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Morphological observations and rates of protein synthesis in rat muscles incubated *in vitro*

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Isolated soleus and extensor digitorum longus muscles from small (40 or 70 g) rats developed a central and substantial (13–57%) loss of glycogen and α-glucan phosphorylase activity after incubation for up to 2 h *in vitro*. The central 'core' of the muscles showed a marked decrease in the rate of protein synthesis. It is suggested that during brief periods of incubation the central core of isolated rat muscles becomes hypoxic, and that consequently the viability of such muscles must be in question.

INTRODUCTION

Isolated skeletal-muscle preparations have been widely used to study a variety of physiological processes, in particular protein turnover (Li & Goldberg, 1976; Libby & Goldberg, 1980; Seider *et al.*, 1980; Shangraw & Turinsky, 1982; Tischler & Fagan, 1983; Goldspink *et al.*, 1983; Baracos *et al.*, 1984; Wagenmakers & Veerkamp, 1984; Segal & Faulkner, 1985). In order to obtain results that are meaningful as a model for the situation *in vivo*, it is essential that the physiological viability and integrity of the muscle is retained. However, although the need for such a viable preparation may be well recognized, the criteria generally used are based on biochemical measurements of metabolites in deproteinized homogenates. Possible regional variations within the muscle, such as might occur if oxygen diffusion to the central area were limited, have not been considered.

In the present study a variety of histochemical techniques have been used to assess the viability of incubated muscles at the level of individual fibres or groups of fibres. These assessments have been compared with measurements of protein synthesis made on fibre groups taken from the periphery or core of individual muscles.

EXPERIMENTAL

Methods

Soleus and extensor digitorum longus muscles from fed Rowett Hooded Lister rats (40–70 g body wt.) were examined either *in vivo* or *in vitro*. The muscles were incubated in 1 ml of a modified Trowell's complete medium without insulin (Reeds *et al.*, 1980) which was saturated with O_2/CO_2 (19:1). The muscles were maintained at fixed resting length with 2 g weights. The maximum diameter of muscles did not exceed 2 mm when measured from cryostat sections. This diameter represents the relaxed state; the stretched state would give a smaller diameter (approx. 1.5 mm). Furthermore, this was the diameter of the major axis: the minor axis was much less (Fig. 1). During the last 30 min of incubation, 0.1 ml (10 μCi) of 150 mM-L-[2,6-^{3}H]phenylalanine was added to the medium. At the end of the incubation period the muscles were removed from the incubation assembly, rinsed in cold medium and blotted before being used for the analyses. Rates of muscle protein synthesis *in vivo* were measured from the incorporation of label into protein 10 min after intravenous injection of a flooding dose of [^{3}H]phenylalanine (150 μmol of L-[2,6-^{3}H]phenylalanine/100 g body wt.; 1 μCi/μmol) as described by Garlick *et al.* (1980).

Histochemistry

Samples were taken from the mid-belly region of both the incubated and unincubated muscles to be used solely for histochemical analysis. The muscle pieces were orientated for transverse sectioning, supported between pieces of chilled liver, covered with talc and frozen in liquid nitrogen. Serial transverse sections (10 μm) were cut in a cryostat at −20 °C, and made to react with stains for one of the following: Ca^{2+}-activated myofibrillar ATPase (EC 3.6.1.3) (Hayashi & Freiman, 1966); cytochrome oxidase (EC 1.9.3.1) (Burstone, 1960); α-glucan phosphorylase (EC 2.4.1.1) (Godlewski, 1963, 1964); periodic acid–Schiff reaction for glycogen (modification of the method of Dubowitz & Brooke, 1973).

Measurements of area were made on light micrographs of the sections stained for phosphorylase by using a Hipad digitizer (Bausch and Lomb, Texas Instruments). The phosphorylase stain was used for measurement since it gave better photographic definition. However, since glycogen loss preceded loss of phosphorylase activity (see the Results and discussion section), the area measured may be an underestimate.

Muscles to be used for both biochemical and histochemical analyses were frozen rapidly at fixed length in liquid nitrogen. The peripheral and core fibres (muscle strips) were teased out from the thawing muscles by using micro-dissection techniques. A sample from each group was checked for its histochemical staining reactions.

