Ca²⁺-uptake properties of two populations of mitochondria from normal and denervated rat soleus muscle

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Ultraturrax- and Nagarse-released populations of mitochondria were characterized with respect to their Ca²⁺-uptake activities (i) by means of the indirect polarographic technique and (ii) directly by the ⁴⁵Ca Ruthenium Red-quench method of Reed & Bygrave [(1974) *Biochem. J.* **140**, 143–155]. The denervated-muscle subsarcolemmal and intermyofibrillar mitochondrial fractions displayed markedly decreased rates and capacities for Ca²⁺ uptake compared with their respective controls. The implications of these findings with respect to the process of cell necrosis are discussed.

Hulsmann *et al.* (1968) originally reported the isolation of two functionally distinct mitochondrial fractions from skeletal muscle. Subsequent studies performed on cardiac material (Palmer *et al.*, 1977; McMillin-Wood *et al.*, 1980; Wolkowicz & McMillin-Wood, 1980) have shown that the so-called intermyofibrillar population displays a greater rate and capacity for Ca²⁺ uptake than the subsarcolemmal fraction. However, the significance of this observation is as yet uncertain.

It is well documented in the literature that altered mitochondrial structure and function are associated with many neuromuscular diseases (Luft *et al.*, 1962; Shy *et al.*, 1966; Haydar *et al.*, 1971; Di Mauro *et al.*, 1976; Lee *et al.*, 1978; Barron *et al.*, 1979; Jacobson *et al.*, 1980; Martens *et al.*, 1980; Joffe *et al.*, 1981a). Whether these structural and functional defects are associated with one or both of the above mentioned mitochondrial populations has not been ascertained. This is because the mitochondrial isolation procedures generally employed for these pathological studies release either exclusively subsarcolemmal mitochondria or a mixed population of subsarcolemmal and intermyofibrillar mitochondria.

Below we report the Ca²⁺-uptake capacities of the Ultraturrax- and Nagarse-released populations of mitochondria isolated from normal and day-21-denervated rat soleus muscle. Our results suggest that impaired Ca²⁺ uptake by both mitochondrial populations may facilitate or increase the cytosolic Ca²⁺ overload that occurs in denervated skeletal muscle (Joffe *et al.*, 1981b).

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Materials and methods

Female Wistar rats weighing 200–250 g were used. Each assay was performed with the soleus muscle pooled from 12 rats. Denervation atrophy was produced in the left hind leg of the rats for a period of 21 days by transection of the sciatic nerve in the hip region before it gives rise to the tibial and popliteal branches. To prevent re-innervation, a 1 cm length of nerve was always removed around the point of transection. In each case the right leg was used as the control.

Preparation of the mitochondria

Skeletal-muscle mitochondria were isolated from the soleus muscles of the rat based on the method of Van Jaarsveld *et al.* (1981) for heart muscle. A 1:12 (w/v) homogenate of the muscle was used. The homogenization medium consisted of 100 mM-KCl, 10 mM-sodium EDTA, 0.5% bovine serum albumin and Tris base, which was adjusted to pH 7.4 at 2°C with HCl. The minced tissue was homogenized on ice twice for a period of 5 s each with intermittent cooling, using an Ultraturrax PT-10 homogenizer at a rheostat setting of 6. The homogenate was centrifuged at 1000 g in a Beckman J.21 centrifuge (17.1 rotor) for 10 min. The resulting supernatant was kept on ice and the nuclear pellet was resuspended to the original volume and centrifuged at 1000 g for 10 min. The two supernatants were combined and the subsarcolemmal mitochondrial pellet was obtained by centrifugation at 17 500 g for 10 min. The pellet was washed twice (with resuspension) in the homogenization medium in the absence of EDTA and resuspended in this medium

at a protein concentration of 5–6 mg/ml. The remaining nuclear pellet was resuspended as a 1:3 (w/v) homogenate in the original homogenization medium. Nagarse was added to a final concentration of 1.92 mg/g wet wt. of tissue, directly in a Potter homogenizing vessel. After one pass with a loosely fitting pestle the homogenate was immediately diluted 5-fold with the homogenizing medium and centrifuged at 10 000 *g* for 5 min. The resulting pellet was resuspended in 12 vol. of homogenizing medium and sedimented at 1500 *g* for 15 min. The nuclear pellet resulting was resuspended in this medium and centrifuged at 1500 *g* for 10 min. The supernatants from the 1500 *g* spins were combined and re-centrifuged at 17 500 *g* for 15 min. The intermyofibrillar pellet so obtained was washed twice in EDTA-free homogenizing medium and resuspended in this medium at a concentration of 5–6 mg/ml.

