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RAPID KINETICS OF CALCIUM ION TRANSPORT AND ATPase ACTIVITY IN THE SARCOPLASMIC RETICULUM OF DYSTROPHIC MUSCLE

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Summary

Vesicular fragments of sarcoplasmic reticulum were isolated from pectoralis muscle of normal and dystrophic chicken. Purification of both preparations was equally satisfactory, as shown by a prominent ATPase band in electrophoresis gels.

Measurements of ATPase phosphorylation, Ca^{2+} transport and P_i cleavage by rapid quench methods revealed a lower specific activity of the dystrophic vesicles with respect to all of these functions. On the other hand, Ca^{2+}-independent ATPase activity was found to be increased in dystrophic vesicles. It is suggested that a fraction of ATPase units of dystrophic sarcoplasmic reticulum is not activated by Ca^{2+}, owing to an altered protein assembly within the membrane bilayer. In fact, when the membrane structure is perturbed by detergents normal and dystrophic preparations acquire an equally high Ca^{2+}-dependent ATPase.

Introduction

Biochemical studies of contractile proteins obtained from dystrophic muscle [1—7] do not show abnormalities which could explain the alterations in contractile behavior observed in physiological studies [8—14]. On the other hand, the possible involvement of the sarcotubular membrane system is suggested by structural alterations which are well evident in the electron microscopic appear-

Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; Mops, morpholinopropanesulfonic acid.

ance of dystrophic muscle [15—19]. Furthermore, sarcoplasmic reticulum vesicles isolated from dystrophic muscle display ultrastructural abnormalities and reduced levels of Ca^{2+} uptake in the presence of ATP [19—25].

The mechanism for the reduced levels of Ca^{2+} uptake observed in isolated sarcoplasmic reticulum vesicles is not understood. In fact, kinetic characterization of Ca^{2+} transport ATPase in conditions permitting maximal steady-state activity, such as in the presence of detergents, fails to reveal any difference in preparations obtained from dystrophic as compared to normal muscle [19,26].

The present study was designed to define rapid kinetic parameters of Ca^{2+} uptake and ATPase activity in an attempt to clarify the nature of the impairment in Ca^{2+} accumulation by dystrophic vesicles.

Materials and Methods

Sarcoplasmic reticulum vesicles were isolated from breast chicken muscle as described by Scales et al. [19] for the standard preparation. Normal (line 412) and dystrophic (line 413) chicken were obtained from the University of California at Davis, Department of Avian Sciences, and killed at 5—7 weeks of age. Preparations were assayed within one day from isolation. Special care was taken in the blender homogenization step so as to keep the pH between 6.8 and 7.0, which insured a good yield of active preparation. In addition, it was found that after the centrifugation which follows the 1-h incubation with 0.6 M KCl, a soft as well as a hard pellet was present at the bottom of the centrifuge tubes. They both represent contaminant fractions. Careful transfer of the supernatant microsomal fraction into new tubes avoided removal of the soft pellet and yielded a pure preparation.

Rapid kinetics measurements were done with a Durrum D-113 multimixing apparatus. Equal volumes of reacting media contained in two syringes were rapidly mixed to start the reaction. The solution in each syringe contained all reactants except ATP and sarcoplasmic reticulum which were present separately in one of the two syringes. Quenching was obtained by addition of an equal volume of either 7% trichloroacetic acid or a 10 mM EGTA quenching solution [27]. Detailed procedures for Ca^{2+} uptake [27] and phosphorylation and P_i production measurements [28] were previously described. All rates were measured at 30° C. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was carried out according to the method of Weber and Osborn [29].

Results and Discussion

The initial phase of Ca^{2+} uptake by sarcoplasmic reticulum vesicles can be monitored with radioactive tracer in rapid quench experiments. This method involves the use of a rapid mixing device with two mixing chambers to start and quench the reaction by sequential additions of ATP and EGTA [27]. Complete quenching of transport activity is obtained upon addition of sufficient EGTA to reduce the Ca^{2+} concentration below 10^{-8} M, which prevents further binding of activating Ca^{2+} to the enzyme transport sites. Following quenching, the leakage of calcium from the vesicles within 30 s is negligible, thereby allowing sufficient time for separation of loaded vesicles from the medium by filtration.

