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Stimulation-induced expression of slow muscle myosin in a fast muscle of the rat

Evidence of an unrestricted adaptive capacity

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Fast muscles of the rat hind limb were stimulated continuously at 10 or 20 Hz for periods of 55–61 days by means of an implantable neuromuscular stimulator. Gel electrophoresis clearly demonstrated the presence in stimulated muscles of slow myosin light and heavy chains, although fast isoforms were still present in all cases. Thus, contrary to previous reports, induction of slow myosin isoforms does occur in this, as in other, mammalian species. The time course of the response to stimulation appears to be more extended than that seen in the rabbit.

Skeletal muscle; Chronic stimulation; Slow myosin isoform; Rat

1. INTRODUCTION

In response to chronic stimulation, fast-twitch muscles of the rabbit hind limb ultimately acquire all the characteristics of slow-twitch muscles [1,2]. An important feature of this transformation of type is the de novo synthesis of slow muscle isoforms of the myosin light chains (MLCs) [3–13] and myosin heavy chains (MHCs) [5,9,12]. Associated transitions in mRNAs encoding fast and slow MLCs and MHCs show that these changes take place at a pretranslational level [14–20].

Attempts to demonstrate a conversion of this extent in the rat have, however, been unsuccessful. Chronic indirect electrical stimulation of fast muscles in this species has been shown to produce a transition from type 2B to type 2A MHC, with changes in the corresponding mRNA species, but the transition to type 1 MHC has not been observed, even after extended periods of stimulation [21–26]. Changes in MLC consisted mainly of an increase in the ratio of $LC1_F$:$LC3_F$; slow MLC, present to a small extent even in control fast muscles in this species, barely increased [22,23,26]. On the basis of this and other studies it is widely believed that rat muscles have a more restricted adaptive range [2,27,28]. Kirschbaum et al. offered an explanation of this species difference based on suppression of slow myosin gene expression by thyroid hormone in these animals [26].

The response to chronic stimulation is not a mere biological curiosity: it is evidence of a mechanism that enables muscle properties to adapt to changes in locomotor and postural demands throughout life. The associated expression of slow myosin isoforms has been reported not only in the rabbit but in a number of other mammalian species, including cat [29], dog [30–32], goat [33], sheep [34,35], and calf [2]. The signalling pathways that are responsible must be an intrinsic feature of gene regulation in these species. It was therefore difficult to accept that the rat could be fundamentally different in this respect.

The rat is a more active species than the rabbit, and slow motor units in rat muscles tend to fire at higher frequencies [36]. Fast-to-slow conversion might therefore be achievable with stimulation patterns that deliver a higher aggregate amount of activity than has been used before. With this possibility in mind we conducted an experiment in which fast hind limb muscles in rats were subjected to prolonged periods of continuous stimulation at either 10 Hz or 20 Hz. We show here that synthesis of type 1 myosin heavy and light chains took place in muscles stimulated at either frequency.

2. MATERIALS AND METHODS

2.1. *Chronic stimulation*

Surgical procedures were carried out with full aseptic precautions on male Wistar rats of 300–345 g body weight anaesthetized with a

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Abbreviations: MLC, myosin light chain; MHC, myosin heavy chain; EDL, extensor digitorum longus; SOL, soleus; TSH, thyroid stimulating hormone; T3, tri-iodothyronine; T4, thyroxine.

combination of atropine sulphate (Sigma Chemical Co. Ltd.; 1.5 mg/kg), diazepam (Valium, Roche Products Ltd.; 5 mg/kg), and Hypnorm (fluanisone 10 mg/ml and fentanyl citrate 0.315 mg/ml; Janssen Pharmaceutica; 0.1 ml/100 g). To avoid the problems and potential limitation of freedom associated with percutaneous stimulation in this highly active species, totally implantable miniature neuromuscular stimulators were used. The circuit design was based on that described by Salmons and Jarvis [37], but with a smaller lithium battery to reduce the overall unencapsulated diameter to 12 mm. The device was placed in the peritoneal cavity. To prevent migration it was suspended from the abdominal wall by a small piece of Dacron mesh, which had been incorporated in the silicone rubber encapsulation (Dow Corning Type 3140). Fine, multistranded stainless steel leads (Cooner Wire Assoc., Chatsworth, CA) were taken subcutaneously to the left hind limb, where the electrodes were fixed in close relationship to, but not in physical contact with, the common peroneal nerve. One week after the operation, the device was activated remotely via an optical link. This initiated stimulation of the fast muscles of the anterior compartment, and in particular the extensor digitorum longus (EDL) muscles used in this study.