Protein content

Mitochondrial protein concentrations were determined by the Bio-Rad protein assay, based on the method of Bradford (1976).

Ca²⁺-uptake studies

(a) Ca²⁺ uptake was determined polarographically as previously described (Joffe *et al.*, 1981b). (b) Ca²⁺ transport was also measured by the EGTA/Ruthenium Red-quench technique (Reed & Bygrave, 1974). The reaction medium consisted of 250 mM-sucrose, 10 mM-Tris/HCl, pH 7.4, 8.5 mM-K₂HPO₄, 5 mM-glutamate, pH 7.4, 5 mM-malate, pH 7.4, and 0.5 mg of mitochondrial protein in a total volume of 0.9 ml. The solution was equilibrated at 25°C after which Ca²⁺, over the concentration range 12.5–100 nmol of CaCl₂ (containing 0.5 μ Ci of ⁴⁵Ca²⁺), was added to a final volume of 1 ml. The reaction was terminated at the times indicated in Fig. 2 by transferring 100 μ l of the assay solution into 1.5 ml Eppendorf centrifuge tubes containing ice-cold reaction medium plus 20 mM-EGTA and 10 μ M-Ruthenium Red. The tubes were immediately centrifuged (Eppendorf Microfuge; 45 s at 12 000 *g*), and 100 μ l samples of the supernatant were transferred to vials containing 10 ml of Instagel II scintillation fluid (Packard Instruments Co.) and counted for radioactivity in a Packard Tri-Carb scintillation counter.

Effect of Nagarse on the Ultraturax-prepared mitochondria

The effect of Nagarse on the functioning of the Ultraturax-prepared mitochondria was determined by adding aliquots of Nagarse to the mitochondria respiring in the oxygen electrode. The reaction medium was as previously described (Joffe *et al.*, 1981a). The mitochondrial pellet was also suspended

in Nagarse at a concentration of 0.25 mg of Nagarse/mg of mitochondrial protein for 25 s and then immediately re-centrifuged to remove the Nagarse.

Results

Polarographic study

Fig. 1 illustrates typical polarographic tracings obtained from repeated additions of 200 or 400 nmol of Ca²⁺ to (a) the Ultraturax- and (b) the Nagarse-released mitochondria from both control and day-21-denervated rat soleus muscles. It is evident that the successive Ca²⁺ additions repeatedly stimulated the mitochondrial respiratory rates during the process of Ca²⁺ uptake, after which the resting State-4 respiratory rate was resumed. Eventually, the mitochondrial Ca²⁺ capacity was reached and thereafter uncoupling or functional damage to the mitochondria occurred. It is also evident that the control Nagarse-treated fraction displayed a greater capacity and rate for Ca²⁺ uptake than the control Ultraturax-treated population. It can be seen that both Nagarse- and Ultraturax-released mitochondria from the denervated muscles displayed very low capacities for Ca²⁺ uptake. Table 1 contains the respiratory parameters associated with the present study. It is evident from Table 1 that the denervated fractions displayed decreased rates and capacities for Ca²⁺ accumulations. Both denervated-muscle Ca²⁺-stimulated respiratory rates decreased to 68% of the corresponding control values and their capacities for Ca²⁺ uptake decreased to 35% of the control values. These results suggest that the denervation process affected the functioning of both mitochondrial populations to the same extent. It is also noteworthy that the denervated Nagarse-prepared mitochondria were relatively uncoupled in that their Ca/O ratio was significantly lower than the corresponding control values.

