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SIMULTANEOUS PREPARATION OF MEMBRANE FRACTIONS FROM SMALL AMOUNTS OF SKELETAL MUSCLE : A STUDY ON MITOCHONDRIAL AND MICROSOMAL FRACTIONS FROM Med^{J} MICE

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This work is the first biochemical study of skeletal muscle membranes isolated from mice displaying an inherited neuromuscular disease: Med^{J} strains. It is focused on the research of a possible alteration of membrane biological activities related to this disease. We describe a procedure which allows the simultaneous preparation of mitochondrial and microsomal fractions from a small amount of skeletal muscle.When EGTA and BSA are present in the buffers, functional mitochondria can be prepared. Under these conditions we found that no major modification occurs for this disease at the mitochondrial inner membrane level. A dramatic impairment of a calcium active transport activity found in the microsomal fraction obtained from Med^{J} is noticed, suggesting that some modification may occur at this level.

In the study of genetic defects of muscular dystrophies and other inherited diseases, it has been proposed that the cellular membrane systems were affected in their permeability properties (1, 2). A limiting step in performing biochemical studies on these systems is the obtaining of homogeneous material from small amounts of skeletal muscles. In this paper we describe a technique which leads rapidly to sufficient quantity of subcellular membrane fractions from low amount of skeletal muscles. Since the main purpose of this study is to know whether or not a modification of membrane biological activities can be detected it is, at this preliminary step of the study, unnecessary to obtain highly purified samples. When this study has been achieved, if a noticeable alteration of an intrinsic activity of a fraction is observed, a more accurate study will then be carried out. We have used

Abbreviations : EGTA : ethylene glycol bis (beta-aminoethyl ether)-N,N'-tetra acetic acid; BSA : bovine serum albumin; RCR : respiratory control ratio; OSATPase : oligomycin sensitive ATPase activity.

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this procedure for the obtaining of membrane systems from animals bearing an inherited neuromuscular disease : mice from Med^{J}(3). This mutation occurs at the motor end plate region.

MATERIALS and METHODS

Med^{J} mice and their controls (6 to 8 week-old) were kindly supplied by Dr. Rieger (INSERM U118 Paris).

Obtaining of the different fractions - The entire procedure was done at 4°C. One g of hind leg muscles freed from fat and cut with scissors were homogenized by a Polytron PT 10/35 for 5 seconds at 18,000 rpm in 10 ml of buffer I (10 mM Tris-maleate, pH 7.2, 3 mM EGTA, 275 mM sucrose and 0.3 % (w/v) BSA. The crude homogenate (H) was centrifuged for 10 min at 1,000g. The supernatant S1 was filtered through four layers of cheese cloth and centrifuged again at 12,000g for 20 min. The pellet P2 and the supernatant S2 were used to prepare fractions F2 and F3 respectively. The pellet resuspended in 20 ml of buffer II (10 mM Tris-maleate pH 7.2, 0.1 mM EDTA, 135 mM KCl and 0.2 % (w/v) BSA) was centrifuged at 12,000g for 20 min. The resulting fraction F2 was resuspended in buffer III (10 mM Tris-maleate pH 7.2, 0.1 mM EDTA, 275 mM Sucrose and 0.1 % (w/v) BSA) and used for activity measurement within two hours.

The supernatant S2 was centrifuged one hour at 37,500g. The resulting supernatant was discarded. The pellet resuspended in 10 to 15 ml of buffer IV (10 mM Hepes pH 7.2, 300 mM sucrose) was treated for one hour by 600 mM KCl and centrifuged again at 37,500g for one hour. The pellet (fraction F3) was resuspended in buffer IV.

Activity measurements - The oxygen consumption of the mitochondrial fraction was measured by means of a Clark electrode. The respiratory rate and the ADP/O ratio were calculated according to Chance and Williams (4). Calcium uptake by mitochondria was determined by means of a Ca^{2+} electrode (5). In 4 ml of 10 mM Hepes-K pH 7.2 buffer containing 60 mM KCl, 156 mM sucrose and 0.15 % (w/v) BSA in which were successively added 46 to 64 µM free calcium, 12 µg of rotenone, 3 µg of oligomycin and 1 to 1.4 mg of mitochondria. The reaction was initiated by addition of 1 mM K-succinate final concentration. OSATPase activity was determined by measuring the amount of released inorganic phosphate according to Somlo (6). Calcium uptake activity of sarcoplasmic reticulum containing fractions was obtained using oxalate as already described (7). Acetylcholinesterase activity was measured according to Ellman et al. (8), succinodehydrogenase according to Singer et al. (9) and 5' nucleotidase with the procedure of Wallis et al. (10). The protein concentration was obtained by the Lowry method (11) with BSA as a standard.