Measurement of protein synthesis

Whole muscles and muscle strips were treated in a similar manner, whole muscles receiving more extensive washing (see below). The tissue was homogenized in 2 ml of 0.5 M-$HClO_4$ and the precipitate was washed with at

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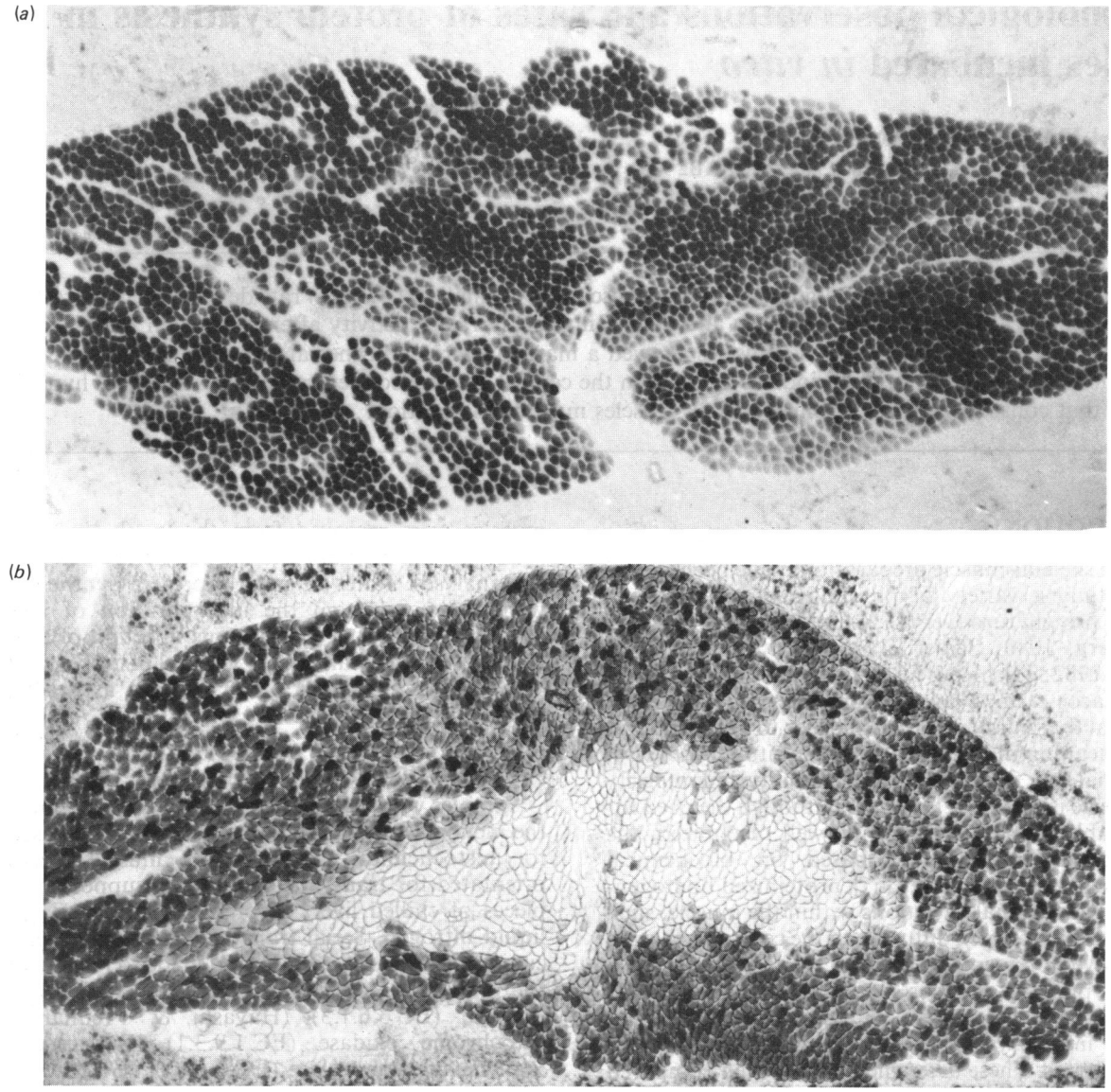

Fig. 1. Transverse sections through (*a*) unincubated control rat soleus and (*b*) rat soleus muscle incubated for 1 h at 37 °C, stained for phosphorylase activity

The central core of the muscle is largely devoid of activity. Magnification × 120 for both (*a*) and (*b*).

least 5 × 1 ml of 0.5 M-$HClO_4$ (eight washes for whole muscle homogenates), followed by four washes (2 ml each) with ethanol and three washes (2 ml each) with diethyl ether. The protein powder was air-dried before hydrolysis in 6 M-HCl for 21 h at 100 °C.

