Ca^{2+} TRANSPORT AND ASSEMBLY OF PROTEIN PARTICLES IN SARCOPLASMIC MEMBRANES ISOLATED FROM NORMAL AND DYSTROPHIC MUSCLE

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1. Introduction

Vesicles of fragmented sarcoplasmic reticulum (SR) membranes have one major activity: Ca^{2+} uptake coupled to ATP hydrolysis. Their simplicity make them particularly suitable for studying structure–function relationships. In this paper we compare the structural and the biochemical properties of normal and dystrophic SR obtained from chicken pectoralis muscle. We show that electrophoretic patterns of solubilized protein and specific ATPase activity are very similar in normal and dystrophic SR, whereas the density of protein particles revealed by freeze fracture and the occurrence of fracture faces containing particles is lower for dystrophic microsomes. Associated with this we find a reduction of ATP dependent calcium uptake in dystrophic SR.

2. Methods

Normal (line 412) and dystrophic (line 413) chickens were obtained from the University of California at Davis. Chickens were sacrificed at 6–8 weeks of age. Fragmented SR was prepared from the pectoralis muscles using methods previously applied to rabbit muscle [1]. The 45 000 *g* SR pellet was resuspended in a stock solution containing 30% sucrose and 20 mM MOPS buffer, pH 6.8, and brought to a final protein concentration of 4–5 mg/ml. Protein was determined by the biuret method.

Calcium transport was determined by the isotopic distribution methods [2] or spectrophotometrically in the presence of the metallochromic dye murexide [3].

ATPase activity of SR membranes was measured by determining the production of inorganic phosphate [4]. SDS solubilization of SR vesicles and the electrophoresis of SR proteins were carried out as described earlier [5].

Samples for freeze fracturing were prepared by washing 8–10 mg SR protein in 80 mM KCl and 10 mM MOPS (pH 6.8), and resuspending the sediments in 0.10 ml of 60 mM KCl, 10 mM MOPS and 20% glycerol. Replicas were made in a Balzers 360M device at −100°C after freezing drops of the suspension in liquid Freon 22. The replicas were examined in a Philips EM 200 electron microscope at 80 kv with a cold contamination device surrounding the specimen.

Electron microscopic observations were also made on SR vesicles negatively stained with a 0.5% uranyl acetate.

3. Results and discussion

Fragments of sarcoplasmic membranes (SR) were obtained from normal and dystrophic muscle with yields of 0.326 mg and 0.153 mg protein/g wet muscle respectively. Electron microscopy of negatively stained material revealed the vesicular shape of the membrane fragments and the absence of contaminants, such as rough mitochondrial membranes.

The preparations were satisfactorily pure, as indicated by gel electrophoresis of solubilized membrane proteins (fig.1). It is apparent in the gels that the major component corresponds to a protein of mol. wt approx. 106 000, which was previously identified with the Ca^{2+} transport ATPase [6,7]. Faint bands may be

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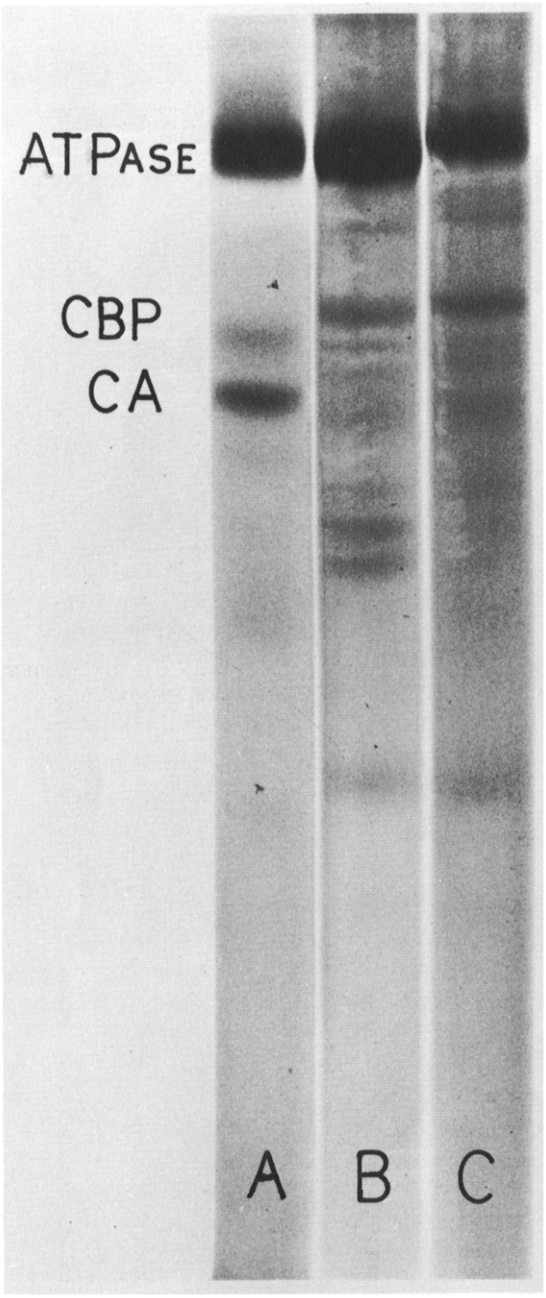

