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Separation of active and inactive (nonphosphorylating) Ca^{2+}-ATPase in sarcoplasmic reticulum subfractions from low-frequency-stimulated rabbit muscle

Shigeki Matsushita, László Dux and Dirk Pette

Fakultät für Biologie, Universität Konstanz, Postfach 5560, D-7750 Konstanz, Germany

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Chronic low-frequency stimulation elicits in rabbit fast-twitch muscle a partial inactivation of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase and Ca^{2+}-uptake activities. Inactive Ca^{2+}-ATPase was enriched in a light microsomal fraction by sucrose density gradient centrifugation after calcium oxalate loading in the presence of ATP. This fraction showed a reduced specific activity and phosphoprotein formation of the Ca^{2+}-transport ATPase. These results suggest that the inactivation of the Ca^{2+}-ATPase as induced by increased contractile activity, is confined to a specific SR vesicle population.

Low-frequency stimulation; Sarcoplasmic reticulum Ca^{2+}-ATPase

1. INTRODUCTION

In previous studies, we have shown that sustained contractile activity as induced by chronic low-frequency stimulation partially reduces Ca^{2+}-sequestration by the sarcoplasmic reticulum (SR) [1–5]. This inactivation affected both the total capacity and initial rate of Ca^{2+}-uptake. The total amount of the immunochemically detectable Ca^{2+}-ATPase protein was unaltered, but the Ca^{2+}-dependent ATP-hydrolysing activity, as well as the formation of the phosphoprotein intermediate, were reduced by approximately 50% [2]. A decreased binding of fluorescein isothiocyanate (FITC), a competitor of ATP at the nucleotide-binding site, indicated that the inhibition might be related to a local structural alteration of the enzyme [2,4,5]. This was corroborated by the observation that the inactive enzyme was less susceptible for tryptic cleavage at Arg^{505} [4,5]. A similar inactivation of the Ca^{2+}-transporting ATPase has also been observed during exercise in fatiguing muscle [6]. The reversible inactivation of the SR Ca^{2+}-ATPase may, therefore, be of general interest with regard to muscle fatigue.

The present study was undertaken in order to investigate whether the overall reduced activity of the SR Ca^{2+}-ATPase results from a partial inactivation of all enzyme molecules or relates to complete inactivation of a selected population. Microsomal preparations from normal and chronically stimulated muscles were separated on a density gradient after Ca^{2+}-loading in the presence of ATP and oxalate. Measurements of protein yield, Ca^{2+}-stimulated ATPase activity, and formation of the phosphorylated enzyme intermediate made possible the identification of a light vesicle subfraction with an increased yield of the inactive enzyme.

Correspondence address: D. Pette, Fakultät für Biologie, Universität Konstanz, Postfach 5560, D-7750 Konstanz, Germany. Fax: (49) (7531) 88 39 40.

2. MATERIALS AND METHODS

2.1. *Animals and chronic stimulation*

Adult male New Zealand White rabbits were indirectly stimulated (10 Hz, 12 h/d) [7,8]. The animals were killed after 4-day stimulation periods and stimulated (left) and control (right) extensor digitorum longus (EDL) and tibialis anterior (TA) muscles were processed for isolation of SR according to Nakamura et al. [9].

2.2. *Calcium oxalate loading and isolation of SR subfractions*

Ca^{2+}-loading was performed as described [2] 60 mg crude SR was incubated in 20 ml of a medium containing 50 mM HEPES (pH 6.8), 100 mM KCl, 10 mM $MgCl_2$, 20 mM potassium oxalate, 5 mM NaN_3, 10 mM ATP and 100 μM $CaCl_2$ at 37°C for 15 min. The chosen Ca^{2+}-to-Ca^{2+}-ATPase ratio was in the range of the physiological storage capacity. Thereafter, the vesicles were sedimented at 55 000 × g for 60 min. The pelleted microsomes were suspended in 3 ml of 0.25 M sucrose containing 0.3 M KCl, 0.05 M sodium pyrophosphate, and 0.1 M Tris/HCl, pH 7.2. After measuring the protein concentration, this suspension was layered on top of a discontinuous sucrose density gradient [10] with consecutive layers of 1.5, 1.0, 0.8 and 0.6 M sucrose, 7 ml each containing 0.3 M KCl, 0.05 M sodium pyrophosphate and 0.1 M Tris/HCl, pH 7.2. Gradient centrifugation was performed for 2 h in a SW27 Beckman rotor at 27 000 rpm. The fractions were separated at the interfaces of the layers. Fraction FI was between 0.25 and 0.6 M, fraction FII between 0.6 and 0.8 M, fraction FIII between 0.8 and 1.0 M, and fraction FIV between 1.0 and 1.5 M sucrose, respectively. The fractions were diluted with 0.1 M KCl, 10 mM MOPS, and centrifuged for 90 min at 60 000 × g. The pellets were suspended in 0.5–1 ml 0.25 M sucrose, 10 mM Tris/HCl, pH 8.0.