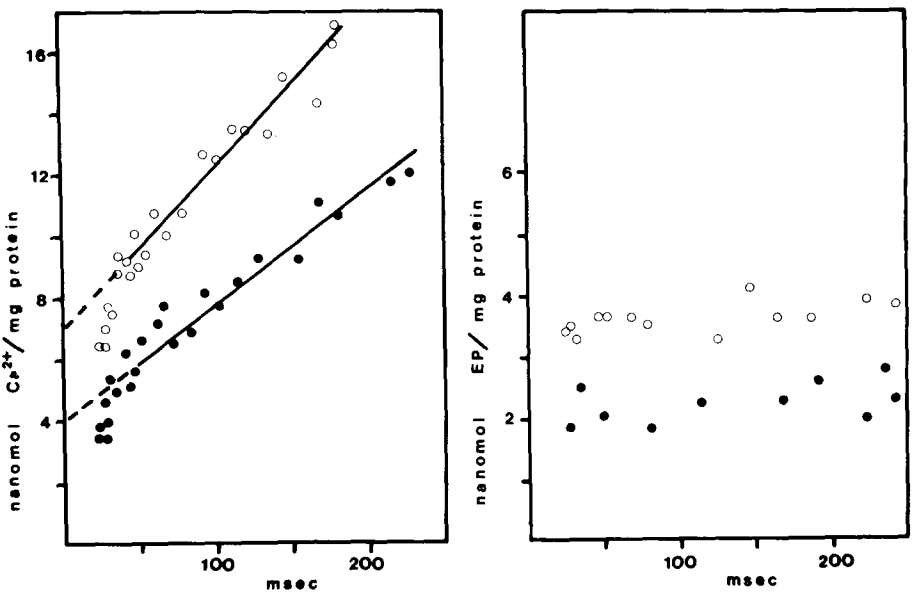

Fig. 1. Initital velocity of Ca^{2+} transport and phosphoenzyme formation. Ca^{2+} transport was initiated by addition of ATP in the presence of $^{45}CaCl_2$ and the reaction was quenched by EGTA (left). Phosphoenzyme intermediate formation was followed by starting the reaction with [γ-^{32}P]ATP in the presence of nonradioactive $CaCl_2$ and the reaction was quenched by acid (right). Reaction medium was 20 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 40 μM $CaCl_2$ and 0.25 mg protein/ml. Sarcoplasmic reticulum vesicles from normal (○) and dystrophic (●) muscle were used.

The residual calcium in the medium is then estimated by determination of radioactivity.

A very interesting feature of the EGTA quench method is that it allows detection of an early burst of Ca^{2+} uptake upon addition of ATP. The time course of the Ca^{2+} burst is identical to that of enzyme phosphorylation from ATP [30]. The burst corresponds to the translocation of calcium already bound to the active sites, immediately following phosphorylation. The size of the burst is related with a 2 : 1 stoichiometric ratio to the level of phosphorylated intermediate [27,30]. It is apparent that the Ca^{2+} burst represents the first cycle of uptake of each activated enzyme unit. Thus, measurements of Ca^{2+} burst and phosphorylated enzyme can indicate the number of activated transporting units per mg of protein.

In Fig. 1 it is shown that enzyme phosphorylation reaches maximal levels within 20 ms at saturating ATP concentrations and is maintained constant in both normal and dystrophic preparations. In the latter the levels of phosphoenzyme varied from 1.5—2.4 nmol/mg protein in four different preparations tested which is lower than in the normal preparations (2.8—3.8 nmol/mg protein). Ca^{2+} uptake measured in identical conditions reveals that in dystrophic microsomes there is also a reduction in the size of early Ca^{2+} burst (Fig. 1, left). The 2 : 1 stoichiometric ratio of Ca^{2+} translocated to phosphorylated enzyme is maintained in both preparations. This is an indication that fewer enzymic units are functioning in dystrophic microsomes, but those that are activated will couple phosphorylation to Ca^{2+} translocation in a normal way.

