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Increased muscle calcium

A possible cause of mitochondrial dysfunction and cellular necrosis in denervated rat skeletal muscle

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Mitochondrial preparations derived from denervated rat skeletal muscle and paired controls were characterized with respect to their ability to take up externally added Ca^{2+}. The denervated and control muscle homogenates and mitochondrial $[Ca^{2+}]$ were also determined. Our data indicate that the denervated mitochondria are able to take up less Ca^{2+} than the controls before uncoupling occurs. This defect is associated with elevated $[Ca^{2+}]$ in homogenate and mitochondrial fractions in the denervated state. The causal relationship between Ca^{2+} overload, mitochondrial functional damage and cell necrosis is discussed.

The structural and functional abnormalities of skeletal muscle associated with diseases of the lower motor neuron have been widely reported and reviewed in the literature (Dubowitz & Brook, 1973; Adams, 1975; Cullen & Pluskal, 1977). The functional mitochondrial defects are often thought to arise from a lack of energy reserves within the affected muscles (Schotland *et al.*, 1976; Malvey *et al.*, 1977; Pichey & Blaise-Smith, 1979). This defect is associated with increased mitochondrial $[Ca^{2+}]$ in dystrophic tissue (Wrogemann *et al.*, 1970; Mezon *et al.*, 1974; Wrogemann & Pena, 1976; Duncan, 1978; Jasmin *et al.*, 1979). It has been postulated (Wrogemann & Pena, 1976) that this excess Ca^{2+} arises from the extracellular space via a damaged sarcolemma. This idea is in accordance with the generalized plasma defect found to occur in dystrophic tissue (Ringel *et al.*, 1976; Bradley, 1980; Rowland, 1980). Alternatively, it has been suggested (Publicover *et al.*, 1978) that the increased mitochondrial $[Ca^{2+}]$ arises from Ca^{2+} released from the sarcoplasmic reticulum. Either way, the mitochondria are believed to take up this excess Ca^{2+} to maintain cytosolic Ca^{2+} homoeostasis (Bragadin *et al.*, 1979; Carafoli, 1979; Fiskum & Lehninger, 1980; Nicholls & Scott, 1980; Panfili *et al.*, 1980). This regulatory process would presumably be possible because the mitochondria have separate influx and efflux pathways for Ca^{2+}, which can be independently controlled by the ambient cytosolic conditions (Lotscher *et al.*, 1979; Pfeiffer *et al.*, 1979; Carafoli, 1979; Crompton, 1980; Denton *et al.*, 1980; Panfili *et al.*, 1980; Fiskum & Lehninger, 1980). We have observed (M. Joffe, N. Savage & H. Isaacs, unpublished work) that denervated-skeletal-muscle mitochondria display many of the respiratory chain defects found to occur in dystrophic muscle tissue (Lee *et al.*, 1978). In both situations the mitochondria are not able to oxidize NAD-linked substrates efficiently, a defect associated with a decreased NADH oxidase activity. This defect is caused by increased mitochondrial $[Ca^{2+}]$ in dystrophic tissue, but has not been investigated in denervation atrophy. We therefore undertook an investigation of the Ca^{2+}-uptake properties and concentrations in denervated-skeletal-muscle mitochondria to discover a possible cause for their impaired functioning. Our findings support the proposal of Wrogemann & Pena (1976) that cytosolic Ca^{2+} overload provides a general mechanism for cell necrosis in muscle disease. However, whether mitochondrial Ca^{2+} overload is directly involved in the necrotic process is questionable.

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Materials and methods

Female rats weighing 200–250g were used. Each assay was performed with muscle pooled from two rats. Denervation atrophy was produced in the left hind leg of rats by transection of the sciatic nerve in the hip region behind the protruding spine of the iliac crest. This ensured that the sciatic nerve was transected at a point proximal to the origin of the popliteal and tibial nerves. To prevent reinnervation, a 1 cm length of nerve was always removed about the point of transection. In each case, the right leg was used as the control at the time of study of the denervated limb.

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Preparation of the mitochondria

Skeletal-muscle mitochondria were isolated from the combined gastrocnemius, tibialis anterior peroneus, semimembranosis, semitendinosis, adductor and soleus muscles of the denervated and control legs by a modification of the method of Sordahl *et al.* (1971). The homogenization medium used consisted of 100mM-KCl, 0.5% bovine serum albumin and 25mM-Tris/HCl, pH 7.4. The minced tissue was homogenized on ice four times for a period of 5s each with intermittent cooling using an UltraTurrax PT-10 homogenizer. The homogenate was centrifuged at 1000*g* for 15min and the resulting supernatant was centrifuged at 17500*g* for 15min. The mitochondrial pellet was resuspended and washed in homogenizing medium and finally suspended in this medium at a concentration of 10–14mg/ml.

