ESTIMATION OF FUNCTIONAL PARAMETERS OF MITOCHONDRIA IN SITU BY RECORDING RESPIRATION OF SKINNED HEART FIBERS

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Investigation of parameters of mitochondrial respiration usually involves isolating these organelles from a tissue homogenate. In that case, several different mitochondrial fractions are as a rule obtained [8, 13, 14]. In turn, the method of isolating mitochondria from tissue affects their properties and their distribution among the various fractions [11, 12], but the total mitochondrial yield is usually only 10-20% [4]. This creates serious difficulties for the objective evaluation of mitochondrial function in a tissue, especially when properties of mitochondria are compared under normal and pathological conditions. With this in mind we developed a new method of estimating the functional parameters of mitochondria without isolating them from the tissue. Treatment of bundles of muscle fibers with saponin is known to destroy the sarcolemma of the cells [3, 9]. Conditions of processing and compositions of the incubation media used in this work enabled preparations of skinned fibers to be obtained, containing the whole population of morphologically and functionally intact mitochondria. This paper gives the results of a study of the respiratory characteristics of saponin-treated bundles of rat myocardial fibers under normal and pathological conditions.

EXPERIMENTAL METHOD

Bundles of myocardial fibers 0.3-0.4 mm in diameter and 5-7 mm long were isolated from the endocardial surface of the left ventricle of Wistar rats and incubated for 20 min in solution A (composition, see below) containing 50 mg/ml of saponin. The bundles of fibers were then washed for 10 min in solution B (composition, see below) to remove saponin. All the above procedures were undertaken in the cold with vigorous shaking of the solutions. Completeness of skinning after saponin treatment was verified by measuring the lactate dehydrogenase (LDH) content in the preparations as described previously [7]. Mitochondria were determined quantitatively in the fibers by determining cytochrome aa_3 by a spectrophotometric method [1]. The protein content in the fibers was measured after homogenization [10]. Preparations for electron microscopy were fixed with 3% glutaraldehyde in 0.1M phosphate buffer, pH 8.0. A group of seven or eight bundles of chemically skinned fibers was transferred into an oxygraphic cell, equipped with a mixer and filled with 3 ml of solution B. The rate of oxygen absorption was measured by oxysymmetry, using a Clark electrode and "Yellow Spring Instruments" oxygraph (USA), equipped with thermostatically controlled mixer at 22°C. Solubility of oxygen was taken to be 460 ng·at/ml. All solutions used in the work had 10 mM EGTA-CaEGTA buffer (free Ca^{2+} concentration 0.1 μM), 3 mM free Mg^{2+}, 20 mM taurine, 0.5 mM dithiothreitol, and 20 mM imidazole, pH 7.0, and an ionic strength of about 0.16M, controlled by the addition of 2(N-morpholino)ethanesulfonate. Free concentrations of Ca^{2+} and Mg^{2+} were calculated on the basis of equations described by Fabiato and Fabiato [5], using values of dissociation constants obtained from [6]. Solution A also contained 5 mM MgATP and 15 mM creatine phosphate. Solution B, besides the other ingredients, contained 5 mM glutamate, 2 mM malate, 3 mM phosphate, and 5-10 mg/ml of bovine serum albumin (BSA), but contained no free fatty acids. To study the effect of ischemia on mitochondrial function in skinned myocardial fibers the heart was placed in a humid chamber at a temperature of 37°C. Periods of total ischemia lasted 15 and 30 min after which bundles of fibers were isolated from the hearts.

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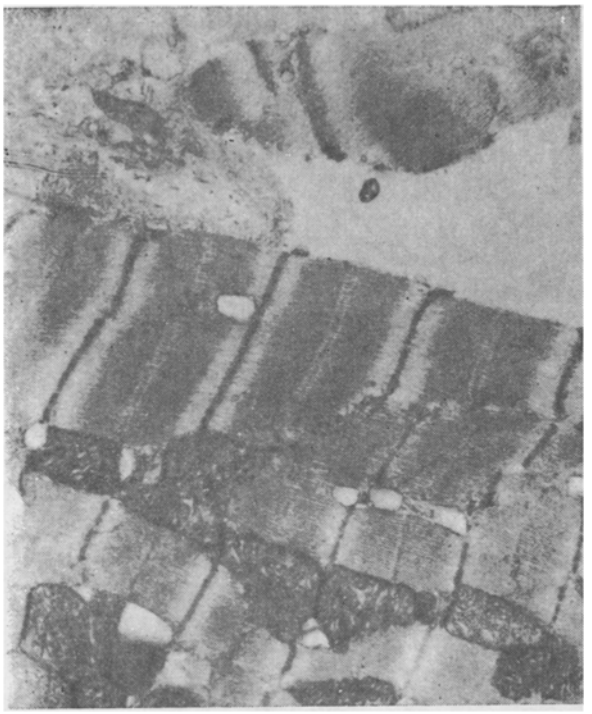

Fig. 1

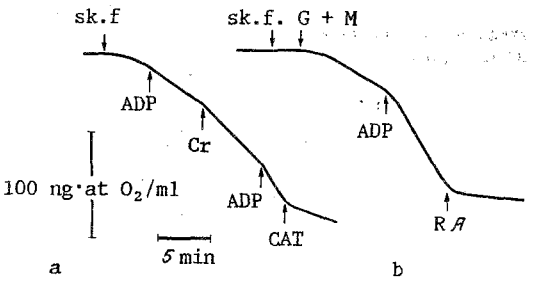

Fig. 2

Fig. 1. Cardiomyocyte of a skinned fiber. Lateral sarcolemma completely absent. Contractile structures show no visible changes: all disks and bands in the sarcomeres are clearly distinguishable. Sarcoplasmic reticulum as a whole is unchanged. Mitochondria completely preserve their structural integrity. Magnification ×12,000.