RESULTS

The membrane fractions obtained are characterized on the basis of their usual positive or negative enzymic activities (Table 1). Fraction F2, which contained the highest succinodehydrogenase specific activity, no detectable calcium uptake, and low acetylcholinesterase and 5' nucleotidase activities, was mitochondrial fraction. It has to be noticed that a relatively important oligomycin insensitive ATPase activity remained in these samples (in both cases, i.e. control and dystrophic animals this activity represents 0.35 µmol Pi min^{-1} mg^{-1} of protein corresponding to

Table 1: Marker enzyme activities of subcellular fractions obtained from mice skeletal muscle

Fractions	Yield▲	Succinodehydrogenase activity (■)	Active calcium transport (□)	Acetylcholinesterase activity (●)	5'-nucleotidase activity (○)
H	106	0.17	n.d	n.d	n.d
S1	2.2	0.25	0.70	10.5	1.7
F2	1.3	1.60	n.d	8.5	2.0
S2	1.9	0.25	0.22	11	3.5
F3	0.8	0.17	1.40	77.5	22.5

▲ : mg of protein per gram of wet muscle; (■) $\Delta OD_{578\ nm}$ per minute per mg of protein
(□): µmol Ca^{++} transported per minute per mg of protein; n.d: non detectable
(●): nmol acetylthiocholine iodide hydrolysed per minute per mg of protein; (○): nmol Pi appeared per minute per mg of protein. Values obtained from a typical experiment.

25 % of the total ATPase activity of these fractions). The values obtained for respiratory rates and RCR are good enough and comparable or sometimes even better than those obtained from Nagarse treatment (12).

Under the mild conditions of membrane disruption that we have used and after a short treatment by 600 mM KCl a relatively large amount of a microsomal fraction F3 was obtained. On the basis of the enzymatic markers used (calcium uptake, acetylcholinesterase and 5'nucleotidase activities) it may be considered as a mixture of sarcoplasmic reticulum and tightly bound sarcolemmal membrane fragments. At this stage of the study, no attempt has been done to further purify this fraction.

This procedure has been used to the study of membrane samples obtained from small amount of muscle from mice. The yields of the fractions obtained were comparable between (+/+) and Med^J/Med^J strains, suggesting that we are not selecting only functional material.

On Table 2 comparisons are done between the respiratory activities of the mitochondrial fractions obtained with (+/+) and Med^J/Med^J animals respectively. When using either pyruvate or glutamate plus malate or succinate plus rotenone as respiratory substrates the different fractions do not show any significant difference in their respiratory rates and

Table 2: Respiratory activities of the mitochondrial fractions F2 obtained with control and Med^J mice

Respiratory substrate	strains	Respiratory rates		RCR	ADP/O
		State III	State IV		
Pyruvate-malate	+/+ (19)	150 ± 15	25 ± 5	6	2.8 ± 0.2
	Med^J/Med^J (6)	140 ± 25	20 ± 5	7	3
Glutamate-malate	+/+ (4)	77 ± 12	16 ± 2	5	3.0 ± 0.2
	Med^J/Med^J (4)	65 ± 5	10 ± 6	6.5	3
Succinate-rotenone	+/+ (14)	100 ± 25	55 ± 10	1.8	1.7 ± 0.3
	Med^J/Med^J (6)	110 ± 20	80 ± 15	1.4	1.6 ± 0.3

Respiratory rates in nanomoles oxygen per minute per mg of protein) are measured in 3 ml of buffer III containing 5 mM Tris-Pi, pH 7.2; 5 mM pyruvate or 5 mM glutamate plus 1 mM malate or 5 mM succinate plus 1.25 µg of rotenone; 100-300 µg of protein in the presence and the absence of 13 µM ADP. The figures in the parentheses represent the numbers of preparations tested.

consequently in their RCR. In addition, the ADP/O ratios never show significant modification with any kind of fraction studied whatever the respiratory substrate. Therefore, one can conclude that F2 is fully functional if we only consider the respiratory chain. At this point, we have to emphasize the role of BSA and EGTA added to the buffer. If one omits EGTA and/or BSA, the mitochondria obtained are always uncoupled. EGTA alone cannot restore optimal coupling. BSA at 0.3 % in buffer I is necessary to bring back the RCR at its optimal level for Med^J animals (0.1 % being sufficient for full restoration of the optimal RCR of their control littermates). In addition the calcium uptake activity of mitochondria was measured with succinate as respiratory substrate. There was no significant difference in this uptake for mitochondria obtained from +/+ and Med^J/Med^J animals (60 ± 5 and 70 ± 10 nanomoles of Ca^{2+} min^{-1} mg^{-1} of protein for +/+ and Med^J/Med^J respectively).