Assays

Ca^{2+} uptake was determined at 30°C by the indirect polarographic technique as described by Chance (1965), using the Clark oxygen electrode. The reaction medium consisted of 250mM-sucrose, 10mM-Tris/HCl, pH 7.4, 8.5mM-K_2HPO_4, 5mM-glutamate + malate and ±4mg of mitochondrial protein in a final volume of 2.4ml. The mitochondrial respiratory rate was repeatedly stimulated with additions of 100 or 200nmol of Ca^{2+} until mitochondrial uncoupling occurred. The rates of Ca^{2+}-stimulated respiration the Ca^{2+}/O ratio and the mitochondrial Ca^{2+} capacity, which reflects the total amount of Ca^{2+} accumulated/mg of mitochondrial protein before uncoupling occurred, were determined, assuming that the Ca^{2+} added was taken up.

The $[Ca^{2+}]$ in muscle homogenate and mitochondrial fractions were determined by the atomic-absorption spectroscopy method of Pleasure *et al.* (1979), after overnight digestion at 37°C in 8M-HNO_3 and 1% (w/v) $LaCl_3$.

Protein content was determined by the Bio-Rad protein assay method, which is based on the method of Bradford (1976).

Results

Fig. 1 illustrates typical polarographic tracings obtained from Ca^{2+}-stimulated mitochondrial respiratory rates of skeletal muscle 3 and 28 days after denervation and of the contralateral control muscles. As can be seen, for both control and

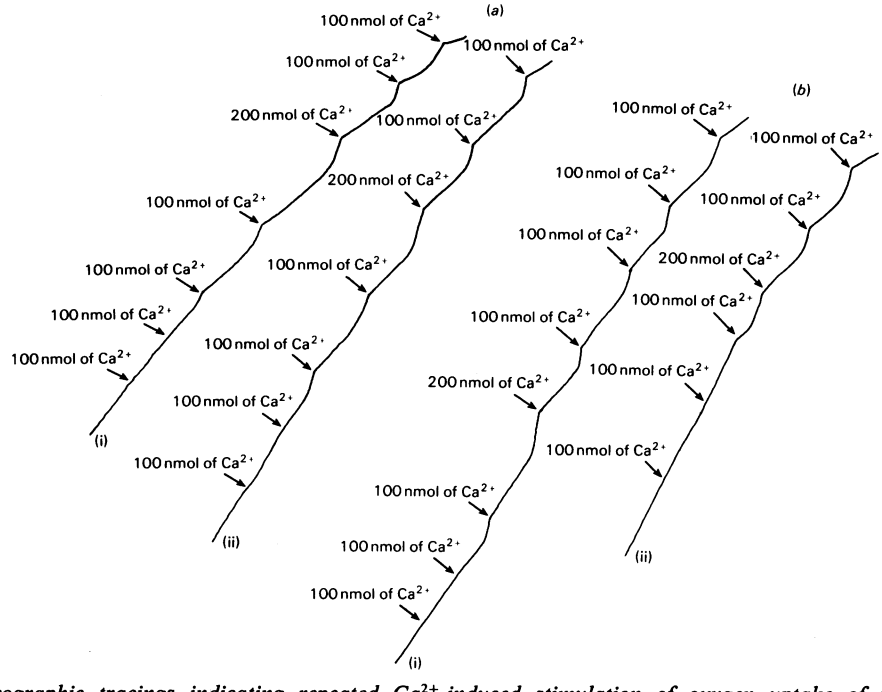

Fig. 1. *Polarographic tracings indicating repeated Ca^{2+}-induced stimulation of oxygen uptake of skeletal-muscle mitochondria from muscle (a) 3 days and (b) 28 days after denervation and from the contralateral controls*

Mitochondria (3–4mg) were suspended in 250mM-sucrose/10mM-Tris/HCl (pH 7.4)/8.5mM-K_2HPO_4/5mM-glutamate/5mM-malate (pH 7.4) in a final volume of 2.4ml. The respiratory rate was stimulated with 100 or 200nmol of $CaCl_2$ in (i) control and (ii) denervated mitochondria as indicated.

