Enzyme Distribution and Transport Activities in Electrophoretically Isolated Fractions of the Muscle Sarcotubular System

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Abstract. A simple electrophoretic method is introduced allowing to isolate five fractions of skeletal muscle ST-system vesicles. In a previous study differences in lipid content, ^{3}H-ouabain binding and in presence of triads in individual fractions (Lehotský et. al. 1986) were analysed. In the present study biochemical characterization was extended, and (in accordance with previous results) major differences were observed to exist between fraction 1 and fractions 3 and 4. SDS-PAGE showed that fractions 3 and 4 were enriched in a protein with m.w. 100 kD, these fractions showing the highest specific activities of $(Mg^{2+} + Ca^{2+})$-ATPase and oxalate-supported Ca^{2+}-uptake; activities of Mg^{2+}-ATPase and surface membrane marker enzymes were the lowest in these fractions. On the other hand, in fraction 1 the highest activities of Mg^{2+}-ATPase and marker enzymes of the surface membrane were observed together with a decreased content of the 100 kD protein and activities of Ca^{2+} transport. It could be concluded that the method is suitable to differentiate between relatively pure SR (fractions 3 and 4) and fractions rich in sarcolemma or T-tubules components (fractions 1 and 5).

Key words: Skeletal sarcotubular system — Vesicle fractionation — Enzyme and transport activities

Introduction

Ca^{2+} transport mechanism in sarcoplasmic reticulum and its changes under various conditions requires the isolation of sufficiently pure membrane fractions. Fractionation in a density gradient centrifugation is the presently most commonly used approach to separation of muscle membrane vesicles (Meisner 1975; Lau et al. 1977; Dyadyusha and Kursky 1985; Beeler et al. 1983). Principally different methods have been introduced recently, allowing to obtain membrane fractions which can not be yielded by centrifugation; these include

free flow electrophoresis as a preparative or analytical tool (Omengi and Snyder 1983; Debanne et al. 1984) or chromatography on glass beads or molecular sieving (Fröman et al. 1980; Nagy et al. 1976).

We introduced a simple preparative method of electrophoresis on starch granules to separate skeletal sarcotubular (ST)-system membrane vesicles (Lehotský et al. 1985, 1986). By this method vesicles of the ST-system yield five fractions differing in their lipid contents, morphological image and ^{3}H-ouabain binding. The present study extends biochemical characterization of fractions by describing their $(Mg^{2+}+Ca^{2+})$-ATPase activities, Ca^{2+} accumulation, sarcolemma-bound enzymes, and protein contents.

Materials and Methods

"Prima A" type starch was from Chynorany Starch Works (Czechoslovakia), sucrose, SDS and acrylamide were from Serva (FRG), ATP sodium salt and AMP sodium salt from Reanal (Hungary), $^{45}CaCl_2$ (197 MBq . mg^{-1}) from INR (Poland); all other chemicals of analytical grade were from Lachema (Czechoslovakia).

Rabbit ST-system vesicles were prepared according to Caswell et al. (1976). Electrophoresis on starch granules (Lehotský et al. 1986) was performed with: 0.025 mol . l^{-1} Na-veronal, pH = 8.5, 0.180 mol . l^{-1} glycerol and 0.01 mol . l^{-1} KCl of the ionic strength 0.04 mol . l^{-1}, at 330 V and potential gradient of 7.33 V . cm^{-1}. Various electrophoretic time intervals were tested; 40 hours proved to be an optimum interval for the vesicles to get separated while keeping sufficient enzyme activities. SDS-PAGE was performed according to King and Laemmli (1971) using 20 to 10 per cent gradient gels at 220 V during 16 hours with 50 μg protein loading. Protein was determined according to Lowry et al. (1951).