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2.3. *Analytical procedures*

The Ca^{2+}-ATPase activity in mirosomal fractions was measured at 37°C by the coupled optical assay [11]. Tryptic cleavage was performed as previously described [5]. After tryptic digestion for different time periods, microsomal fractions were phosphorylated at 4°C using [γ-^{32}P]ATP at a final concentration of 0.2 μM in a medium containing 75 mM KCl, 30 mM HEPES, pH 7.0, and 20 μM $CaCl_2$ for 1 min. The reaction was stopped by adding a two-fold volume of 6% trichloroacetic acid containing 1 mM ATP, 10 mM phosphate. The precipitate was washed by centrifugation with the same solution three times and then dissolved in lysis buffer composed of 60% sucrose, 10 mM EDTA, 2.5% SDS, 0.014% Bromphenol blue, 0.15 M Tris/HCl, pH 6.8. In order to stabilize the phosphorylated intermediate, electrophoresis was performed using the acidic system described by Sarkadi et al. [12]. Phosphorylated peptides were detected by autoradiography using Hyperfilm MP (Amersham, Braunschweig, FRG) exposed at −70°C for 24 h. After electrophoresis, proteins were transferred to nitrocellulose filters. The Ca^{2+}-ATPase protein, as well as immunoreactive fragments, were identified with a polyclonal antibody raised against fast SR Ca^{2+}-ATPase [13] Horse radish peroxidase-conjugated goat anti-rabbit IgG served as the secondary antibody.

3. RESULTS

Sarcoplasmic reticulum vesicles isolated from normal, and 4-day-stimulated muscles were subfractionated on a sucrose density gradient after loading with calcium oxalate. The total yield was found to be unaltered between the two experimental conditions, indicating that contamination with non-SR membranes [2] had not yet occurred after this short stimulation period. Marked differences existed between the protein amounts recovered in the different subfractions (Table I). The lightest fraction (FI) of the unstimulated muscle contained 8.5% of the total protein, whereas the FI fraction of the stimulated muscle contained 18.6% of the total protein applied to the gradient. The higher protein amount of FI was compensated for by a lower yield in the heavier fractions, particularly in FIII. Its protein amount was 35% in the unstimulated, but only 26% in the stimulated muscle (Table I).

The FI fraction of the stimulated muscle exhibited the lowest Ca^{2+}-ATPase specific activity, amounting to only 43% of that detected in FI of the unstimulated muscle. No differences were found between the Ca^{2+}-ATPase specific activities of fractions FIII and FIV from unstimulated and stimulated muscles.

The possibility existed that the vesicles with lower Ca^{2+}-accumulating activity contained preferentially the inactive enzyme. Therefore, the vesicle fractions separated after calcium oxalate loading were subjected to tryptic digestion. Thereafter, we examined the ability of the trypsin-treated vesicles to form phosphorylated intermediates. The phosphorylated peptides were separated electrophoretically and their identity was established by immunoblotting. The radioactive peptides were visualized autoradiographically on the same nitrocellulose membranes.

Compared with the contralateral (unstimulated) muscle (Fig. 1A), the phosphate incorporation into FI was reduced in the stimulated muscle (Fig. 1B). As judged from the radioactivity of the first cleavage product A and the second cleavage product A1 (Fig. 1A,B), this difference existed also after tryptic cleavage. The immunostaining of the same nitrocellulose membranes indicated that this difference was not due to the application of different amounts of the Ca^{2+}-ATPase protein (Fig. 1C,D). No difference in radioactive phosphate incorporation was observed between the heavy fractions FIII from contralateral unstimulated and stimulated muscles (Fig. 2).