Table 1. *Polarographic Ca^{2+}-stimulated respiratory parameters of denervated and control rat skeletal-muscle mitochondria*

The mitochondria (3–4 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) in a final volume of 2.4 ml. Mitochondrial respiratory rate was stimulated with 100 or 200 nmol of $CaCl_2$. Values are expressed as means ± S.E.M. The numbers of experiments are given in parentheses.

	Ca^{2+}/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)
Control	5.25 ± 0.09 (14)	49.70 ± 3.45 (11)	277.24 ± 14.79 (8)
Day-3 denervated	5.03 ± 0.19 (6)	47.60 ± 6.88 (5)	266.53 ± 17.50 (4)
Day-28 denervated	5.35 ± 0.11 (5)	[b]38.55 ± 8.03 (6)	[a]112.38 ± 22.11 (5)

[a] Statistically different from controls ($0.01 > P > 0.001$ for unpaired analysis and $0.02 > P > 0.01$ for paired analysis by Student's *t* test).

[b] Statistically insignificant ($0.01 > P > 0.05$ for both unpaired and paired analyses by Student's *t* test).

Table 2. *Ca^{2+} content of normal and denervated skeletal-muscle homogenate and mitochondrial fractions*

The fractions were digested in a final concentration of 8 M-HNO_3 containing 1% $LaCl_3$ overnight at 37°C in a total volume of 2 ml. The digests were centrifuged and the Ca^{2+} concentrations were determined on the supernatants by atomic-absorption spectroscopy. Values are expressed as means ± S.E.M. The numbers of experiments are given in parentheses.

	Homogenate Ca^{2+} (μmol/g wet wt. of muscle)	Mitochondrial Ca^{2+} (nmol/mg of mitochondrial protein)
Control	1.26 ± 0.15 (12)	26.15 ± 1.16 (14)
Day-3 denervated	1.19 ± 0.04 (4)	[a]34.95 ± 1.04 (8)
Day-28 denervated	[b]3.08 ± 0.39 (10)	[b]71.30 ± 9.66 (5)

[a] Statistically insignificant ($0.1 > P > 0.05$) for an unpaired analysis by the Student's *t* test.

[b] Statistically different from controls ($P < 0.001$) for an unpaired analysis by the Student's *t* test.

denervated states, repeated cycles of respiratory-rate stimulation were induced by Ca^{2+} in agreement with the findings of Crompton & Carafoli (1979), until eventually uncoupling occurred. However, the day-28 denervated mitochondrial fractions uncouple much sooner than do the controls. This indicates that they are able to accumulate much less Ca^{2+} than their contralateral controls before uncoupling occurred. The day-3 denervated fractions respond normally to Ca^{2+} uptake. Table 1 summarizes the various respiratory parameters obtained that were associated with the present study. It can be seen that after 3 days there was no significant difference between the denervated and control mitochondria with respect to the Ca^{2+}/O ratio, Ca^{2+}-stimulated respiratory rates and Ca^{2+}-uptake capacities. However, by day 28 there is a significant decrease in the denervated mitochondrial uptake capacities, i.e. 102.38 ± 22.11 nmol of Ca^{2+}/mg of mitochondrial protein as against 177.24 ± 14.79 nmol of Ca^{2+}/mg of mitochondrial protein for the controls. The day-28 denervated Ca^{2+}/O ratios and Ca^{2+}-stimulated respiratory rates did not differ significantly from the control values.

The mitochondrial and muscle Ca^{2+} concentrations determined in day-3 and day-28 denervated and control samples are presented in Table 2. Although the day-3 denervated mitochondrial Ca^{2+} concentrations are higher than the control values (34.55 ± 1.94 nmol/mg of mitochondrial protein as against 26.15 ± 1.16 for the controls), there is no significant difference between day-3 denervated and control homogenate and mitochondrial Ca^{2+} concentrations. By day 28, the denervated homogenate and mitochondrial fractions have significantly elevated Ca^{2+} concentrations, i.e. 3.10 ± 0.31 μmol/g of muscle as against 1.25 ± 0.15 μmol/g of muscle for the control homogenates and 71.30 ± 9.66 nmol/mg of mitochondrial protein as against 26.15 ± 1.16 nmol/mg of mitochondrial protein for the control mitochondrial fractions.

Discussion

Our results suggest that decreased mitochondrial Ca^{2+} accumulation is associated with elevated homogenate and mitochondrial Ca^{2+} concentration found to occur in the day-28 rats.