Determination of enzyme activities: Mg^{2+}-dependent Ca^{2+}-stimulated ATP-ase-$(Mg^{2+}Ca^{2+})$-ATPase (E. C. 3. 6. 1. 3) was determined by the method of Chiesi and Martonosi (1979) at 37 °C. One ml of the reaction mixture contained (in mmol . l^{-1}): 25 Tris-HCl; pH = 7.4; 50 KCl; 4 $MgCl_2$; 4 ATP; 0.5 EGTA; 0.5 $CaCl_2$; and 75 – 100 μg protein. The reaction was stopped by adding 1 ml of 0.073 mol . l^{-1} trichloracetic acid, and P_i released to the medium was measured by the method of Taussky and Shorr (1953). Ca^{2+}-stimulated ATPase was estimated as the difference between total $(Mg^{2+} + Ca^{2+})$-ATPase activity and Mg^{2+}-ATPase activity measured in the presence of EGTA, oligomycin and in absence of Ca^{2+}.

$(Na^+ + K^+)$-ATPase activity was determined by the method of Fedelešová et al. (1976), P_i released to the medium was measured according to Taussky and Shorr (1953). $(Na^+ + K^+)$-ATPase activity (60 – 80 % inhibitable with 1 mmol . l^{-1} ouabain) was calculated as the difference between total $(Mg^{2+} + Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase activity measured in the presence of EGTA and that in absence of the activating ions.

5'-nucleotidase was determined by the method of Glastris and Pfeiffer (1974), P_i released to the medium was measured by the method of Taussky and Shorr (1953).

Ca^{2+}-uptake into the vesicles was determined by the filtration method of Martonosi and Feretos (1964) with $^{45}CaCl_2$ in the presence of oxalate. Six ml of reaction mixture contained (in mmol . l^{-1}): 10 Histidine-NaOH; pH = 7.0; 100 KCl; 5 $MgCl_2$; $^{45}CaCl_2$(3.7.10^4 Bq . ml^{-1}); 4 ATP; temperature: 22 °C. The reaction was stopped by filtrating aliquots of the reaction mixture through a Synpor membrane filter with a pore diameter of 0.4 μm (Chemapol, Czechoslovakia). Radioactivity on the filter was measured in SLT-41 scintillation coctail (Spolana, Czechoslovakia) using a Packard 300 C

scintillation spectrophotometer. The purity of the obtained ST-fraction membranes was checked by measuring the activities of succinate dehydrogenase, oligomycin-sensitive Mg^{2+}-ATPase, NADPH-cytochrome c-reductase, 5'-nucleotidase and by electron microscopic characterization. The results show that the ST-fraction has been contaminated to less than 6 per cent by other subcellular membrane components (Lehotský et al. 1986).

Results

A simple electrophoretic method on starch granules was developed in our laboratory for the separation of membrane vesicles. This method was used to separate rabbit skeletal muscle ST-system vesicles into several fractions (Fig. 1) differing in their lipid contents, ^{3}H-ouabain binding and morphological image (Lehotský et al. 1985, 1986).

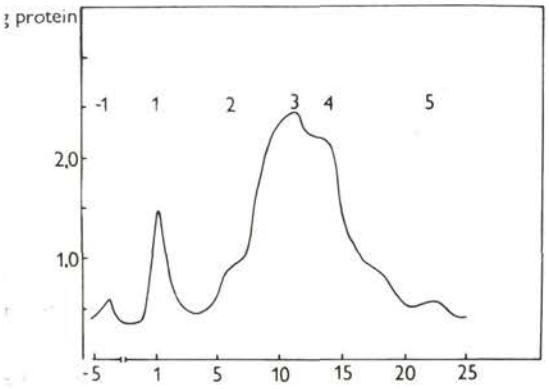

Fig. 1. Protein separation profile of ST-system vesicles. *Abscissa*: Slice number and collected fraction number (−1 to 5) *Ordinate*: Protein absorbance at 720 nm (Lowry et al. 1951)

Biochemical characterization of isolated fractions showed the highest difference in protein content between fraction 1 and fraction 3 and 4 (Fig. 2). A 100 kD protein was a major component in fractions 3 and 4 (probably Ca^{2+}-pump protein) making up 39 and 41 per cent of total proteins, respectively. In fraction 1 this protein participated in total proteins by 32 per cent only. In the latter fraction 20 kD protein found in fractions 3 and 4, could not be detected.

