were subjected to SDS-polyacrylamide gel electrophoresis (27),

with a linear concentration gradient of the gel from 3% to 20%. Gels were stained with Coomassie brilliant blue (CBB).

Proteins in one part of the gel were transferred to a nitrocellulose sheet by electro-blotting (50), and one part of the blotted proteins was stained with amido black. Another part was coated with 3% bovine serum albumin (BSA) in phosphate buffer, pH 8.0, for 60 min at 40°C, and the sheet was washed five times in phosphate buffer containing 0.2% Tween. The sheet was then incubated with a specific antiserum diluted in phosphate buffer for 1 h at 37°C. After washing, the sheet was incubated with horseradish peroxidase (HRP)-conjugated rabbit antiserum against guinea-pig immunoglobulins. The antigen-antibody complex was visualized with diaminobenzidine (0.5 mg/ml) in 50 mM Tris-HCl, pH 7.4, containing 0.01% H_2O_2 . The staining was stopped by washing the sheet in water, and the sheet was then dried.

Glycoproteins blotted on the sheet were detected essentially as described by Hawkes (24). The sheet was incubated for 10 min in TBS (50 mM Tris-HCl, pH 7.4, in 200 mM NaCl) containing 3% BSA to block the remaining protein binding sites. Then the sheet was incubated for 30 min in TBS containing concanavalin A (ConA; 50 µg/ml), washed in TBS for 15 min, and incubated in HRP solution (50 µg/ml in TBS-BSA). After washing, bound HRP was detected by immersing the sheet in 0.06% 4-chloro-1-naphthol and 0.01% H_2O_2 in TBS. ConA-binding proteins appeared as blue bands within 1 to 2 min.

Antisera

Antiserum against purified dog kidney NaK-ATPase was raised in rabbits, and that against rat cardiac muscle myosin in guinea pigs (30). Antiserum against bovine liver 5'-nucleotidase was raised in rabbits (33). All antisera were used at 1/200 dilution.

RESULTS

Characterization of the Isolated Sarcolemma

Specific activity of 5'-nucleotidase, AChE and NaK-ATPase was high in the F1 fraction which was enriched with the sarcolemma (Table 1). 5'-Nucleotidase and AChE were 80-100 times more concentrated in the F1 fraction than in the homogenate. NaK-ATPase was 60 times more concentrated in the F1 fraction than in the supernatant; this enzyme activity was not detectable

Table 1 *Distribution of Marker Enzymes in Various Subcellular Fractions from the Mouse Skeletal Muscle*

		Homogenate	Pellet	Sarcolemma (F1)	Sarcoplasmic reticulum (F2)	Mitochondria (F3)
5'-Nucleotidase	Control	0.25 (0.056)	1.3 (0.52)	20.2 (3.3)	—	—
	Dyst.	0.14 (0.031)	0.58 (0.13)	9.5 (2.21)	—	—
Ouabain sensitive [Na ⁺ + K ⁺] ATPase	Control	1.68 (0.6)	20.4 (5.4)	100.2 (18.6)	—	—
	Dyst.	1.69 (0.22)	16.8 (2.93)	99.4 (20.6)	—	—
Acetylcholinesterase	Control	0.332 (0.10)	3.78 (0.52)	25.12 (7.6)	—	—
	Dyst.	0.295 (0.06)	3.35 (0.42)	25.02 (4.6)	—	—
Cytochrome c oxidase	Control	—	—	2.90 (0.21)	3.78 (0.98)	96.5 (2.56)
	Dyst.	—	—	2.83 (0.17)	4.02 (0.92)	99.6 (3.12)
NADPH cytochrome c reductase	Control	—	—	0.11 (0.03)	5.43 (1.32)	—
	Dyst.	—	—	0.13 (0.04)	4.89 (1.46)	—
Glucose 6 phosphatase	Control	—	—	0.027(0.007)	1.18 (0.66)	—
	Dyst.	—	—	0.022(0.01)	1.06 (0.47)	—

For fractionation procedures, see Fig. 1. Enzyme activity is expressed in $\mu\text{mol product/mg protein/h}$. Values are means of six determinations, with SE in parentheses. *Assayed with the supernatant.

Fig. 2 Proteins of myofibrils (a and b), and of the sarcolemma from normal (c-f) and dystrophic (g-j) skeletal muscles. Proteins in each fraction (40 μg) were electrophoresed in SDS-gel, and after blotting on a nitrocellulose sheet, myosin, NaK-ATPase and 5'-nucleotidase were immunologically visualized. Myofibril proteins stained with amido black (a), and reacted with anti-myosin antiserum (b). Normal sarcolemmal proteins stained with amido black (c), and reacted with anti-myosin antiserum (d), with anti-NaK-ATPase antiserum (e), and with anti-5'-nucleotidase antiserum (f). Dystrophic sarcolemmal proteins stained with amido black (g), and reacted with anti-myosin antiserum (h), with anti-NaK-ATPase antiserum (i) and with anti-5'-nucleotidase antiserum (j). Molecular masses in kDa are indicated on the left.

with the homogenate. Contamination of mitochondria in the F1 fraction, as evaluated by the cytochrome *c* oxidase activity, was less than 3%. Also, less than 2% of the sarcoplasmic reticulum contaminated the F1 fraction, as estimated by the NADPH cytochrome *c* reductase and glucose 6-phosphatase activity (Table 1).