Fig.1. Gel electrophoresis of SR membrane proteins after SDS solubilization. SR protein was obtained from rabbit hind limb muscles (A), normal chicken pectoralis muscle (B), and dystrophic chicken pectoralis muscle (C). Each gel contained 25–30 μg protein. The identified bands correspond to ATPase, Ca^{2+} binding protein (CBP) and calsequestrin (CA).

visualized which correspond to the 'high affinity Ca^{2+} binding protein' found in rabbit SR [6]. Other faint unidentified bands seem to be more pronounced in normal SR. Hardly noticeable is the 55 000 mol. wt protein found in rabbit SR and named 'calsequestrin' [6]. It should be pointed out that the ATPase protein of normal and dystrophic SR displays identical electrophoretic behavior, and no evidence of proteolytic digestion is apparent in either case.

No difference was found either with regard to specific ATPase activity in normal and dystrophic SR. It is shown in fig.2 that ATP hydrolysis is catalyzed at comparable rates by normal and dystrophic SR, and the enzymatic activity is Ca^{2+} dependent.

On the other hand, a profound difference in the assembly of protein particles in normal and dystrophic SR membranes was revealed by electron microscopy on freeze-fracture preparations. The presence of 90 Å

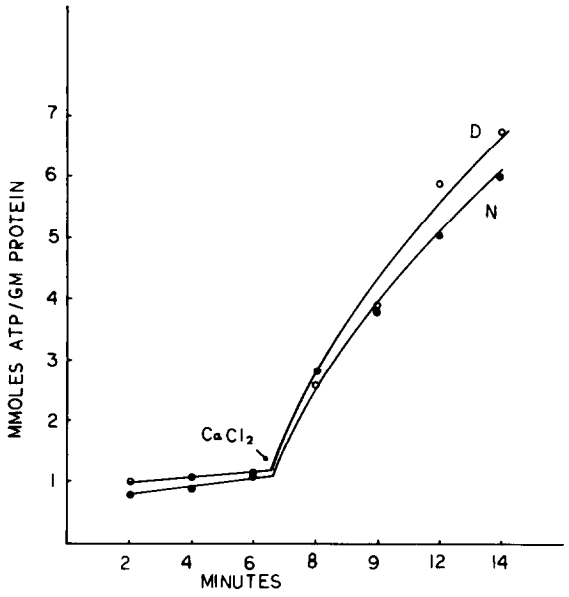

Fig.2. ATPase activity of normal SR (•) and dystrophic SR (○). Reaction mixture: 20 mM MOPS (pH 6.8), 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA and 0.2 mg SR protein/ml and 0.01% Triton X-100. The reaction was started by the addition of ATP to a final concentration of 5 mM. After 7 minutes incubation, calcium stimulated ATPase activity was initiated by the addition of $CaCl_2$ to a final concentration of 0.1 mM. At 2 min intervals, 1 ml samples of the reaction medium were taken and mixed with TCA (final concentration 5%) to stop enzymatic activity.