The Ca^{2+}-ATPase and Ca^{2+} transport of F3 prepared from Med^J strain are presented in Table 3. A decrease in the rate of calcium uptake and in the ability to sequester this cation in the presence of oxalate, was

Table 3 : Calcium transport and Ca^{2+}-ATPase activities of F3 obtained from control and Med^J/Med^J mice

Strains		Ca^{2+} transport			Ca^{2+} ATPase activity		
		V_{Ca} (■)	C_{Ca} (□)	V_{ATP} (●)	V_{ATP}^{EGTA} (△)	V_{ATP}^{Ca} (○)	V_{ATP}^{Ca}-IO (▼)
+/+	(a)	1.6	3.8	0.10	0.12	0.61	1.7
	(b)	1.4	3.2	0.22		1.12	2.0
Med^J/Med^J	(a)	0.5	1.0	0.68	0.12	0.63	2.1
	(b)	∿ 0	-	0.58		0.61	1.8

(■) rate of calcium uptake (µmole calcium per minute per mg of protein) measured at 20°C in 10 mM Hepes, pH 7.2; 100 mM KCl; 5 mM $MgCl_2$; 5 mM K-oxalate; 0.1 mM $CaCl_2$; 5 mM ATP and 25 µg/ml of protein. (□) µmole of calcium accumulated per mg of protein.
Rate of ATP hydrolysis (µmoles Pi per minute per mg of protein) in absence of added calcium (●); with 0.1 mM EGTA (△); with 0.1 mM calcium (○); with 0.1 mM calcium and 2 µM of A23187 (▼).
(a) and (b) represent values obtained with freshly prepared or frozen (one night at -22°C) microsomes respectively. All these activities were obtained at ± 10 % from 6 experiments.

observed when we compared the data obtained with F3 from control (+/+) and Med^J/Med^J mice. At the same time, an increase in the ATPase activity (V_{ATP}) is noticed suggesting that the SR fraction obtained from Med^J/Med^J mice is a mixture of coupled and non functional material displaying an uncoupled ATPase activity as it is observed for "leaky" vesicles. Moreover, V_{ATP}^{EGTA}(obtained in presence of 0.1 mM EGTA) and V_{ATP}^{Ca}-IO (obtained in presence of 2 µM A23187 ionophore) did not show significant difference among the SR from +/+ and Med^J/Med^J animals. This strongly suggests that the amount of Ca^{2+}-ATPase per mg of protein is nearly the same in each fraction.

In addition, the calcium transport activity is rapidly destroyed upon storage at low temperature leading to total insensitivity towards calcium of the ATPase.

A very important decrease in the acetylcholinesterase and 5'nucleotidase specific activities is observed for F3 obtained with the mutant (38.7 % and 24.6 % for acetylcholinesterase and 5'nucleotidase respectively). At this stage of the work, two explanations may be raised : (i) either the preparation procedure more or less destroyed these activities

when it was applied to the mutant; (ii) or this result may indicate that the two enzymatic markers are altered due to plasma membrane modification.

DISCUSSION

Taken together, these results show that it is possible to obtain in the same experiment and from a low amount of skeletal muscle, biologically active subcellular fractions showing degree of purity allowing the study of their modification, if any, in the case of muscular diseases. Thus, we obtained two fractions : F2, enriched in mitochondria, and F3, enriched in sarcoplasmic reticulum. The rate of calcium uptake (V_{Ca}) indicates that this fraction was enriched enough in sarcoplasmic reticulum.

This allows a preliminary study of the behaviour of this entity in the case of neuromuscular disease. In this work, we do not consider possible functional interactions between the surface membranes and the Ca^{2+}-transport activity localized in sarcoplasmic reticulum.

Used for the first time in the study of mitochondrial and microsomal fractions obtained from Med^{J} mice, this procedure shows that only sarcoplasmic reticulum seems to be altered by the mutation. Obviously, this does not imply that the first target of the mutation in the cell is sarcoplasmic reticulum but does indicate that some specific modifications of the environment of the Ca^{2+} pump in this membrane may induce the instability observed for this system. With a high calcium ATPase activity (V_{ATP}) and very low coupling and rate of transport this sample behaves as skeletal sarcoplasmic reticulum membranes in which detergent molecules have been incorporated. One hypothesis can be raised. This assumes that the structural modification of the motor end plate mutation at the level of nodes of Ranvier observed for this strain (13, 14) modifies the calcium pumping efficiency of this system.

The application of this technique to the study of muscular diseases in mice shows the limits and the usefulness of this procedure. Firstly

we have to assume that the hind leg muscles from which we obtained the biological samples have been modified to the same extent and that the disease affects identically all the animals used in our experiments. This limitation has been partially overcome, since this work was undertaken on a limited number of mice. Secondly, the question whether or not the impairment eventually observed was created during homogenization or already existing in vivo will remain to be elucidated. The advantage of this approach is obvious : we are able in one experiment to get a general and comparative view of the state of biological membranes activities purified from a small amount of muscle.

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