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PREPARATION OF SARCOPLASMIC RETICULUM WITH HIGH CALCIUM-SENSITIVE ATPase AND STABLE CALCIUM TRANSPORT FUNCTION FROM RAT SKELETAL MUSCLE

RICARDO L. BOLAND *, EBERHARD RITZ and WILHELM HASSELBACH

Max-Planck-Institut für Medizinische Forschung und Medizinische Universitätsklinik, D-6900 Heidelberg (F.R.G.)

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A method was developed for the isolation from rat skeletal muscle of sacroplasmic reticulum vesicles in which the calcium transport function does not decay during storage. High initial and maximum uptake of calcium and calcium-dependent ATPase activity were obtained for membranes isolated from mixed muscles or pure red fibers. Unstable vesicles resulted when 2 mM EDTA was included in the isolation medium. The calcium uptake activity was lost upon ageing at 0°C, probably due to conversion of the calcium-dependent ATPase to a calcium-independent form. Addition of Ca^{2+} counteracted the effects of EDTA, suggesting their involvement in maintaining the structure of the calcium transport system. This is supported by the fact that different structural states of the ATPase in stable and unstable vesicles were detected by DEAE-cellulose column chromatography.

Introduction

It has been reported that sarcoplasmic reticulum isolated from red or mixtures of red and white skeletal muscles in several animal species is characterized by low calcium uptake function and high calcium-independent ATPase activity (basal or Mg^{2+}-ATPase) as compared to white fast skeletal muscle [1–4]. The differences have been attributed to denaturating processes associated with the preparative procedure [5] and/or to an intrinsically lower concentration of transport sites as measured by [γ-^{32}P]ATP phosphorylation [2].

The activity of membranes isolated from rat muscle is particularly labile. The in vitro capacity and initial rate of calcium accumulation of sarcoplasmic reticulum from hindlimb mixed muscles decay in a few hours, even when stored in the cold. This has in many instances precluded the use of the rat as a convenient experimental model where several determinations on sarcoplasmic reticulum should be made after isolation. In addition, we have observed that a muscle-extracting buffer solution containing 2 mM EDTA, used routinely as described by De Meis and Hasselbach [6] to obtain stable rabbit sarcoplasmic reticulum, proved to be especially deleterious to rat preparations.

The objective of this work was to develop optimal methodological conditions for preserving calcium transport and Ca^{2+}-ATPase activity in sarcoplasmic reticulum during and after its isolation. Simultaneously, it was attempted to gain some understanding about the factors required for stability of the membranes.

Materials and Methods

Isolation of sarcoplasmic reticulum. Rats of either sex, weighing 120–180 g, were killed by stunning and

* To whom reprint requests should be sent at (present address): Departamento de Ciencias Naturales, Universidad Nacional del Sur, 8000 Bahia Blanca, Argentina.

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetate; PMSF, phenylmethylsulfonyl fluoride.

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bled through the carotid arteries. Whole muscle or in some cases pure red fibers from hindleg musculi soleus semitendinosus were quickly excised and placed in ice-cold distilled water. Two different extracting solutions were used to prepare the homogenates. Solution A contained 0.1 M KCl, 2 mM EDTA and 5 mM potassium phosphate, pH 7.0. Solution B was composed of 0.1 M KCl, 10 mM imidazole, pH 7.4, 0.3 M sucrose and 0.1 mM PMSF. The muscles were freed of connective tissue and fat and homogenized with an Omni-Mixer (Ivan Sorval, Inc., U.S.A.) in a 4-fold volume of extracting medium during 90 s at 15 700 rev./min. Thereafter, the homogenate was centrifuged for 30 min at 9 900 $\times g$ in a preparative ultracentrifuge (Beckman, model L2-65). The supernatant was passed through cheesecloth prewashed with extracting medium. The centrifugation and filtration steps were repeated once. The filtrate was then centrifuged for 60 min at 80 000 $\times g$. The sediment was resuspended in 0.6 M KCl, 2 mM ATP, and centrifuged as before to remove contaminating actomyosin. The final pellet was resuspended in 0.1 M KCl to give a protein concentration of approx. 10–15 mg/ml. The determination of protein concentration was performed according to the method of Lowry et al. [7].

