Calcium Transport and Phospholipid Composition of Microsomal Fractions from Denervated Rat Gastrocnemius

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Total calcium uptake, but not the initial rate, and non-calcium-stimulated (basic) adenosine triphosphatase activity of rat gastrocnemius microsomal fractions 3, 7 and 14 days after denervation increased compared with contralateral controls. Microsomal sphingomyelin also increased but phosphatidylcholine plus choline plasmalogen was unchanged in amount. These results are contrasted with those reported for vincristine myopathy.

Yasin *et al.* (1973) described alterations in the phospholipid composition and function of rat skeletal-muscle microsomal fractions 5 days after a single intraperitoneal injection of vincristine (0.6 mg/kg body wt.) which are analogous to those reported for certain hereditary myopathies. The microsomal changes observed included a decrease in calcium-accumulating ability and a lowering of the phosphatidylcholine plus choline plasmalogen content. Several workers have reported nerve lesions in both types of disorder (Bradley, 1970; Gottschalk *et al.*, 1968; Harris & Wilson, 1971), so that the biochemical changes could be due to nerve damage, since the influence of the motor nerve on muscle biochemistry is well documented (Guth, 1968).

Our aim was to examine the phospholipid composition, calcium transport and ATPase (adenosine triphosphatase) activity of microsomal fractions from rat muscle deprived of motor innervation so as to compare changes in these parameters with those produced by vincristine.

Although the effect of denervation on the calcium-accumulating ability of rat muscle microsomal fractions has been previously examined the findings are not clear cut. Sreter (1970) reported an increase in calcium-accumulating capacity of microsomal fractions from 'fast' muscles (gastrocnemius) and no change in 'slow' muscle (soleus) after short-term denervation. Margreth *et al.* (1972), however, described little change in the calcium-accumulating capacity for 'fast' muscle (extensor digitorum longus) and an enhancement in 'slow' soleus muscle microsomal fractions.

In the present study we have examined the initial rate and total calcium accumulation in the presence of oxalate, the ATPase activity and the phospholipid composition of microsomal fractions from the denervated rat gastrocnemius.

Materials and methods

Male Wistar rats [type CFHB, Carworth (Europe)

Ltd.] weighing initially 300–330 g were used in this study. The animals were fed on Oxoid 5941B pellets and water *ad libitum*. Denervation of the right gastrocnemius was performed under ether anaesthesia by removing 1 cm of the sciatic nerve high in the thigh. Four to nine rats were killed by decapitation 3, 7 and 14 days after denervation. Both sham-operated and unoperated rats were used as controls.

Gastrocnemii from the left contralateral leg, the right denervated and sham-operated legs were removed and cleaned of fat, connective tissue, blood vessels and nerves. The muscles from corresponding legs in each animal were pooled and used for the preparation of the microsomal fractions as described previously (Yasin *et al.*, 1973). The microsomal fractions containing vesicular elements of the sarcoplasmic reticulum were washed with 10 vol. of suspension medium (0.3 M-sucrose, 1 mM-Tris, 5 mM-histidine hydrochloride, 5 mM-potassium oxalate, pH 7.4) by hand homogenization with an all-glass homogenizer and resedimented at 105 000 g for 60 min. After resuspension in the above medium these fractions were stored on ice and used within 24 h for calcium-uptake and ATP measurements, a portion also being freeze-dried for phospholipid extraction.

Mitochondrial contamination of these fractions varied between 8 and 15% as determined by succinate-2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-tetrazolium chloride oxidoreductase activity (Pennington, 1961). Protein was measured by the method of Lowry *et al.* (1951) with human serum albumin (Dade) as a standard and the phospholipid composition of the microsomal fractions was determined by the method of Owens & Hughes (1970).

Calcium transport and ATPase activities were determined as described previously (Yasin *et al.*, 1973) except for the following modifications. Initial and total calcium uptake were measured at 1 and 20 min and the calcium-concentrating ability at 5 min. This last assay measures the ability of the microsomal fractions to lower the calcium concentration of the

medium to a low value, when they are not fully saturated, and reflects the balance between calcium influx and efflux (Peter & Worsfold, 1969) where calcium concentration is rate-limiting. Filtrate (1 ml) was collected into cooled test tubes containing 50 μ l of 15 mM-EGTA [ethanedioxybis(ethylamine)tetra-acetate], which served as a calcium trap. Basic ATPase activity assays were conducted in the presence of 0.5 mM-EGTA to remove contaminating calcium. The above assays were performed at 20°C.