The highest specific (Mg^{2+} + Ca^{2+})-ATPase activity (Fig. 3), was found in fractions 3 and 4. The lowest specific activity was measured in fractions 1 and 5, while fraction 2 reached only about 65 per cent of activity of fractions 3 and 4. In spite of 40 hours lasting electrophoresis and unfavourable pH during the run (pH = 8.5), the specific activity of fractions 3 and 4 was similar to that of

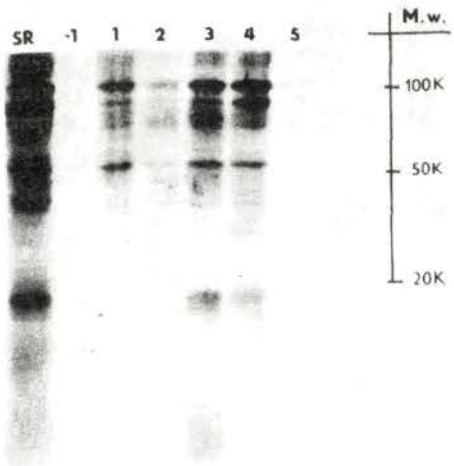

Fig. 2. SDS-PAGE of isolated fractions of ST-system vesicles. SDS-PAGE was performed according to King and Laemmli (1971). Standards used: phosphorylase b (94 kD); bovine serum albumin (67 kD); ovalbumin (43 kD); carbonic anhydrase (30 kD); soyabean trypsin inhibitor (20.1 kD); lactalbumin (14.4 kD). (Pharmacia Fine Chemicals, Sweden).

$(Mg^{2+} + Ca^{2+})$-ATPase in the original ST-system membranes (stored at pH = 7.4). Specific activities of the basal Mg^{2+}-ATPase (insensitive to oligomycin) from the individual ST-system fractions showed an inverse pattern as compared to $(Mg^{2+} + Ca^{2+})$-ATPase activity. The highest activity was found in fractions 1 and 5, the lowest one in fractions 3 and 4; in the latter fractions it reached only 10 to 17 per cent of the activity measured in membrane fractions 1 and 5. The fractions with the highest activity had by 20.5 per cent higher activity than the original ST-system.

The sarcoplasmic reticulum is a complex membrane system that serves as both a sink for calcium ions during muscle relaxation and calcium source during excitation. The transport protein, Ca^{2+}-pump, plays a major role in those processes (Hasselbach et al. 1985). Estimation of enzyme activity and particulary of active oxalate-supported Ca^{2+} accumulation by SR vesicles allows to characterize vesicle integrity, permeability and also functional capacity of the Ca^{2+}-pump. Figure 4 shows active Ca^{2+} accumulation by isolated vesicles in the presence of oxalate. The highest activity of Ca^{2+}-activated ATPase was associated with the highest uptake ability in fractions 3 and 4; the activity of fractions 1 and 2 reached only 35 to 41 per cent of that of the most active fractions. Only minimum net Ca^{2+} uptake was observed in fractions 1 and 5.

Activities of $(Na^+ + K^+)$-ATPase and 5'-nucleotidase are mostly bound to the plasmalemma (Akera and Brody 1978; Daniel et al. 1982; Hidalgo et al.

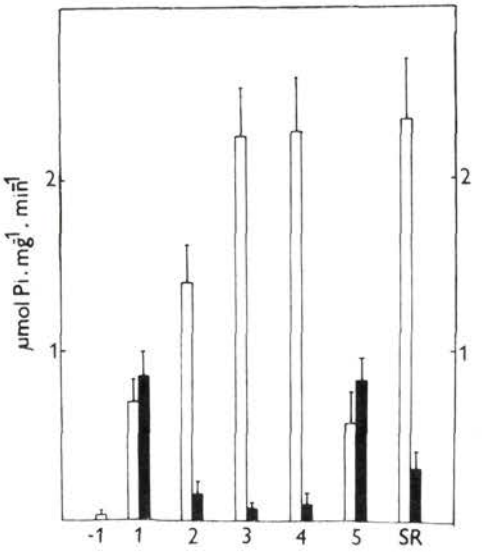

Fig. 3. Specific activities of $(Mg^{2+} + Ca^{2+})$--ATPase and Mg^{2+}-ATPase in electrophoretically isolated fractions of the ST-system. For experimental conditions, see Materials and Methods. Results are means ± double SD from 6 experiments. Empty columns: $(Mg^{2+} + Ca^{2+})$--ATPase; filled columns: Mg^{2+}-ATPase.