Sarcolemmal proteins blotted on a nitrocellulose sheet were stained by anti-myosin antiserum. In contrast to myofibrils that showed a heavy myosin band (Fig. 2, lane b), myosin was not detectable in the sarcolemmal fraction (Fig. 2, lane d), suggesting that the amount of myosin in this fraction was less than 12 $\mu\text{g}/\text{mg}$.

In agreement with the results in Table 1, NaK-ATPase (Fig. 2, lane e; α -subunit, 90 kDa; and β -subunit, 45 kDa), and the dimeric form of 5'-nucleotidase (Fig. 2, lane f) were detected by immuno-blotting.

Sarcolemmal proteins visualized by CBB stain of the gel are shown in Fig. 3, lane 1, and sarcolemmal glycoproteins bound to ConA on a nitrocellulose sheet in Fig. 3, lane 3. Banding patterns greatly differ with the single exception of a band with an apparent molecular mass of 30 kDa.

Comparison between the Normal and Dystrophic Sarcolemma

The dystrophic sarcolemmal fraction was purified to the same extent as the normal sarcolemmal fraction (Table 1; Fig. 2, lane h).

Specific activity of NaK-ATPase and AChE did not appreciably differ between the normal and dystrophic sarcolemma (Table 1). On immuno-blotting, however, immunoreactivity of α - and β -subunits of NaK-ATPase was found weaker in the dystrophic sarcolemma (Fig. 2, lane i) than in the control (Fig. 2, lane e). In addition, γ -subunit with an apparent molecular mass of 200 kDa was seen only in the dystrophic sarcolemma (Fig. 2, lane i).

In sharp contrast to NaK-ATPase and AChE, specific activity of 5'-nucleotidase was decreased to half in both the homogenate and sarcolemma from the dystrophic mouse (Table 1). Immunoreactivity of this enzyme was weaker in the dystrophic sarcolemma than in the control (Fig. 2, lanes f and j). In addition, the monomeric form (55 kDa) of the enzyme was found only in the dystrophic sarcolemma (Fig. 2, lane j).

In the electrophoretic banding pattern stained with CBB, two proteins (100 kDa and 30 kDa) appear to decrease, and other two proteins (200 kDa and 45 kDa) appear to increase in the dystrophic sarcolemma (Fig. 3, lanes 1 and 2).

Fig. 3 Normal and dystrophic sarcolemmal proteins. A: Visualized by CBB stain of the gel. B: Visualized by glycoprotein stain after proteins were blotted on nitrocellulose. Lanes 1 and 3, sarcolemma from the control mice. Lanes 2 and 4, sarcolemma from the dystrophic mice. Arrowheads indicate the difference observed between the control and dystrophic sarcolemma. Molecular masses in kDa are shown on the left.

With respect to glycoproteins capable of binding to ConA, disappearance of a 30 kDa protein is a noteworthy finding in the dystrophic sarcolemma (Fig. 3, lanes 3 and 4). On CBB staining, this 30 kDa protein appears to be composed of two bands, and only the smaller band seems to be missing in the dystrophic sarcolemma (Fig. 3, lanes 1 and 2).

Fig. 4 shows electron microscopic appearances of the sarcolemmal fraction purified from the control mouse. The sarcolemmal fraction from the dystrophic mouse had similar appearances (not shown). Sealed membrane vesicles ranging in diameter from 0.2 μm to 0.5 μm predominate. Flattened vesicles (transverse tubules), mitochondria, muscle filaments and collagen were not observed.

DISCUSSION

The sarcolemmal preparation we have purified from the skeletal muscle shows the high specific

Fig. 4 Electron micrograph of sarcolemmal vesicles isolated from the control mouse. Appearances of dystrophic sarcolemma did not seem to differ from those in the control (not shown).

activity of 5'-nucleotidase, AChE and NaK-ATPase, comparable to that reported for the cardiac muscle (13, 14, 25, 30, 51), but not for the skeletal muscle (1, 4, 7, 17, 20, 21, 26, 29, 37, 40, 44, 45). The extent of purification was comparable between the normal and dystrophic muscles (Table 1), which enabled us to compare the enzyme changes on a sound basis.

Changes in immunoreactivity of NaK-ATPase without concomitant decrease in its specific activity are difficult to explain, but may suggest an alteration in the relative proportion of subunits with different reactivity to the antiserum used in the present study.

Though we did not find any appreciable alteration in the specific activity of AChE in the dystrophic sarcolemma, this does not exclude the possible differences in its molecular subspecies between the normal and dystrophic muscles, such as those observed with the whole muscle from other strain of dystrophic mice (23).

The most important finding in this study is a striking decrease in the specific activity of 5'-nucleotidase in the dystrophic sarcolemma. The decrease is not due to a decrease in the incorporation of this enzyme into the sarcolemma, since the specific activity of this enzyme is also decreased in the homogenate to a similar extent. Decrease in 5'-nucleotidase activity can regulate

the intracellular level of nucleotides, and may ultimately affect the protein synthesis as a result of alterations in the nucleic acid metabolism. Also, a reduction of this enzyme activity may account for disturbances in neurotransmission (10, 15, 16, 42, 43). Recent observations (12, 18) indicate that the binding of lectin to the muscle biopsied from the patient with Duchenne muscular dystrophy is decreased, suggesting alterations in the composition of sarcolemmal glycoproteins and/or glycolipids. Our present observations on the altered glycoprotein pattern in the purified sarcolemma are in line with the above observations obtained with biopsied muscles.

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