The supernatant of the homogenate was combined with that of the first $HClO_4$ wash, chilled to 4 °C, adjusted to pH 3.0 with 2 M-KOH and centrifuged to remove most of the $HClO_4$, and freeze-dried. This fraction was analysed for the specific radioactivity of the free phenylalanine in muscle. Protein hydrolysates and free phenylalanine samples were dissolved in 0.1 M-HCl and analysed for phenylalanine by ion-exchange chromatography in a Locarte amino acid analyser and counted for radioactivity in a Beckman LS 345 scintillation counter by using Lumax scintillator (LKB, Bromma, Sweden). Muscles from animals injected with [^{3}H]phenylalanine *in vivo* were treated similarly, but in addition they were chromatographed preparatively, to isolate phenylalanine before analysis. Because of the very small amount of free phenylalanine in muscle strips weighing only 5 mg, the specific radioactivity of free phenylalanine in plasma was used in the calculations. The fractional rate of protein synthesis (k_s), as a percentage of the protein mass synthesized per day, was calculated as $k_s = 100 \times S_B/S_A t$, where S_A and S_B are the specific radioactivities of phenylalanine in the precursor pool and protein respectively, and t is the time in days.

RESULTS AND DISCUSSION

All the muscles incubated at 37 °C for up to 120 min showed staining patterns for cytochrome oxidase and Ca^{2+}-activated myofibrillar ATPase which were com-

Table 1. Effect of the duration and temperature of incubation on the size of cores in soleus and extensor digitorum longus muscles from 70 g rats

The mean values of core areas as determined from transverse cryostat sections stained for phosphorylase are expressed as a percentage of the total muscle area. Results are means ± S.D., with the numbers of observations in parentheses. The significance of the difference in values from muscles incubated for 1 h at 37 °C compared with values from muscles incubated for 2 h or at 40 °C was assessed by a two-tailed *t* test based on the pooled estimate of variance: ***$P < 0.001$, *$P < 0.05$.

	Core (% of total muscle cross-sectional area)	
Incubation conditions	Extensor digitorum longus	Soleus
1 h, 30 °C	0	0
1 h, 37 °C	20.4 ± 4.1 (7)	13.4 ± 6.9 (7)
2 h, 37 °C	51.5*** ± 3.3 (4)	57.5*** ± 6.2 (4)
1 h, 40 °C	28.0 ± 7.6 (4)	29.0* ± 13.0 (4)

parable with those seen in unincubated control muscles. However, in muscles incubated for 60 min or more there was a central loss of histochemically demonstrable α-glucan phosphorylase activity (Fig. 1) and glycogen, which at times was substantial. Serial sections revealed that the loss of glycogen preceded the loss of α-glucan phosphorylase activity in affected fibres. This suggested that the affected fibres had become depleted of intrinsic cellular glycogen, which is known to act as a primer for the phosphorylase staining reaction (Martin & Engel, 1972). This differential staining or 'core' effect showed a significant increase in magnitude with both time and temperature (Table 1). It was observed that muscles could be incubated for up to 180 min at room temperature (20 °C) before a core became evident (C. A. Maltin, unpublished work).

The absence of stainable glycogen in the core after incubation for 60 min implies that oxygen had already been deficient (Kauffman & Albuquerque, 1970; Meijer, 1968), and that glycogen had been metabolized anaerobically to supply energy for processes including protein synthesis. Such a deficiency of oxygen would also exclude fatty acid oxidation as an energy source. The appearance of cores after 60 min at 37 °C suggested that the initial utilization to exhaustion of glycogen occurs during this period, and Table 1 suggests a progression with time. The increasing core area suggests that, during the incubation, oxygen is not reaching the centre of the muscle in amounts sufficient to sustain aerobic metabolism.