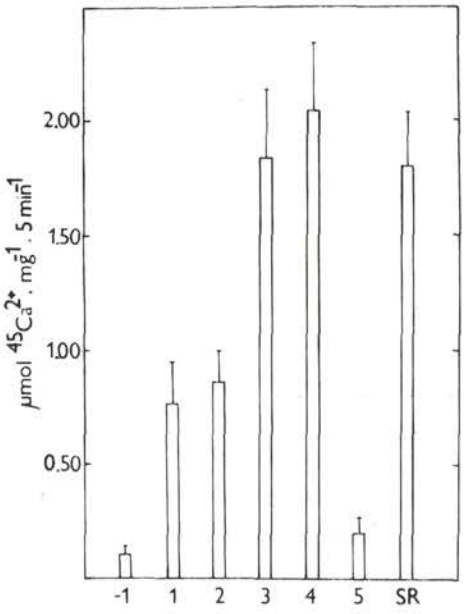

Fig. 4. Calcium uptake by ST-system membrane fractions in the presence of oxalate. $^{45}Ca^{2+}$-uptake was determined by the filtration method according to Martonosi and Feretos (1964). For experimental conditions, see Materials and Methods. Results are means ± double SD from 6 experiments.

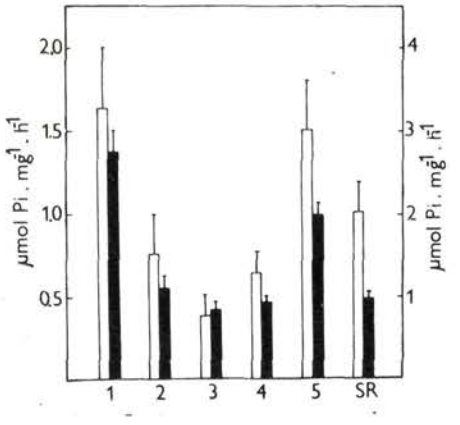

Fig. 5. Specific activities of $(Na^+ + K^+)$--ATPase and 5'-nucleotidase in isolated ST--system fractions. For experimental conditions, see Materials and Methods. Results are means ± double SD from 4 experiments. Empty columns: 5'-nucleotidase; filled columns: $(Na^+ + K^+)$-ATPase.

1986) and the distribution of their activities in individual fractions isolated by electrophoresis can provide an image of the presence of fragments of sarcolemma or T-tubules in these fractions. Fig. 5 shows that activities of both enzymes were highest in fractions 1 and 5, and lowest in fractions 3 and 4, with only 30 per cent of activity found in the most active fractions present.

Discussion

At present, membrane vesicles are most commonly separated by the method of differential or density gradient centrifugation (Meissner 1975; Caswell et al. 1976; Dyadyusha and Kursky 1985; Hidalgo et al. 1986). These methods are most commonly used to prepare comparatively pure membrane fractions. Other approches, such as free flow electrophoresis (Omengi and Snyder 1983; Debanne et al. 1984), gel chromatography, chromatography on glass beads, or HPLC are also often used (Fröman et al. 1980; Nagy et al. 1976; Lüdi and Hasselbach 1984).

A simple separation method, namely electrophoresis on starch granules, developed by us to fractionate ST-system vesicles is a new approach to membrane vesicles separation (Lehotský et al. 1985, 1986). The method is based on size- and charge-dependent separation, while the surface of starch granules and pH is also playing an important role. Based on high phospholipid and cholesterol content, ^{3}H-ouabain binding and the presence of triads in isolated fraction 1, we concluded that this method allows to purify sarcoplasmic reticulum (fractions 3 and 4) from original unfractionated ST-system membranes and to separate from it a plasmalemma-enriched fraction (T-tubules) (Lehotský et al. 1986).