Radioactive Ca²⁺-uptake studies

The polarographic findings were confirmed by an initial-rate study for ⁴⁵Ca²⁺ uptake in the presence of P_i at various concentrations of Ca²⁺ using the Ruthenium Red-quench method of Reed & Bygrave (1974). The results are presented in Figs. 2 and 3. Fig. 2(a) represents a typical rate curve for Ca²⁺ uptake by the denervated and control Ultraturax-released mitochondrial fractions in the presence of 50 μ M-Ca²⁺. The normal mitochondria displayed a greater rate for Ca²⁺ accumulation than did the denervated fraction. Figs. 2(b) and 2(c) represent similar curves for the Nagarse-released mitochondrial fractions in the presence of 50 μ M- (b) and 75 μ M-Ca²⁺ (c). Clearly there was very little difference between the normal and denervated curves in the presence of 50 μ M-Ca²⁺. However, in the presence

Fig. 1. Polarographic tracings indicating repeated Ca²⁺-induced stimulation of O₂ uptake in Ultraturrax- and Nagarse-released mitochondrial populations from control and day-21-denervated rat soleus muscle Mitochondria (±1 mg) were suspended in 250 mM-sucrose/10 mM-Tris/HCl (pH 7.4)/8.5 mM-K₂HPO₄/5 mM-glutamate/5 mM-malate (pH 7.4) in a final volume of 2.4 ml. Respiratory rates were stimulated with 200 or 400 nmol of CaCl₂ for (a) Ultraturrax- and (b) Nagarse-released mitochondria. (i) represents the normal mitochondria traces; (ii) represents the denervated-muscle mitochondrial traces.

Table 1. Polarographic Ca²⁺-stimulated respiratory parameters of Ultraturrax- and Nagarse-released mitochondrial populations from control and day-21-denervated rat soleus muscle

The mitochondria (±1 mg) were suspended in 250 mM-sucrose/10 mM-Tris/HCl (pH 7.4)/8.5 mM-K₂HPO₄/5 mM-glutamate/5 mM-malate (pH 7.4) in a final volume of 2.4 ml. Mitochondrial respiratory rates were stimulated with 200 or 400 nmol of CaCl₂ as indicated. The assay temperature was 30°C. Values are means ± s.e.m. The numbers of experiments performed are given in parentheses.

Source	Ca/O	Ca ²⁺ -stimulated respiratory rate (ng-atoms of O/min per mg of mitochondrial protein)	Ca ²⁺ capacity (nmol of Ca ²⁺ /mg of mitochondrial protein)
Normal Ultraturrax-released mitochondria	5.9 ± 0.2 (6)	98.1 ± 9.3 (8)	507.8 ± 41.6 (7)
Denervated-muscle Ultraturrax-released mitochondria	6.7 ± 0.3 (6)§	65.1 ± 5.6 (5)‡	182.6 ± 11.2 (6)*
Normal Nagarse-released mitochondria	6.1 ± 0.4 (7)	206.6 ± 20.1 (8)	1286.3 ± 104.0 (9)
Denervated-muscle Nagarse-released mitochondria	3.7 ± 0.5 (5)†	141.1 ± 16.1 (5)‡	448.4 ± 29.9 (6)*

* Significantly different from controls at *P* < 0.001.
† Significantly different from controls at 0.01 > *P* > 0.001.
‡ Significantly different from controls at 0.05 > *P* > 0.02.
§ Insignificantly different from controls at 0.10 > *P* > 0.05

Fig. 2. Typical rate curves for Ca^{2+} transport by Ultraturrax- and Nagarse-released mitochondria from normal and day-21-denervated rat soleus muscle

The reaction medium contained 250 mM-sucrose/10 mM-Tris/HCl (pH 7.4)/8.5 mM- K_2HPO_4 /5 mM-glutamate (pH 7.4)/5 mM-malate (pH 7.4) and 0.5 mg of mitochondrial protein in a final volume of 1.0 ml. The reaction was started by the addition of 50 nmol of Ca^{2+} (containing 0.5 μCi of $^{45}\text{Ca}^{2+}$) in (a) and (b) and 75 nmol of Ca^{2+} in (c). At the times indicated, 100 μl of the reaction mixture was removed and rapidly added to the Ruthenium Red (10 μM)/EGTA (20 mM) quench medium and the extent of Ca^{2+} transport was determined for (a) Ultraturrax- and (b and c) Nagarse-released mitochondria (see Reed & Bygrave, 1974). The assay temperature was 25°C. Symbols: ●, normal muscle; ○, day-21-denervated muscle.

of 75 μM - Ca^{2+} the denervated mitochondria were unable to retain the Ca^{2+} and spontaneously released it.