4. DISCUSSION

Crude microsomal preparations can be separated by density gradient centrifugation into subfractions of different densities corresponding to different sarcomeric regions of the SR. This purely membrane-derived sedimentation pattern is shifted by the calcium oxalate pre-loading procedure which leads to a separation primarily based on different Ca^{2+}-accumulating activities. Because of the loading at an approximately physiological Ca^{2+}-to-membrane protein ratio, i.e. 30-50 nM Ca^{2+} per mg protein, the effects of native vesicle

Table I

The distribution of protein yield and Ca^{2+}-ATPase specific activity between sarcoplasmic reticulum subfractions of contralateral unstimulated and 4-day-stimulated rabbit fast-twitch muscles

Subfraction	Protein amount (% of total protein)		Ca^{2+}-ATPase activity (μmol ATP × mg protein^{-1} × min^{-1})		
	Unstimulated	Stimulated	Unstimulated	Stimulated	Stimulated / Unstimulated (%)
FI	8.5	18.6	1.40	0.60	43
FII	48.5	48.6	2.54	1.70	67
FIII	35.4	25.5	2.30	2.25	98
FIV	7.6	7.3	1.01	1.03	102

Sarcoplasmic reticulum subfractions were seperated by density gradient centrifugation after calcium oxalate loading.

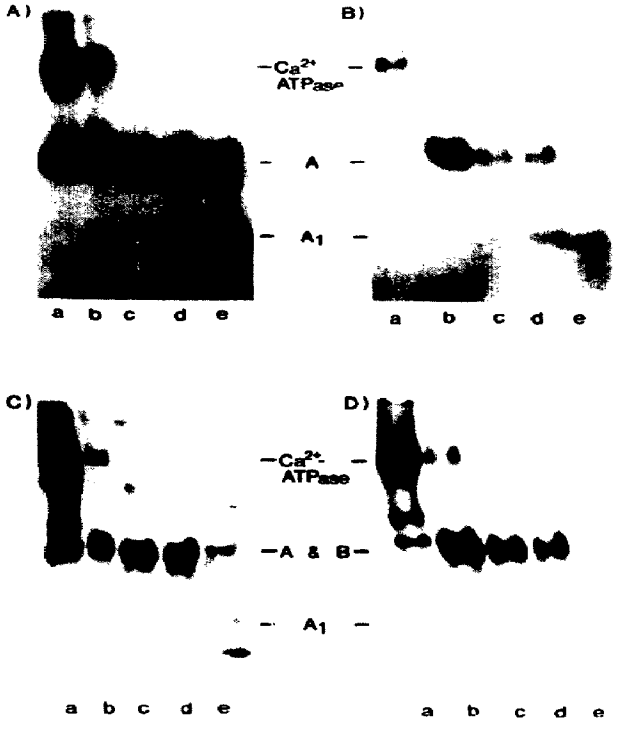

Fig. 1. SDS/gel electrophoretic separation at pH 6.0 of the lightest sarcoplasmic reticulum fraction (FI) after tryptic digestion and phosphorylation. The fraction isolated by density gradient centrifugation after calcium oxalate loading was subjected for different time periods to tryptic digestion at a rato of 1:20 (mg/mg protein) and, thereafter, incubated with [γ-^{32}P]ATP at 4°C for 1 min. After electrophoretic separation was completed, proteins were transferred electrophoretically to nitrocellulose membranes for autoradiography and Western blot analysis with a polyclonal antibody against Ca^{2+}-ATPase. (A and B) autoradiographs of samples from unstimulated contralateral and 4-day stimulated fast-twitch muscles, respectively. (C and D) immunoblots of (A) and (B). (Lanes a–e) tryptic fragment after digestion periods of (a) 0 s; (b) 15 s; (c) 1 min; (d) 5 min; (e) 30 min. A, B, primary tryptic cleavage fragments, A1, secondary tryptic cleavage fragment.