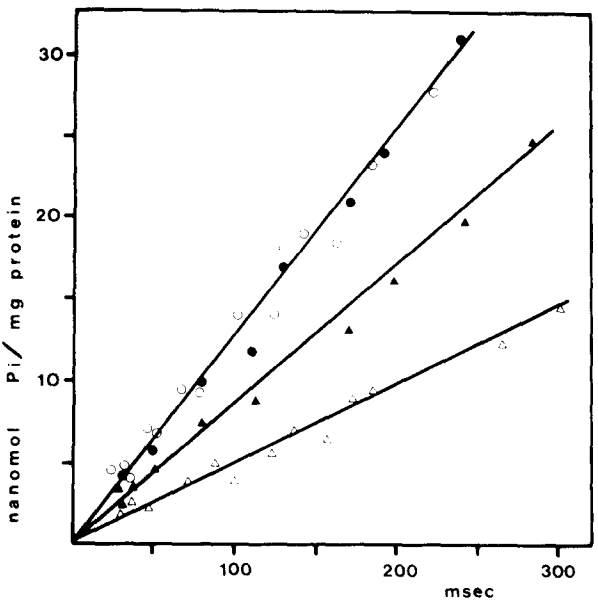

Fig. 2. Initial velocities of P_i production in the presence of Mg^{2+} and Ca^{2+} (○,●) and in the presence of Mg^{2+} and EGTA (△,▲). Sarcoplasmic reticulum vesicles from normal (open symbols) and dystrophic (full symbols) muscle were used. Reaction started by addition of ATP and quenched with acid, in a medium identical to Fig. 1. For Mg^{2+}-dependent P_i production (△,▲) $CaCl_2$ was omitted and 1 mM EGTA was included.

The linear initial velocity of Ca^{2+} uptake following the burst is 52.0 ± 5.6 nmol $Ca^{2+} \cdot mg^{-1} \cdot s^{-1}$ in normal, and 24.3 ± 5.4 nmol $Ca^{2+} \cdot mg^{-1} \cdot s^{-1}$ in dystrophic microsomes (3 preparations tested). Under identical conditions, i.e., in the presence of both Ca^{2+} and Mg^{2+}, the P_i production during this phase is also linear. Fig. 2 shows a typical experiment where the velocity is 120 nmol $P_i \cdot mg^{-1} \cdot s^{-1}$, for both normal and dystrophic preparations. However, there is

TABLE I

Ca^{2+} UPTAKE AND ATPase ACTIVITY

	Normal (N)	Dystrophic (D)	D/N	*P*
	(nmol · mg^{-1} · s^{-1})	(nmol · mg^{-1} · s^{-1})		
Initial velocity of Ca^{2+}-dependent P_i production *	73.2 ± 5.3 (3)	32.4 ± 4.1 (3)	0.44	<0.01
Initial velocity of Ca^{2+} uptake **	52.0 ± 5.6 (3)	24.3 ± 5.4 (3)	0.47	<0.01
	(nmol · mg^{-1})	(nmol · mg^{-1})		
Steady-state level of phosphoenzyme	3.13 ± 0.31 (4)	1.95 ± 0.37 (4)	0.62	<0.05
Burst of Ca^{2+} uptake ** (Ca^{2+} translocated within first 20 ms)	6.5 — 7.5	3.5 — 4.5	0.57	
Steady-state levels of Ca^{2+} uptake ***	43.4 ± 8.5 (4)	29.9 ± 6.5 (4)	0.69	<0.05

* Determined from experiments similar to that of Fig. 2.
** Determined from experiments similar to that of Fig. 1, left.
*** Determined from experiments similar to that of Fig. 3.

a large fraction of this hydrolytic activity which is not Ca^{2+} dependent. This activity is 50 nmol $P_i \cdot mg^{-1} \cdot s^{-1}$ in the normal preparation and 80 nmol $P_i \cdot mg^{-1} \cdot s^{-1}$ in the dystrophic. Therefore, the calculated velocity of Ca^{2+}-dependent ATPase is 70 and 40 nmol $P_i \cdot mg^{-1} \cdot s^{-1}$ for normal and dystrophic microsomes, respectively.

Table I summarises the significant differences found. The reduction in phosphoenzyme level and in Ca^{2+}-dependent P_i production suggests fewer active enzyme units per protein unit weight of dystrophic microsomes.

The phosphoenzyme levels obtained under conditions expected to yield maximal phosphorylation, i.e., saturating ATP and Ca^{2+} concentrations, can be taken as a good estimate of the number of total activated sites per mg of protein. From these levels and the initial velocities of Ca^{2+}-dependent P_i production obtained one can calculate the turnover of the enzyme to be 18—20 s^{-1} at 30° C for both preparations.

It should be pointed out that in spite of a normal 2 : 1 ratio of initially translocated Ca^{2+} to phosphorylated enzyme (Fig. 1) the efficiency of net transport, expressed as the ratio between velocities of net Ca^{2+} uptake and Ca^{2+}-dependent P_i production is 0.7 in both preparations. This is an indication that translocated Ca^{2+} leaks back at a considerable rate. The presence of a high passive outward diffusion of Ca^{2+} could explain the lower levels of accumulated Ca^{2+} which are obtained in dystrophic preparations (Fig. 3). In fact, a high passive Ca^{2+} permeability of chicken microsomes becomes evident when one compares the half-time of passive Ca^{2+} efflux in both normal and dystrophic chicken preparations [31], i.e., 0.5 min, to that of rabbit preparations [32], which is 5 min.