Fig. 2. Typical oxygrams of respiration of skinned fibers. Medium (3 ml) initially contains (a) or does not contain (b) substrates for mitochrondrial respiration. Arrows indicate time of addition: of skinned fibers (sk.f.), 5 mM glutamate and 2 mM malate (G + M), 60 μM ADP (ADP), 20 mM creatine (Cr), 1 mM ADP (ADP), 20 μM rotenone (R) or 3 μg/ml antimycin (A); 35 μM carboxyatractyloside (CAT).

The action of isoproterenol on the respiratory characteristics of the mitochondria was studied in experiments in which the rats were killed 3 h after subcutaneous injection of 80 mg/kg of the drug.

EXPERIMENTAL RESULTS

Electron-microscopic study of saponin-treated myocardial fibers (Fig. 1) revealed a good state of preservation of the intracellular organelles, including mitochondria, and complete destruction of the sarcolemma. The LDH content in the myocardium after saponin treatment was about 1% of its original content, evidence of disturbance of the integrity of the sarcolemma of the cells virtually throughout the preparation. The concentration of cytochrome aa_3 is skinned fibers (20.4 ± 1.8 nmoles/g wet weight) was virtually the same as its concentration in heart tissue measured in the present investigation (22.5 ± 2.0 nmoles/g) and cited in the literature (21 ± 1.5 nmoles/g) [1]; consequently, skinned fibers preserved the entire mitochondrial population.

A typical oxygram obtained during measurement of respiration of saponin-treated bundles of fibers is given in Fig. 2. Clearly respiration is appreciably intensified by those factors which stimulate respiration of isolated mitochondria, namely ADP and creatine, and it is inhibited by rotenone, antimycin A, and by carboxyatractyloside (CAT), an inhibitor of ATP–ADP translocase. Averaged relative data on oxygen absorption by skinned myocardial fibers in the control, in ischemia, and after administration of isoproterenol are given in Table 1. The percentage increase in the velocity of respiration after addition of creatine in the presence of 60 μM ADP reflects mitochondrial creatine kinase activity. The ratio of the maximal velocity of respiration and of basal respiration in the absence of ADP is to some extent an analog of the respiratory control for isolated mitochondria and characterizes coupling of respiration with oxidative phosphorylation. Finally, the third parameter – the percentage

TABLE 1. Respiratory Characteristics of Skinned Fibers (n = 9) from Rat Heart

Experimental conditions	Parameter		
	V(ADP)/VO	[VCr − V(adp)]/V(adp)	% CAT
Control	3,2+0,2	0,34+0,04	69,0+2,0
Ischemia for 15 min	2,9+0,1	0,18+0,01**	65,0+1,2
Ischemia for 30 min	2,2+0,2**	0,08+0,02***	39,0+2,8
Isoproterenol	2,6+0,1*	0,11+0,02**	64,1+1,0

Legend. VO) Velocity of respiration without adenine nucleotides; V(adp) the same in the presence of 60 μM ADP, V(Cr) the same after addition of 20 mM creatine; V(ADP) the same addition of 1 mM ADP; %CAT) percentage inhibition of respiration by 35 μM CAT (*$p < 0.05$; **$p < 0.1$; ***$p < 0.001$ compared with control.

inhibition of the velocity of maximal respiration by CAT – is evidence of the state of the inner mitochondrial membrane [2]. It follows from the data given in Table 1 that creatine-stimulated respiration undergoes the greatest changes. The ratio of the maximal velocity of respiration to the velocity of basal respiration after ischemia for 30 min also was considerably reduced. This same period of ischemia also had a powerful effect on the degree of inhibition of respiration by CAT. Catecholamine stress also had a marked effect on creatine- and ADP-stimulated respiration. Isoproterenol caused a marked decrease in the ratio of the maximal velocity of respiration to the basal velocity, but reduced the value of stimulation of respiration by creatine particularly strongly (threefold compared with the control).

The results indicate that the suggested method is sufficiently sensitive to evaluate the functional state of the mitochondria in the cell without having to isolate them. By means of the method it is possible to study the state of mitochondria in very small pieces of tissue weighing only a few milligrams. To isolate a sufficient number of mitochondria to study their respiratory characteristics, grams of tissue at least are required. Another advantage of the method is that the whole population of cellular mitochondria is investigated in their natural environment and in a medium similar in its ionic composition to the intracellular medium.

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