Fig. 3 represents the concentration dependence of Ca^{2+} on the initial rate of Ca^{2+} uptake for (a) Ultraturrax- and (b) Nagarse-released mitochondria in the control and denervated states. Fig. 3(a) shows that the denervated-muscle Ultraturrax-released-mitochondrial Ca^{2+} -uptake system was saturated at a lower initial Ca^{2+} concentration and displayed a significantly lower rate for Ca^{2+} uptake than the controls. Fig. 3(b) shows that up to an initial 50 μM - Ca^{2+} concentration the denervated-muscle Nagarse-released mitochondria displayed similar Ca^{2+} -uptake properties to the controls. However, thereafter the denervated-muscle uptake rate decreased markedly, indicating Ca^{2+} release from these fractions. Fig. 3 also shows that the Ultraturrax-released mitochondrial populations displayed much lower rates for Ca^{2+} uptake than did the Nagarse-released fractions. Our results support the findings for heart muscle mitochondria (Wolkowicz & McMillin-Wood, 1980). Fig. 3 further shows that the normal Nagarse-released mitochondria displayed a sigmoidal rate curve for Ca^{2+} uptake as opposed to the hyperbolic curve displayed by the Ultraturrax-

released mitochondria. Furthermore, this sigmoidal effect was not as obvious for the denervated-muscle Nagarse-released population. This suggests that co-operative sites are involved in Ca^{2+} binding and uptake into Nagarse-released mitochondria, whereas Ca^{2+} uptake and binding occurs via independent sites in Ultraturrax-released mitochondria.

Effect of Nagarse on Ultraturrax mitochondria

It could be argued that Nagarse itself might be activating the intermyofibrillar mitochondrial population. As shown in Fig. 4(a) normal Ultraturrax-released mitochondria that were resuspended in medium containing 0.25 mg of Nagarse/mg of mitochondrial protein for 25 s and then re-centrifuged to remove the Nagarse were slightly damaged by this treatment in that their ADP/O ratio and State-3 respiratory rate was decreased compared with the controls. The respective Nagarse-treated and control ADP/O ratios were 2.6 and 3.0 and the respective State-3 respiratory rates were 81.4 and 142.7 ng-atoms of O/min per mg of mitochondrial protein. As shown in Fig. 4(b), Nagarse added directly to the normal Ultraturrax-released mitochondria respiring in the oxygen electrode had no stimulating effect on the State-3 respiratory rate. In

Fig. 3. Respiration-supported Ca^{2+} transport by Ultraturrax- and Nagarse-released mitochondria from normal and day-21-denervated rat soleus muscle

Mitochondria (0.5 mg) were incubated in 250 mM-sucrose/10 mM-Tris/HCl (pH 7.4)/8.5 mM- K_2HPO_4 /5 mM-glutamate (pH 7.4)/5 mM-malate (pH 7.4) in a final volume of 1.0 ml at 25°C . The reaction was initiated by the addition of Ca^{2+} (containing $0.5 \mu\text{Ci}$ of $^{45}\text{Ca}^{2+}$) at the various concentrations employed. At specific time intervals within a 2 min period, $100 \mu\text{l}$ of the reaction mixture was removed and rapidly added to the Ruthenium Red ($10 \mu\text{M}$) and EGTA (20 mM) quench medium and the extent of transport was determined (see Reed & Bygrave, 1974). The initial rates of Ca^{2+} transport at the various Ca^{2+} concentrations indicated were then plotted for (a) Ultraturrax- and (b) Nagarse-released mitochondrial fractions in control (●) and denervated (○) states. For all conditions the number of experiments performed was two to six (S.E.M., calculated where $n > 2$, is indicated by the bars).

fact, as shown, it caused a slight but progressive reduction of the State-3 respiratory rate.