Results and discussion

Similar wet weights were obtained for unoperated, sham-operated and the contralateral gastrocnemii, with the values in the range 0.44–0.46 g/100 g body wt. The denervated muscle showed progressive loss in weight, of 8, 18 and 50% at 3, 7 and 14 days after denervation respectively. No differences were observed in the yield of microsomal protein for the control, contralateral and denervated muscles; this is contrary to the findings of Sreter (1970) but consistent with the results reported by Margreth *et al.* (1972), for extensor digitorum longus. The yields ranged between 0.39 and 0.48 mg/g of muscle.

Table 1 lists the results for calcium transport and ATPase activity of denervated and contralateral microsomal preparations. The mean values obtained for six preparations from unoperated and five preparations from sham-operated animals did not vary

significantly from that of the combined contralateral samples and are therefore omitted.

Microsomal fractions from denervated gastrocnemii exhibited a significant increase in total calcium uptake at 7 and 14 days after denervation, but the initial rate showed no change (Table 1). By 14 days the total calcium-accumulating ability was almost twice that of the contralateral samples.

With regard to calcium-concentrating ability only the 14-days-denervated samples showed a significantly increased ability to lower the calcium concentration in the medium to values below that observed for the contralateral preparations.

No change was observed in extra (calcium-stimulated) ATPase or in the transport ratio (Table 1). However, the basic ATPase, which is unrelated to the calcium-transport mechanism (Makinose & The, 1965), showed a progressive increase in activity during denervation (Table 1), similar to that reported by Sreter (1970) but not as stated by Margreth *et al.* (1972).

In comparing microsomal phospholipids (Table 2), since no trend with time after denervation was observed, results for muscle microsomal fractions from gastrocnemii contralateral to the denervated muscle have been pooled. Comparison of the phospholipid composition of microsomal fractions from denervated and contralateral gastrocnemii reveals certain differences. The percentage of sphingomyelin is increased at each time-interval after denervation, but

Table 1. *Effect of denervation on microsomal calcium transport and ATPase activity*

For details see the text. Transport ratio means μ mol of calcium accumulated/ μ mol of ATP hydrolysed. Values represent means $\pm$ s.d. for the numbers of samples in parentheses. Differences between denervated and contralateral controls significant by Student's *t* test or, where appropriate, by Cochran's (Snedecor & Cochran, 1967) approximation are indicated by: * 0.05 > *P* > 0.02; † 0.01 > *P* > 0.001; ‡ *P* < 0.001.

	Contralateral	Time after denervation (days)		
		3	7	14
Calcium accumulation (μ mol/mg of protein)				
1 min	0.89 $\pm$ 0.24 (14)	0.77 $\pm$ 0.20 (5)	1.03 $\pm$ 0.21 (5)	1.21 $\pm$ 0.46 (5)
20 min	1.93 $\pm$ 0.44 (14)	1.78 $\pm$ 0.60 (5)	2.72 $\pm$ 0.49† (5)	3.85 $\pm$ 0.85‡ (5)
Calcium concentration (μ M remaining in filtrate)				
5 min	0.20 $\pm$ 0.08 (14)	0.20 $\pm$ 0.08 (5)	0.12 $\pm$ 0.03 (5)	0.10 $\pm$ 0.03* (5)
Transport ratio	1.13 $\pm$ 0.37 (14)	1.03 $\pm$ 0.09 (5)	1.23 $\pm$ 0.16 (5)	1.15 $\pm$ 0.15 (5)
ATPase activity (μ mol of ATP hydrolysed/min per mg of protein)				
Basic	1.07 $\pm$ 0.23 (13)	1.54 $\pm$ 0.39† (5)	1.95 $\pm$ 0.53* (4)	2.38 $\pm$ 0.38‡ (5)
Total	1.87 $\pm$ 0.33 (13)	2.29 $\pm$ 0.42* (5)	2.74 $\pm$ 0.57† (4)	3.44 $\pm$ 0.49‡ (5)
Extra	0.80 $\pm$ 0.19 (13)	0.74 $\pm$ 0.15 (5)	0.79 $\pm$ 0.13 (4)	1.06 $\pm$ 0.37 (5)

Table 2. *Phospholipid composition of microsomal fractions from denervated muscle*

Phospholipid P is expressed as the percentage of the total lipid P. Values represent means $\pm$ S.D. for the numbers of samples in parentheses. Differences between denervated and contralateral controls significant by Student's *t* test or, where appropriate, by Cochran's (Snedecor & Cochran, 1967) approximation are indicated by: * $0.05 > P > 0.02$; † $0.01 > P > 0.001$; ‡ $P < 0.001$.