In a preliminary experiment, not reported in detail here, muscles were stimulated at 5, 10 or 20 Hz for 9 days in order to assess the level of damage induced at these frequencies of stimulation. We went on to stimulate the muscles of 7 rats for periods of 55–61 days continuously at frequencies of 10 or 20 Hz. The devices and the stimulation were well tolerated by the animals, but it was difficult to avoid some stimulus spread to muscles in the posterior compartment.

2.2. *Terminal procedure*

At the end of each experiment, physiological measurements were carried out under deep anaesthesia, after which the stimulated EDL muscles were removed, weighed, frozen in liquid N_2, and stored at −77°C pending biochemical analysis. The corresponding muscles from the unstimulated contralateral limbs served as controls; they were removed at the same time and treated identically to the stimulated muscles. The soleus (SOL) muscles of the contralateral limbs served as slow muscle controls. Results are presented for all 7 animals used in the study.

2.3. *Myosin extraction and protein assay*

Myosin was extracted from the muscle samples according to Mayne et al. [31]. Protein was assayed by the Lowry–Folin method [38], and sodium dodecyl sulphate (SDS, Biorad Laboratories Ltd.) was added to the extracts to a final concentration of 0.5% before electrophoresis.

2.4. *SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of myosin heavy chains*

MHC were separated, essentially by the method of Laemmli [39], in a 7 cm vertical slab unit (Hoefer Scientific Instruments). Glycerol was included in both the resolving and stacking gels: the resolving gel contained 7% polyacrylamide/44% glycerol at pH 8.8 and the stacking gel contained 4% polyacrylamide/33% glycerol at pH 6.8. Approximately 2 μg of myosin was loaded per well, and electrophoresis was carried out at 250 V for 3 h. The gels were stained with 2.5% w/v Coomassie blue R250 in methanol/water/acetic acid (5:5:1) and destained in the same solvent.

2.5. *SDS-PAGE of myosin light chains*

MLC were analysed according to methods described by Brown et al. [9]. Approximately 2.5 μg of myosin was loaded per well, and electrophoresis was carried out at 30 mA for 4 h. The gel was silver-stained by the method of Morrissey [40].

2.6. *Thyroid assays*

During the terminal procedure, blood was collected by cardiac puncture for analysis of thyroid-related hormones. The blood samples were maintained at 4°C for 2 h, centrifuged for 10 min at 6000 × *g* and the sera stored at −77°C pending analysis for basal thyroid stimulating hormone (bTSH), tri-iodothyronine (T3), and thyroxine (T4) by radioimmunoassay.

3. RESULTS

3.1. *Preliminary experiment*

In the preliminary experiment, muscles were stimulated for 9 days, at which stage any damage induced by stimulation should be fully developed [41,42]. Morphometric analysis of transverse cryostat sections from the muscles showed that the incidence of damaged fibres in muscles stimulated continuously at 5, 10 or 20 Hz was less than 1%.

3.2. *Gel electrophoresis*

Extracts from all of the stimulated muscles were subjected, together with control samples, to gel electrophoresis for the analysis of heavy chain isoform composition. In Fig. 1, the stimulated muscle samples are arranged in approximate order of their degree of transformation; they are flanked by extracts from control EDL (fast, lane 1) and SOL (slow, lane 9). The results confirm earlier studies in showing that chronic indirect electrical stimulation of fast muscles in the rat produces a transition from type 2B to type 2A MHC [21–26]. They go further in providing clear evidence (lanes 5–8) for the stimulation-induced transition to type 1 MHC, which has not been reported previously. The progressive appearance of type 2A and 1 MHC in the stimulated muscles is associated with a reduction in type 2B MHC. In lane 8, fast MHC isoforms are barely detectable, in spite of slightly heavier loading. There is no evidence of an effect of stimulation frequency on the degree of transformation achieved.