The reduced steady-state level of Ca^{2+} uptake in dystrophic chicken microsomes is probably related to a lower number of actively transporting units per membrane area, in the presence of a significant passive efflux.

Microsomes obtained from normal and dystrophic mice were also found to have a ratio of Ca^{2+} transport/ATP hydrolysis of 0.6—0.8 [25]. A decreased phosphoenzyme level and a reduced steady-state rate of Ca^{2+} uptake in the presence of oxalate were also reported [25], suggestive of fewer transporting units in dystrophic mouse preparations.

Following the procedures described in the Methods, we obtained chicken preparations with a high degree of purity, as evidenced by a predominant 100 000 dalton ATPase band in gel electrophoresis (Fig. 4) of both normal and dystrophic microsomes. Therefore, dilution of ATPase with contaminating proteins does not explain the decreased number of actively transporting units of dystrophic preparations. Rather, a modification in the membrane assembly of the ATPase chains, as expressed by an altered particle distribution in freeze fractured faces [19], may be responsible for the alteration in the functional properties of the ATPase. In fact, following perturbations of membrane structure with detergents, dystrophic and normal microsomes acquire an identical specific activity for Ca^{2+}-dependent ATPase, while the Ca^{2+}-independent ATPase is reduced [24].

In view of an increased Ca^{2+}-independent and a decreased Ca^{2+}-dependent ATPase in dystrophic sarcoplasmic reticulum, it is tempting to speculate that a mechanism of interconversion of the former to the latter enzyme, similar to

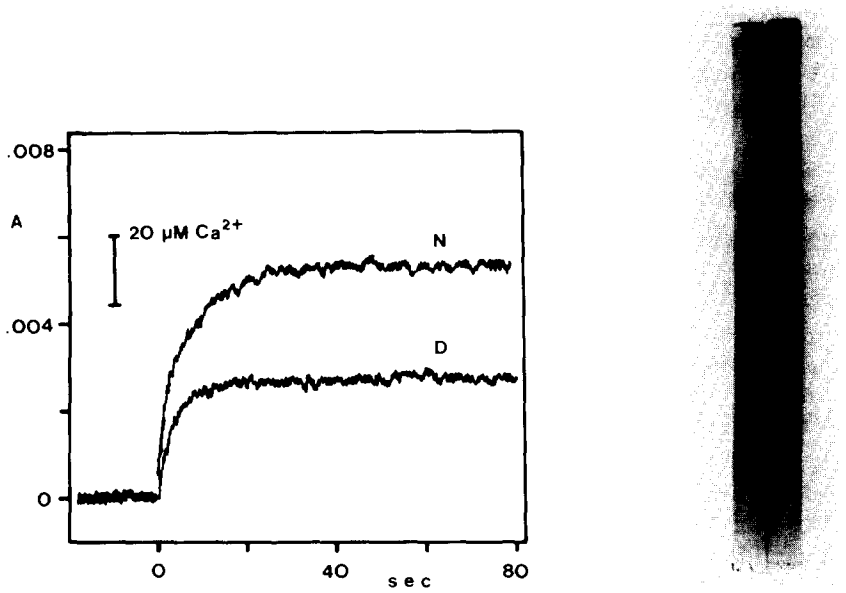

Fig. 3. Steady-state levels of Ca^{2+} uptake measured spectrophotometrically in the presence of the indicator murexide. Reaction mixture: 20 mM Mops (pH 6.8), 80 mM KCl, 10 mM $MgCl_2$, 100 μM $CaCl_2$, 100 μM murexide and 0.8 mg protein/ml. The reaction was started by the addition of ATP to a final concentration of 0.67 mM. Differential light absorption was monitored with an Aminco dual wavelength spectrophotometer, set at 507 and 540 nm wavelength. Maximal levels of uptake are 58 and 37 nmol Ca^{2+}/mg protein for normal (N) and dystrophic (D) sarcoplasmic reticulum, respectively.

Fig. 4. Gel electrophoresis of sarcoplasmic reticulum membrane proteins after SDS solubilization. The 100 000 dalton calcium-ATPase represents over 80% of the total protein in both normal (right) and dystrophic (left) preparations. The amount of protein per gel was 25 μg (normal) and 19 μg (dystrophic).

that suggested by Inesi et al. [33], may be impaired in the dystrophic membrane, due to abnormal protein assembly in a membrane of higher lipid content [19]. With regard to muscle mechanics and impairment of excitation-contraction coupling, we have here provided further evidence for the involvement of sarcotubular membranes by the dystrophic process.

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