Assays of calcium uptake and ATPase activity. The initial rate of calcium uptake was estimated as described before [8] using the following standard conditions: 5 mM ATP, 10 mM $MgCl_2$, 20 mM histidine, pH 7.0, 40 mM KCl, 5 mM oxalate, 0.1 mM $CaCl_2$ (plus $^{45}CaCl_2$) and 0.05 mg vesicular protein/ml. The uptake reaction was stopped after 30 s incubation by filtration through Millipore filters (pore diameter: 0.45 μm). Storage capacity was measured after 30 min incubation under conditions of excess calcium (0.2 mM) and lower vesicular protein concentration in the medium (0.005 mg/ml). In a few experiments, ^{45}Ca permeability of sarcoplasmic reticulum membranes was also estimated. Vesicles (5 mg/ml) were incubated in a medium containing 20 mM $^{45}CaCl_2$, 20 mM KCl. The efflux was initiated upon 30-fold dilution with 40 mM KCl, 10 mM EGTA, pH 7.3. Aliquots were sampled from 0 to 20 min, filtered through a Millipore filter and the radioactivity of the filter measured.

The assay mixture for determining ATPase contained 20 mM histidine, pH 7.0, 40 mM KCl, 5 mM ATP, 5 mM oxalate, 5 mM $MgCl_2$, 0.5 mM EGTA and 0.5 mM $CaCl_2$. For assay of Mg^{2+}-ATPase no calcium was added to the medium. The reactions were started by addition of the membranes (0.5 mg protein/ml) and after 1, 2, 3 and 5 min incubation aliquots were taken and mixed with an equal volume of 6% trichloroacetic acid. The mixture was centrifuged and P_i determined in the supernatant. Calcium uptake and ATPase activity were measured at 25°C. Assays in the presence of 5 mM NaN_3 were run to estimate mitochondrial contamination.

Column chromatography of solubilized sarcoplasmic reticulum membranes. The vesicles were treated at 0°C with Triton X-100 and calsequestrin was removed by centrifugation according to the method of Hasselbach and Migala [9]. The solubilized protein (50–70 mg) was separated on a 2.5 $\times$ 50 cm DEAE DE 32 cellulose (Whatman Biochemicals Ltd.) column equilibrated and eluted with 30 mM Tris-HCl, pH 8.1, 5 mM $CaCl_2$, 10% glycerol and 2 mg/ml Triton X-100. The elution profile was monitored at 280 nm by a 2089 Uvicord II (LKB, Sweden).

Results

Fig. 1 shows that vesicles isolated from rat muscle homogenates prepared with solution A ('A vesicles') lose their calcium uptake function rather rapidly upon storage in ice. The initial rate of uptake decays to 15–18% of the initial values at 44 h, while the storage capacity is affected to a lesser degree (approx. 45%). It is known that rabbit membranes maintain constant activity for 1 week when extracted with a similar solution [6]. Substitution of solution B for solution A to extract sarcoplasmic reticulum from the rat muscle fibers ('B vesicles') resulted in an improved stability. After 44 h, both the initial rate and the storage capacity were maintained without appreciable decay. Transport values not lower than 80% of the initial ones could be measured even after 5 days of storage.

The loss of calcium transport in 'A vesicles' upon storage was accompanied by a reduction of the calcium-sensitive component of the ATPase (extra ATPase) and a proportional increase of the basal ATPase (Table I). High basal ATPase activity in these preparations could not be attributed to mitochondrial contamination as has been reported by other authors