	Controls		Denervated		
	Unoperated (10)	Contralateral (7)	3 days (4)	7 days (4)	14 days (4)
Sphingomyelin	2.1 $\pm$ 0.7	3.1 $\pm$ 0.5	4.1 $\pm$ 0.9*	5.4 $\pm$ 0.7‡	4.7 $\pm$ 0.5‡
Phosphatidylcholine + choline plasmalogen	62.8 $\pm$ 1.5	63.1 $\pm$ 1.4	61.9 $\pm$ 1.4	61.3 $\pm$ 1.5	62.7 $\pm$ 1.9
Phosphatidylserine	3.1 $\pm$ 0.8	2.7 $\pm$ 0.5	3.4 $\pm$ 0.8	4.6 $\pm$ 0.9†	2.9 $\pm$ 0.2
Monophosphoinositide	6.4 $\pm$ 0.7	6.9 $\pm$ 0.7	7.1 $\pm$ 0.4	7.1 $\pm$ 0.2	8.2 $\pm$ 1.0*
Total ethanolamine phospholipid	22.1 $\pm$ 1.0	21.0 $\pm$ 0.5	20.4 $\pm$ 0.7	20.6 $\pm$ 0.9	20.5 $\pm$ 0.3
Cardiolipin	2.0 $\pm$ 0.9	2.4 $\pm$ 0.3	2.2 $\pm$ 0.0	2.55 $\pm$ 0.8	1.6 $\pm$ 0.4†
Ethanolamine plasmalogen	9.9 $\pm$ 1.4	9.8 $\pm$ 0.5	9.9 $\pm$ 0.7	11.5 $\pm$ 1.5*	12.8 $\pm$ 1.7*
Choline plasmalogen	1.8 $\pm$ 0.6	2.1 $\pm$ 1.0	2.3 $\pm$ 0.6	3.3 $\pm$ 0.6	2.7 $\pm$ 1.4

there is no significant change in the total of phosphatidylcholine plus choline plasmalogen up to 14 days. At 7 days after denervation only, some increase in phosphatidylserine was found, and likewise at 14 days there was an increase in monophosphoinositide. Although the total ethanolamine phospholipid did not change there was an increase in ethanolamine plasmalogen.

Microsomal sphingomyelin content seems to be particularly susceptible to disturbance or alteration of muscle function, since there was a significant increase ($0.01 > P > 0.001$) in the percentage of sphingomyelin in the contralateral as opposed to the unoperated controls (Table 2).

The increased microsomal calcium-accumulating capacity from denervated rat gastrocnemius observed in this study is consistent with previous findings reported by other workers (Brody, 1966; Howell *et al.*, 1966; Sreter, 1970).

The results obtained for microsomal calcium uptake and phospholipid composition during denervation differ from those we reported for vincristine myopathy and which others have described for some naturally occurring myopathies, where a decrease in phosphatidylcholine plus choline plasmalogen, an increase in ethanolamine phospholipid and a fall in both the initial and total calcium uptake were observed. The only similarities between total denervation and these myopathies were a rise in the basic (calcium-independent) microsomal ATPase activity and in the sphingomyelin percentage. Conceivably the differences between vincristine myopathy and denervation for the calcium-uptake activity and phospholipid

composition could result from the different muscles used. In this denervation study only a single 'fast' muscle, the gastrocnemius, was examined, whereas in the vincristine study there was a mixture of 'slow' and 'fast' muscle. However, if denervation plays a primary role in vincristine myopathy, then, on the basis of data for microsomal calcium uptake in 'slow' denervated muscle reported by Sreter (1970), who found no change, or by Margreth *et al.* (1972), who found an increase, the presence of proportionately more 'slow' muscle would tend to leave the calcium accumulation unaltered or enhanced, rather than decreased.

Our results indicate that the short-term effects of total denervation, as achieved by sciatic-nerve section, differ from those that follow vincristine administration. Consequently, in the rat vincristine most probably affects muscle directly. Nevertheless, because of the complex interactions between nerve and muscle, a more subtle disturbance of neural influence could still be a factor both in naturally occurring myopathies and in those caused by vincristine.

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