An assumption in studies with incubated muscles is that oxygen supply is not deficient in the centre of the tissue. Goldberg *et al.* (1975) pointed out that, for complete oxygenation of roughly cylindrical muscles incubated at 37 °C, the cross-sectional diameter should not exceed 1.5 mm, and that muscles of this size are found in rats of body weight 60 g. However, animals larger than this (60–120 g) have usually been used (Li & Goldberg, 1976; Seider *et al.*, 1980; Tischler & Fagan, 1983; Wagenmakers & Veerkamp, 1984). Some investigations have involved periods of growth (Goldspink *et al.*, 1983) where muscles from older groups of animals also appear to have exceeded this limit. Furthermore, experimental manipulations such as tenotomy (Goldspink *et al.*, 1983) involve compensatory muscle growth, as a result of which the diameter of the treated muscle will probably exceed that of the normal control. However, even the size limit of 1.5 mm recommended by Goldberg *et al.* (1975) may be an overestimate, since a core [20.5 ± 6.2 ($n = 5$) or 31.2 ± 14.5 ($n = 3$) % (mean ± S.D.) of the whole muscle area for soleus and extensor digitorum longus respectively] was observed in smaller muscles of < 1.5 mm diameter incubated for 1 h at 37 °C and taken from rats of a mean body weight of only 40 g. Since these rats were younger, it is possible that the higher metabolic rate of the tissue in the younger animals may have contributed to the formation of the core. Cores have also been demonstrated in muscles from both rabbits and mice (Harris *et al.*, 1985). Consequently it is possible that the rate at which cores form is dependent on both the diameter of the muscle and the metabolic rate of the donor animal.

Table 2 shows that tissue in the centre of incubated

Table 2. Fractional rates of protein synthesis (k_s) during incubation *in vitro* or *in vivo* in whole muscles or in strips of soleus and extensor digitorum longus from 70 g rats

The values are expressed as means ± S.D., with the numbers of observations shown in parentheses. The significance of the differences between whole muscles and muscle strips was assessed by a two-tailed *t* test based on a pooled estimate of variance: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Differences between cores and periphery were assessed by paired *t* tests: †††$P < 0.001$, ††$P < 0.01$, †$P < 0.05$.

		k_s (%/day)		
			Muscle strips	
Muscle	Condition	Whole muscles	Core	Periphery
Extensor digitorum longus	*In vivo*		18.3 ± 2.8 (7)	17.9 ± 3.5 (7)
	60 min incubation	7.2 ± 1.8 (8)	5.6 ± 1.6 (6)	11.1***††† ± 2.1 (6)
	120 min incubation	7.9 ± 2.1 (6)	5.3††† ± 1.4 (12)	11.0***††† ± 1.9 (12)
Soleus	*In vivo*		19.9 ± 3.5 (7)	20.0 ± 3.4 (7)
	60 min incubation	10.7 ± 1.4 (8)	10.7 ± 2.7 (6)	16.6***††† ± 3.9 (6)
	120 min incubation	11.4 ± 1.7 (6)	9.4† ± 2.7 (12)	13.7***† ± 1.0 (12)

muscles also has a lower rate of incorporation of labelled amino acids, similar to the effect found by Van Venrooij (1972) in cubes of pancreas when they were larger than 1 mm. The decreased k_s in the core compared with the periphery was not, however, evident when measurements were made in muscle strips *in vivo* (Table 2). The lower incorporation at the centre of the incubated muscles did not result from a failure of the precursor to penetrate: although the concentration of the free phenylalanine was lower in the core, its specific radioactivity was constant throughout the muscle. As with glycogen loss, the low synthesis rate in the core may also have resulted from poor oxygen diffusion, since hypoxia has been shown to inhibit protein synthesis in both perfused (Preedy *et al.*, 1984) and incubated (Palmer *et al.*, 1981) muscle.

Rates of protein synthesis in incubated rat muscles have previously been shown to be lower than those measured *in vivo* (Goldspink *et al.*, 1983), and this is confirmed by the data in Table 2. Hence the depressed rates of synthesis in the cores may in part account for the depressed rates of synthesis in incubated muscles. However, this is not the sole explanation: although peripheral fibres from the incubated muscle have a higher k_s than for either core fibres or the whole muscles (Table 2), the values are still less ($P < 0.001$ for extensor digitorum longus; $P < 0.05$ for soleus) than those measured from muscles *in vivo* (Table 2). The absence of insulin from the incubations *in vitro*, compared with the situation *in vivo*, may also contribute to the differences observed.

Thus this study demonstrates that muscles from young rats do not remain wholly viable when incubated (cf. Goldberg *et al.*, 1975). This conclusion is based on histochemical techniques which show that central fibres lose phosphorylase activity and become depleted of glycogen even in muscles from small animals. Moreover, lower rates of protein synthesis in central than in peripheral fibres provide further evidence for lack of integrity in the central core. These findings suggest caution when interpreting results for rates of protein turnover in incubated muscles.

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