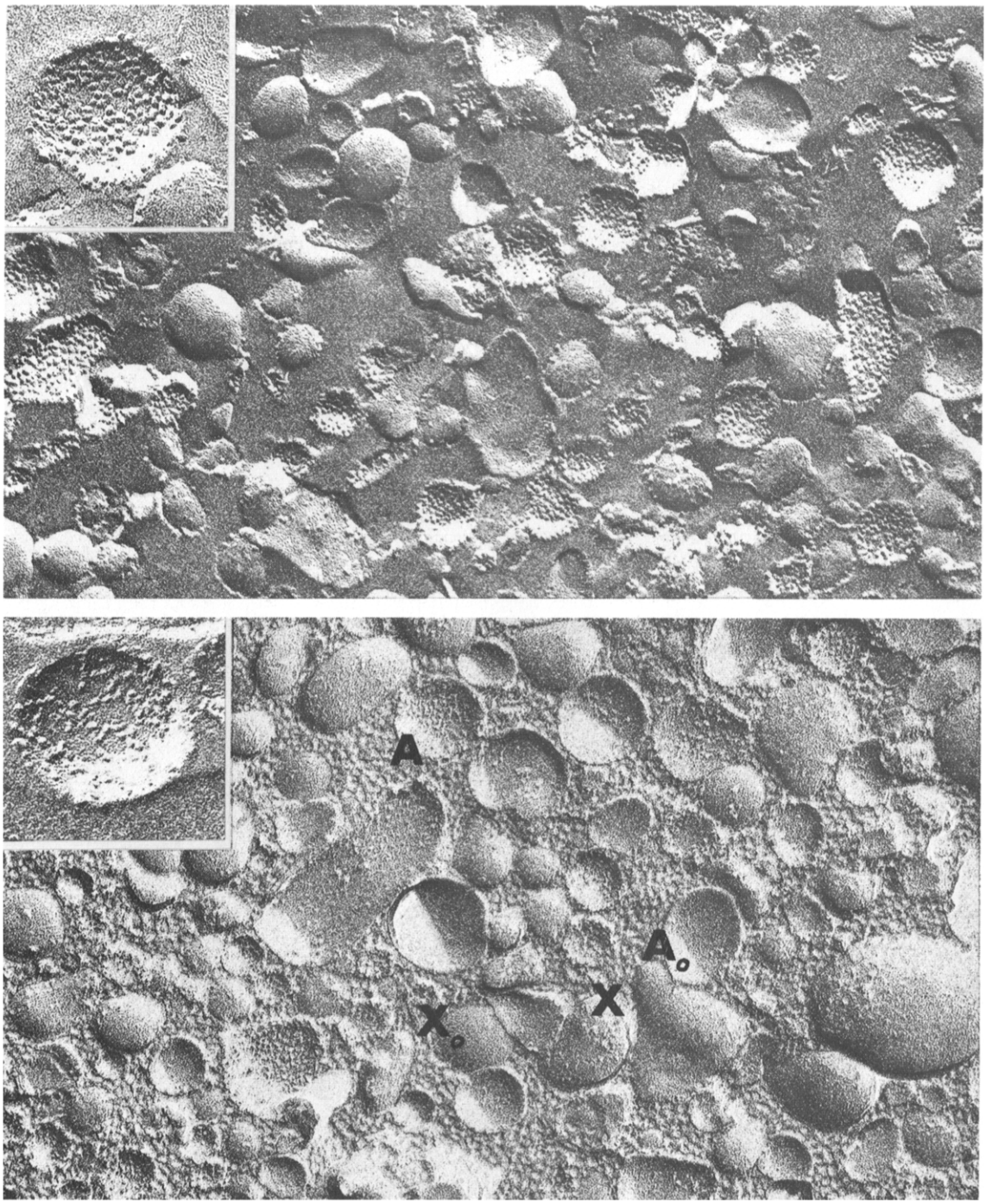

Fig. 3

membrane particles in freeze-fractured SR was first noticed by Deamer and Baskin [8] who tentatively identified the particles with the ATPase protein. Subsequently this identification was confirmed by quantitative studies on the ATPase content of SR and the membrane surface area, and by parallel biochemical and structural observations on the effects of stepwise trypsin digestion [5].

Freeze fracturing of sarcoplasmic reticulum vesicles reveals an equal number of concave outer fracture faces (cytoplasmic leaflet) and convex inner faces. In the present study of both normal and dystrophic SR, 90 Å particles were found predominantly, but not exclusively, on the concave faces. The assessment of the particle distribution was made easier by distinguishing four different types of fracture faces and examining the number of faces of each type.

Of the concave faces, there are some that have many particles (A) and others that are empty or nearly empty (A_o). Similarly, the convex faces are either smooth (X_o) or have particles (X). Fracture faces of each type are indicated on fig.3.

Type assignments and related computations on a total number of 1507 dystrophic and 1132 normal vesicles revealed that 70% of the concave faces contained particles in normal SR, while only 27% of the dystrophic concave faces contained particles.

The particle densities were also measured for both normal and dystrophic SR preparations. Here again there was a striking difference. The average density of particles found in type A fracture faces was 5150 ± 300/μ^2 for normal SR and 3700 ± 530/μ^2 for dystrophic SR. This gives a ratio for dystrophic/normal particle densities of 0.72. Such a reduction in particle density is in agreement with the decreased protein to total lipid ratio found in dystrophic chicken microsomes [9].