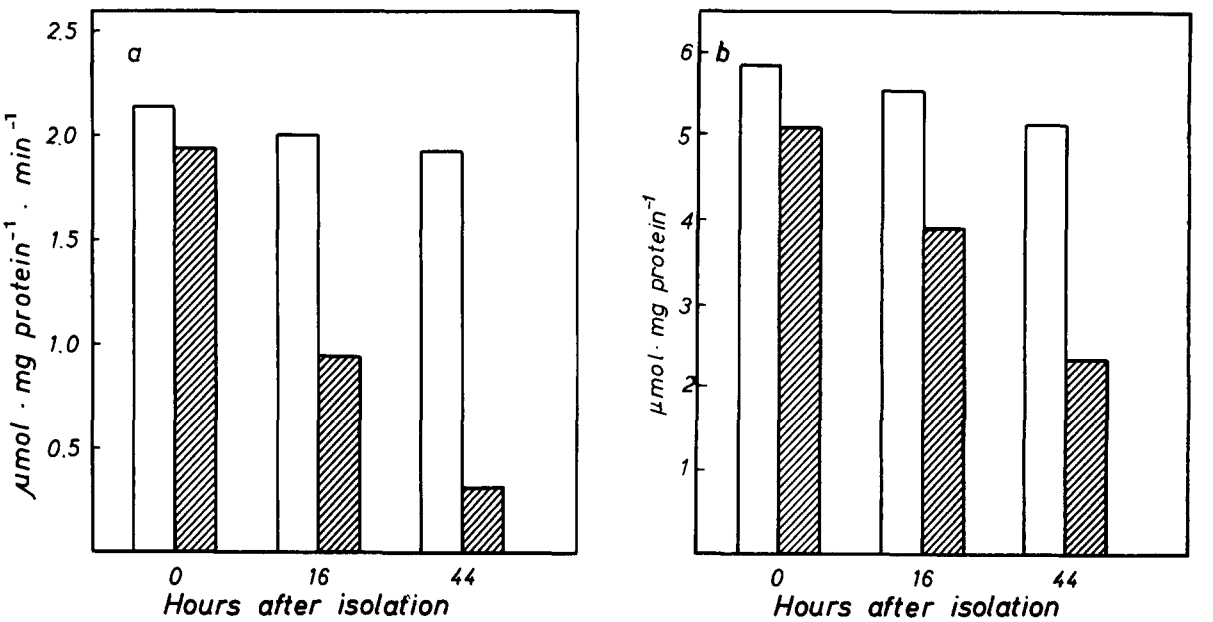

Fig. 1. Stability of calcium uptake in sarcoplasmic reticulum isolated from rat leg mixed muscles employing different extracting solutions. Shaded bars, solution A; unshaded bars, solution B. The composition of bath solutions is described in Methods and Materials. a, rate of calcium uptake; b, calcium storage.

[10], since the addition of NaN_3 to the assay medium was without appreciable effect. The B vesicles which were able to transport higher amounts of calcium displayed higher and more stable Ca^{2+}-ATPase activity. Surprisingly enough, the transport enzyme in vesicular preparations obtained from pure red fibers with solution B is approx. 70% calcium sensitive (Table II).

The factors involved in the rapid decay of the calcium transport function of A vesicles were investigated. It could not be related to the absence of sucrose in the medium, since its addition in the concentration range 0–0.45 M did not result in greater stability. Similarly, the incorporation of PMSF as a proteolytic enzyme inhibitor was without effect. Moreover, comparison of the protein, phospholipid and fatty acid composition by standard procedures [8,11] with that of B vesicles did not reveal any significant difference, as indicative of degradative action by hydrolytic enzymes. The loss of calcium transport activity was not associated either with oxidation of essential thiol groups, since dithiothreitol did not prevent it. Measurements of calcium-

TABLE I

STABILITY OF CALCIUM-DEPENDENT ATPase IN SARCOPLASMIC RETICULUM ISOLATED FROM RAT MIXED LEG MUSCLES EMPLOYING DIFFERENT EXTRACTING SOLUTIONS

Extracting solution	Storage time (h)	Initial rate of calcium uptake (μmol Ca/min per mg protein)	ATPase activity (μmol P_i/min per mg protein)		
			Mg^{2+}	$Ca^{2+} + Mg^{2+}$	Ca^{2+}
Solution A	0	1.560	0.73	1.29	0.56
Solution B	0	1.870	0.41	1.43	1.02
Solution A	16	0.923	1.00	1.19	0.19
Solution B	16	1.815	0.38	1.33	0.95

TABLE II

STABILITY OF CALCIUM TRANSPORT AND ATPase ACTIVITY OF SARCOPLASMIC RETICULUM EXTRACTED FROM RAT WHITE AND RED SKELETAL MUSCLES WITH SOLUTION B (NO EDTA)

Muscle	Storage time (h)	Initial rate of calcium uptake (μmol Ca/min per mg protein)	Calcium storage capacity (μmol Ca/mg protein)	ATPase (μmol P_i/min per mg protein)		
				Mg^{2+}	$Ca^{2+} + Mg^{2+}$	Ca^{2+}
White	18	2.12	5.44	0.39	1.93	1.54
White	40	1.83	5.15	0.39	1.71	1.32
Red	18	1.45	4.30	0.42	1.74	1.32
Red	40	1.27	4.00	0.53	1.68	1.15

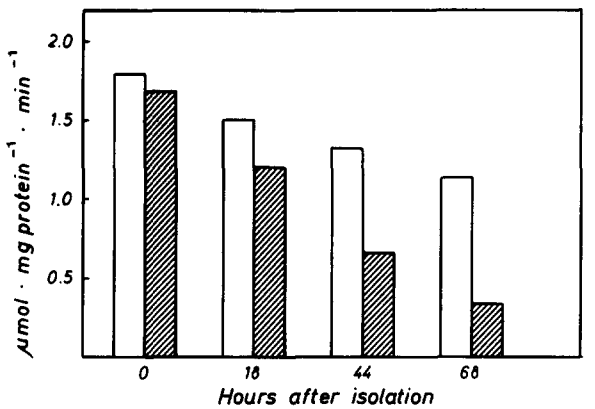

Fig. 2. Effects of the addition of calcium to extraction medium A (EDTA) on the stability of sarcoplasmic reticulum calcium uptake. Unshaded bars, 2 mM $CaCl_2$; shaded bars, no calcium added.