SR is a comparatively simple membrane containing a major protein, the Ca^{2+}-pump (m.w. 100,000 D) making up about 30—60% of the total protein; some other minor proteins, e.g. calsequestrin, M_{55}-protein etc. are also present (Meissner 1983; Tada et al. 1982; Tanford 1984). In our crude preparation of ST-system membranes a 100 kD protein (probably Ca^{2+}-pump) made up about 33 per cent of total protein. The results of SDS-PAGE confirmed previous considerations concerning fractionation of ST-system vesicles. The fractions differed from each other by the content of a 100 kD protein and by the presence of minority 20 kD protein (fractions 3 and 4).

The results of SDS-PAGE were strengthened by the estimation of sarcoplasmic enzyme activities, $(Mg^{2+}+Ca^{2+})$-ATPase and Ca^{2+}-uptake. The specific activities of $(Mg^{2+}+Ca^{2+})$-ATPase of non-purified SR vesicles prepared by the current isolation techniques varied according to the preparation method used (Tada et al. 1978; Moller et al. 1982; Hasselbach and Oetliker 1983) between 1

and 3 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$. Results of ATPase estimation in our ST-system and its fractions were well within this range. An enrichment in 100 kD protein of fractions 3 and 4 was confirmed by an increase in specific activity of Ca^{2+}-activated ATPase as compared to the other fractions.

In contrast Mg^{2+}-ATPase activity in fractions 3 and 4 was the lowest, reaching 8—10 times lower values that in fractions 1 and 5. Surface membrane fractions enriched in T-tubule vesicles have been isolated from different animal species. The T-tubule membranes are characterized by an unusually high cholesterol content and by a very active Mg^{2+}-basal ATPase (Rosemblatt et al. 1981; Beeler et al. 1983; Baskin and Kawamoto 1984). Mg^{2+}-activated ATPase of T-tubules is a simple protein with m.w. 102 kD; it can be distinguished from Ca^{2+}-ATPase by a more acidic isoelectric point and by a unique affinity for iodinated lectins (Hidalgo et al. 1983; Okamoto et al. 1985). These findings as well as reports by Fernandez et al. (1980) of highly purified SR vesicles with almost no Mg^{2+}-ATPase activity, support our previous considerations concerning the presence of surface membrane fragments (T-tubules) in fraction 1, and those concerning the nature of fractions 3 and 4. Values of Mg^{2+}-ATPase activity in fractions 3 and 4 are presumably due to a contamination with T-tubule membranes (Hidalgo et al. 1983).

Ca^{2+} transport into SR is an active process activated by oxalate, in which Ca^{2+} moves against a electrochemical gradient; an ion pump is operative consuming chemical energy derived from the hydrolysis of the terminal phosphate of ATP (Katz 1984; Tada et al. 1978; Tanford 1984). The activity of oxalate supported Ca^{2+}-uptake in the individual fractions of the ST-system showed a similar distribution as did the inherent $(Mg^{2+} + Ca^{2+})$-ATPase activity. The activity in fraction 1 was only 35 to 40 per cent that of the most active fractions 3 and 4; fraction 5 reached only very low activity. This can be explained by a comparatively low content of the transport protein (see SDS-PAGE) or by the presence of untight or leaky vesicles unable to make a net Ca^{2+}-uptake.

$(Na^+ + K^+)$-activated ATPase and 5'-nucleotidase have been considered to be marker enzymes of muscle cell surface membrane or its invaginations-T-tubules (Akera and Brody 1978; Daniel et al. 1982; Hidalgo et al. 1986). The distribution of activities of these enzymes in isolated fractions corresponds well to the respective abilities of fractions to bind active ^{3}H-ouabain (Lehotský et al. 1986). The results suggest that fractions 1 and 5 are enriched in this type of membranes. On the other hand, fractions 3 and 4 contain only low levels of these enzymes (e.g. purified SR).

Based on our results (a high lipid content, ^{3}H-ouabain binding, high activities of $(Na^+ + K^+)$-ATPase, 5'-nucleotidase and Mg^{2+}-ATPase typical for surface membranes, and the presence of triads in fraction 1; protein content, high activities of $(Mg^{2+} + Ca^{2+})$-ATPase and Ca^{2+}-uptake in fractions 3 and 4) the

conclusion can be drawn that the method is suitable to differentiate between relatively pure sarcoplasmic reticulum (fractions 3 and 4) and fractions rich in sarcolemma or T-tubule components (fractions 1 and 5).

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