The convex faces were found to be predominantly smooth. In both normal and dystrophic preparations

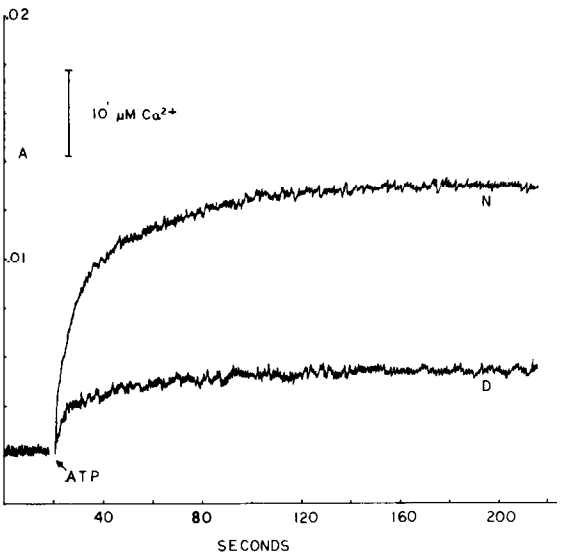

Fig.4. ATP dependent Ca^{2+} uptake by normal (N) and dystrophic (D) SR. Reaction mixture: 20 mM MOPS (pH 6.8), 100 mM KCl, 5 mM $MgCl_2$, 0.12 mM $CaCl_2$, 0.1 mM Murexide and 0.44 mg SR protein/ml. The reaction was started by the addition of ATP to a final concentration of 1.67 mM. Differential light absorption was monitored with the aid of an Aminco double wavelength spectrophotometer, set at 507 and 540 nm wavelengths.

the fraction of convex faces presenting particles was estimated to be 25%. In these faces (type X) the particles density was very low (500 – 900/μ^2), with no significant difference between normal and dystrophic preparations.

Another striking abnormality of the dystrophic SR was found when its ability to accumulate Ca^{2+} was measured in the absence of oxalate. It is shown in fig.4 that ATP dependent Ca^{2+} uptake proceeds very rapidly in normal SR, reaching steady state levels of 72 nmol Ca^{2+}/mg protein in a few seconds. On the contrary, in dystrophic SR Ca^{2+} accumulation stops

Fig.3. Top: Freeze fracture replica of normal SR prepared from chicken pectoralis muscle (× 82 800). Most concave fracture faces contain particles and most convex faces are smooth. The insert shows a single concave face and illustrates the typical particle density of 5150 ± 300 μ^2 found in the normal preparation (× 147 500).
Bottom: Freeze fracture replica of dystrophic SR from chicken pectoralis muscle (× 82 800). The four types of fracture faces described in the text are indicated by the letters. These same types of faces are found in the normal preparation but with a different distribution. The insert shows a single concave face with a lower average particle density (3700 ± 530/μ^2) than the normal fracture faces of type A (× 147 500).

after a short initial burst, reaching maximal levels of 20 nmol/mg protein. Similar results were obtained when Ca^{2+} uptake was measured by isotope distribution methods in the presence of Ca-EGTA buffers.

In conclusion, an altered distribution and a decreased density of membrane protein particles, as well as a reduction of the ability to accumulate Ca^{2+} in the presence of ATP were found in SR isolated from dystrophic muscle. These findings are in agreement with the proposed relationship between particles observed in freeze fracture faces and Ca^{2+} transport activity of SR membrane [5,8]. Furthermore, the profound abnormalities observed in vitro may account for the low tension development and delayed mechanical relaxation exhibited by dystrophic muscle [10].

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References

[1] McFarland, B. H. and Inesi, G. (1971) Arch. Biochem. Biophys. 145, 456–464.
[2] Martonosi, A. and Feretos, R. (1964) J. Biol. Chem. 239, 648–658.
[3] Ohnishi, T. and Ebashi, S. (1963) J. Biochem. (Tokyo) 54, 506–511.
[4] Fiske, H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375–387.
[5] Inesi, G. and Scales, D. (1974) Biochemistry 13, 3298–3306.
[6] Mac Lennan, D. H., Yip, C. C., Iles, G. H. and Seeman, P. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 469–477.
[7] Inesi, G. (1972) Ann. Rev. Biophys. Bioeng. I, 191–210.
[8] Deamer, D. W. and Baskin, R. J. (1969) J. Cell Biol. 42, 296–307.
[9] Hsu, Q. and Kaldor, G. (1971) Proc. Soc. Exp. Biol. Med. 138, 733–737.
[10] Sandow, A. and Brust, M. (1962) Am. J. Physiol. 202, 815–820.