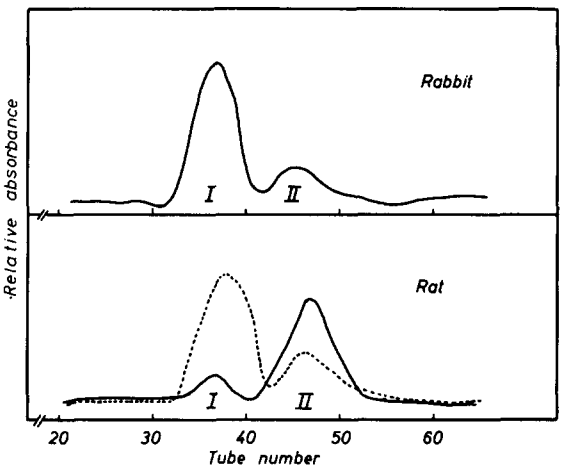

Fig. 3. Chromatographic profiles of solubilized sarcoplasmic reticulum membranes extracted with solutions A (———) and B (- - - - -).

passive permeability in A vesicles loaded with ^{45}Ca did not show higher leakiness than in B vesicles.

As the homogenizing medium to prepare A vesicles contained EDTA, essential divalent cations for maintaining the proper structure of the transport system might have been removed by the chelating agent. In Fig. 2 it is shown that addition of 2 mM $CaCl_2$ to the homogenization buffer avoids to a great extent decay of calcium uptake by A vesicles during storage. It was observed that addition of 2 mM $MgCl_2$ to the homogenates was much less effective than $CaCl_2$ in protecting the calcium transport function in sarcoplasmic reticulum.

DEAE-cellulose column chromatography of vesicles solubilized with 0.2% Triton X-100 permits one to distinguish between different functional and structural states of the ATPase. The elution pattern of sarcoplasmic reticulum extracted from rabbit muscle with medium containing EDTA consists, as shown in Fig. 3, of a major peak I which corresponds to the calcium-activated ATPase and a minor fraction II having magnesium-dependent ATPase activity when the lipids are reconstituted [9]. A similar profile is obtained with rat membranes prepared in the absence of EDTA. However, when EDTA was included the proportions of both peaks were reversed. This change was observed in the presence of 4 mM calcium throughout the elution.

Discussion

The method developed for the isolation of sarcoplasmic reticulum membranes from rat skeletal muscle allows storage of the vesicles for several days

without appreciable decay of the calcium transport function. Moreover, the preparation has ATPase activity which to a large extent is calcium dependent. This finding is particularly interesting in the case of sarcoplasmic reticulum derived from red muscle. According to several studies the sarcoplasmic reticulum from this type of muscle fiber has low activities of calcium uptake and calcium-dependent ATPase, and a high calcium-independent ATPase [1–5,12–14]. In the case of the rat, lack of a sufficient concentration of Ca^{2+} in the isolation medium required to maintain the structure of the enzyme might explain the results obtained by these authors. As in many of these studies extraction solutions without cation-chelating agents were employed, other ways of inactivation of the enzyme should not be disregarded. In our studies it is highly suggestive that the presence of EDTA in the homogenizing buffer resulted in greater lability of the preparations and that addition of calcium counteracted the effects. Deteriorating effects of Ca^{2+} chelators on the membrane have been previously observed [15].

In vesicular fragments of sarcoplasmic reticulum prepared from rabbit white muscle it has been shown [16] that the calcium transport system is in dynamic equilibrium between two states; a stable calcium-bound state and an unstable calcium-free state (obtained by washing the vesicles with EGTA) which is irreversible inactivated. The inactivation could be halted by the addition of Ca^{2+}. In contrast to our preparations, this form incapable of active transport still retains calcium-stimulated ATPase activity [17]. The calcium-binding sites which stabilize the transport system in rabbit vesicles could be of higher affinity than those in rat vesicles. In this case EDTA treatment could not effectively remove bound calcium. This would explain their greater stability when isolated under these conditions.

It also can be speculated that in the absence of calcium a membrane-bound activator of the Ca^{2+}-ATPase is released, shifting the enzyme to a calcium-insensitive state, as has been reported for human erythrocyte membranes [18,19] and more recently for rabbit skeletal muscle [20]. This possibility should, however, be disregarded as no differences in the protein profiles between stable and unstable vesicles were detected.

The measurements of ATPase activity suggested that the progressive loss of calcium uptake upon storage is apparently associated with the conversion of the transport enzyme to a calcium-insensitive form. The different elution patterns of the stable and unstable rat vesicles solubilized with detergent in DEAE-cellulose columns revealed, in addition, structural differences of their ATPase molecules.

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