Discussion

We have shown that the denervated Ultraturrax- and Nagarse-released mitochondrial populations display markedly decreased capacities and rates for Ca^{2+} uptake compared with their corresponding controls. Furthermore, both mitochondrial populations appear to have been affected to the same degree by the denervation process. These results were correlated with the denervated populations displaying decreased State-3 respiratory rates using glutamate as the substrate (M. Joffe, M. Badenhorst, N. Savage & H. Isaacs, unpublished work). This suggests that the mitochondrial respiratory chains were subject to partial inactivation or damage possibly through swelling effects in both denervated populations. Our electron-microscopy studies of the

muscle and mitochondria *in situ* (M. Joffe, M. Badenhorst, N. Savage & H. Isaacs, unpublished work) support the latter suggestion. It is also possible that the decreased Ca^{2+} uptake is associated with elevated Ca^{2+} concentrations observed in denervated-muscle homogenate and mitochondrial fractions (Joffe *et al.*, 1981b). It is noteworthy that in both the normal and denervated states, the Nagarse-released populations display a greater rate and capacity for Ca^{2+} accumulation than do the Ultraturrax-released mitochondria. The reasons for this are as yet unclear. As suggested by McMillin-Wood *et al.* (1980) the Nagarse-released mitochondria may possess a higher negative potential across the inner membrane compared with the Ultraturrax-released population. Alternatively, the Nagarse-released mitochondrial respiratory chain may contain more cytochrome *c* and cytochrome *aa₃* (Matlib *et al.*, 1977; McMillin-Wood *et al.*, 1980). It could of course be argued that Nagarse itself might be

Fig. 4. Effect of Nagarse on the oxidative-phosphorylation profiles of normal Ultraturrax-released mitochondria. Mitochondria (± 1 mg) were suspended in 250 mM-sucrose/10 mM-Tris/HCl (pH 7.4)/8.5 mM- K_2HPO_4 /5 mM-glutamate/5 mM-malate (pH 7.4)/1.5 mM-EDTA in a final volume of 2.4 ml. Respiratory rates were stimulated with 280 nmol of ADP. For (a) the mitochondria used were previously suspended in Nagarse at 0.25 mg/mg of mitochondrial protein for 25 s and then resuspended in Nagarse-free medium. In (b) successive additions of Nagarse were made to the untreated mitochondria respiring in the oxygen electrode. * indicates ADP-induced State-3 respiratory rate (ng-atoms of O/min per mg). Abbreviation used: NUT mitochondria, normal Ultraturrax-released mitochondria.

activating the intermyofibrillar mitochondrial population. However, in agreement with Palmer *et al.* (1977) and McMillin-Wood *et al.* (1980) for heart mitochondria, treatment of our Ultraturrax-released-mitochondrial fractions with Nagarse did not increase their respiratory activities. In fact, as would be expected, excessive Nagarse treatment caused functional damage to the Ultraturrax-released mitochondria.

At this stage, the significance of the impaired Ca^{2+} -uptake capacities of the denervated mitochondria with respect to the process of cell necrosis that occurs is unclear. Wrogemann & Pena (1976) suggest that a muscle energy deficit develops because of mitochondrial Ca^{2+} overload. The subsequent mitochondrial damage results in Ca^{2+} release. This would serve to perpetuate the necrotic process. Alternatively, it could be argued that, because of their depressed respiratory-chain-linked functioning, the denervated-muscle mitochondria are unable to buffer the elevated sarcoplasmic Ca^{2+} concentrations that are thought to arise by way of a leaky sarcolemma (Leonard & Salpeter, 1979). The increased cytosolic Ca^{2+} concentrations that ensue

could activate Ca^{2+} -dependent neutral proteinases (Kar & Pearson, 1976; Neerunjun & Dubowitz, 1979) and neutral lysosomal proteinases (Gerard & Schneider, 1979), which are able to digest myofibrillar material.

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