The decreased respiratory activity that we observed in the denervated mitochondria (M. Joffe, N. Savage & H. Isaacs, unpublished work) is possibly due to inner-membrane damage caused by a Ca^{2+}-induced swelling (Publicover *et al.*, 1978) or transition (Hunter & Haworth, 1979; Haworth & Hunter, 1980). Increased Ca^{2+} concentrations are known to cause NAD^+ leakage out of the mito-

chondria (Wrogemann *et al.*, 1970; Schotland *et al.*, 1976). A decreased mitochondrial NADH concentration would provide another explanation for the marked inhibition of the NADH oxidase activity and State-3 respiratory rates observed in the denervated state. In the last few years, several proteolytic enzymes have been discovered in rat liver mitochondria (Gear *et al.*, 1974; Duque-Magalhaes, 1979; Haas & Heinrich, 1978*a*,*b*, 1979). It has been suggested that the mitochondrial neutral proteolytic enzymes are implicated in autoproteolytic activity. These enzymes, if stimulated by the elevated intramitochondrial Ca^{2+} concentrations, would cause increased autoproteolysis providing a third alternative for the mitochondrial functional abnormalities observed.

Alternatively, although the day-28 denervated mitochondria displayed significantly increased $[Ca^{2+}]$, this increase was much lower than that observed for dystrophic tissue (Wrogemann *et al.*, 1970), where 200nmol of Ca^{2+}/mg of mitochondrial protein was measured as against our 71.30 ± 0.66nmol of Ca^{2+}/mg of mitochondrial protein for the denervated tissue. However, the mitochondria were isolated from the pooled back muscles of the hind leg. We are therefore dealing with a heterogeneous population of red and white muscles, some of which are more sensitive to the denervation effects than others. Also Mezon *et al.* (1974) have shown that in the dystrophic state, different populations of mitochondria are obtained containing various Ca^{2+} concentrations. It is therefore possible that our denervated mitochondrial fraction represents a mixed population of mitochondria with relatively fewer abnormal mitochondria than would be found in the dystrophic state.

The causal relationship between elevated muscle and mitochondrial Ca^{2+} concentrations and cell necrosis observed to occur in the denervated skeletal muscle is unclear. Wrogemann & Pena (1976) suggest that a muscle energy deficiency due to mitochondrial Ca^{2+} overload resulting in its subsequent damage and Ca^{2+} release is directly involved in this process. However, other studies suggest that cytosolic Ca^{2+} activated neutral proteinases (Kar & Pearson, 1976; Neerunjun & Dubowitz, 1979) and neutral lysosomal proteinases (Gerard & Schneider, 1979) are responsible for the myofibrillar digestion that occurs in necrotic areas. However, at this stage the problem is not resolved and our results do not clarify the issue.

The source of the increased denervated muscle $[Ca^{2+}]$ is unknown. It has been shown by Leonard & Salpeter (1979) that inactivation of cholinesterases at mammalian neuromuscular junctions produces extensive muscle necrosis. These workers propose that the esterase inactivation leads to prolongation of the acetylcholine lifetime and thus of Ca^{2+} influx. It has been shown by Ringel *et al.* (1976) that diffuse extra-junctional acetylcholine receptors develop in denervated muscle fibres. It is possible that these extra receptors may be involved in increased Ca^{2+} influx by way of the sarcolemma. Alternatively, we have observed abnormal functioning of the sarcoplasmic reticulum of denervated skeletal muscle (G. Palexas, N. Savage & H. Isaacs, unpublished work). The increased cytosolic Ca^{2+} concentrations observed could result from sarcoplasmic-reticulum Ca^{2+} release due possibly to an energy deficit or absence of specific neurotrophic factors in the denervated state (Cangiano & Lutzemburger, 1980).

It could also be argued that the mitochondria only gain Ca^{2+} on isolation and that the denervated-muscle mitochondria are more susceptible than the controls to this effect. However, in a subsequent study we included 10mM-EDTA in the initial homogenization medium to minimize mitochondrial Ca^{2+} uptake or release during the isolation procedure (M. Joffe, N. Savage & H. Issacs, unpublished work). Under these conditions the denervated mitochondria displayed the same abnormalities observed in the present study. This suggests that the increased denervated mitochondria Ca^{2+} concentration was not artifactually acquired in the isolation procedure.

Our results support the proposal of Wrogemann & Pena (1976) that elevated Ca^{2+} concentrations are associated with cellular necrosis in muscle diseases of widely differing aetiology. However, mitochondrial Ca^{2+} overload itself need not directly mediate this process.

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