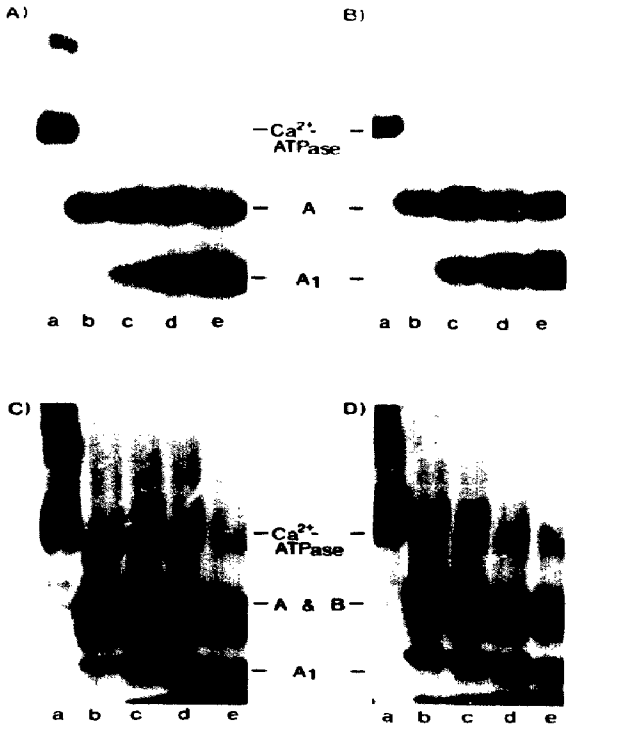

Fig. 2. SDS/gel electrophoretic separation at pH 6.0 of the heavy sarcoplasmic reticulum fraction (FIII) after tryptic digestion and phosphorylation. The same experimental protocol was used as described in Fig. 1. (A and B) autoradiographs of samples from unstimulated contralateral and 4-day stimulated fast-twitch muscles, respectively. (C and D), immunoblots of (A) and (B). (Lanes a–e) tryptic fragment after digestion periods of (a) 0 s; (b) 15 s; (c) 1 min; (d) 5 min; (e) 30 min. A, B, primary tryptic cleavage fragments; A1, secondary tryptic cleavage fragment.

density, vesicle size and Ca^{2+}-accumulating activity most likely overlap in the fractionation process used.

The finding that the lightest microsomal subfraction displays higher protein amounts in the stimulated than in the unstimulated muscle, suggests that a larger portion of the vesicles in this fraction remains unloaded. Obviously, vesicles with inactive or reduced Ca^{2+}-accumulating ability have been enriched in this fraction. This is in agreement with both the lower specific Ca^{2+}-ATPase activity and the reduced phosphoprotein formation. The lower phosphorylation of the first tryptic cleavage product may be taken as an additional proof of the modification of the enzyme species enriched in the light subfraction. Thus, it is conceivable to correlate the reduced Ca^{2+}-uptake activity of this fraction with a predominance of inactive Ca^{2+}-ATPase. This interpretation is supported by the finding that no differences exist between the heavier microsomal subfractions from stimulated and contralateral unstimulated muscles with regard to Ca^{2+}-accumulation, Ca^{2+}-ATPase activity or phosphoprotein formation.

These results suggest that the inactivation of the SR Ca^{2+}-ATPase is confined to a specific vesicle population. This could result from an inactivation of selected regions of the SR in all muscle fibers or from an inactivation of the SR of a specific fiber population. Because TA and EDL muscles are composed of approximately 95% fast-twitch (type IIA and type IIB) fibers which are identical in their fast Ca^{2+}-ATPase isoform composition [14,15], it appears unlikely that the inactivation affects, selectively, fast or slow isoforms. Also, a fast-to-slow Ca^{2+}-ATPase isozyme transition does not occur after 4-day low-frequency stimulation [15]. However, the possibility exists that type IIA and IIB fibers which differ in their fatigability and acrobic-oxidative capacity [16], respond metabolically in a different manner to persistently increased contractile activity. Therefore, the Ca^{2+}-ATPase might be exposed to con-

ditions which lead to inactivation in the one, but not in the other fiber type. Measurements of the SR Ca^{2+}-ATPase activity in defined single fibers of stimulated muscles will provide an answer to this question. The method established in the present study for isolating an enriched population of inactive Ca^{2+}-ATPase offers the possibility to investigate in more detail the underlying mechanism of the inactivation process.

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