Fig. 2 shows the results of gel electrophoretic analysis of light chain isoform composition. In this gel, extracts from muscles that were stimulated at the same frequencies were loaded in adjacent lanes (see legend). One myosin extract was exhaused before its light chain composition could be analysed: for this reason only 6 stimulated muscle extracts are represented on this gel. They are flanked by control samples: EDL (fast, lane 1), SOL (slow, lane 8), and a mixture of EDL and SOL (lane 9). $LC1_F$, $LC2_F$, $LC3_F$ are the MLC isoforms that normally predominate in fast-twitch muscle; $LC1_S$, $LC2_S$ are the MLC isoforms that predominate in slow-twitch muscle. Control fast muscle (lane 1) contained a small amount of $LC1_S$ and $LC2_S$, but these isoforms comprised a noticeably larger proportion of the total light chain content in the stimulated fast muscles. This is seen to its greatest extent in lane 7 (corresponding to the heavy chain sample in lane 8 of Fig. 1), in which the MLC isoforms are predominantly those characteristic of slow muscle. However, none of the stimulated muscle samples showed the complete disappearance of fast MLCs that has been seen in the rabbit [9] and the dog [31].

3.3. *Thyroid status*

Analysis of T3, T4, and bTSH levels confirmed that all animals in this study were of euthyroid status.

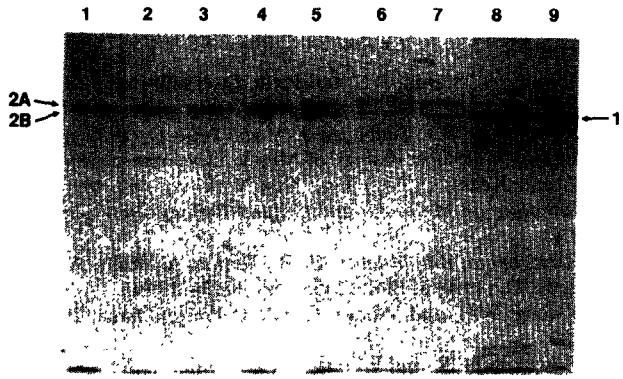

Fig. 1. SDS-PAGE analysis of MHCs in in extracts from rat hind limb muscles. Lanes 1 and 9 correspond to control EDL and control SOL muscles, respectively. Lanes 2–8 are myosin extracts from individual rats stimulated continuously at 10 Hz or 20 Hz for 55–61 days. The gel demonstrates an increase in the amount of MHC2A in relation to the total MHC content in all the stimulated muscle extracts and, in particular, induced synthesis of MHC1 in lanes 5–8. The level of MHC2B decreased in the stimulated muscles, and in one case (lane 8) both MHC2B and MHC2A were reduced to nearly undetectable levels. (Lanes 2, 6, 7: 10 Hz; lanes 3, 4, 5, 8: 20 Hz.)

4. DISCUSSION

This study was designed to address the question: can synthesis of the slow isoforms of MHC and MLC be induced by chronic indirect stimulation in the rat? The unequivocal answer is that it can. Carraro et al. [43] have demonstrated stimulation-induced synthesis of slow MHC in denervated fast muscles of the rat hind limb, but this is the first time that it has been shown to take place in fully innervated muscles. We report elsewhere that the changes were associated with a marked reduction in contractile speed ([44], and in preparation).

Studies of motor unit activity in freely moving rats show that the predominant firing frequency in the soleus muscle is about 20 Hz [36]. We reasoned that this frequency would be more likely to transform muscles in the rat than continuous or intermittent stimulation at 10 Hz, which had proved adequate in the rabbit. Stimulation at 10 Hz was included in the study for purposes of comparison. We were therefore surprised to find that stimulation at either 10 or 20 Hz was sufficient to induce the synthesis of slow myosin isoforms.

Kirschbaum et al. [26] have shown that slow MHC is expressed in fast muscles of rats if they are rendered hypothyroid, and that this expression is enhanced at both protein and mRNA levels if hypothyroidism is combined with chronic stimulation. We were therefore careful to check the thyroid status of animals used in this study. All were normal. Thyroid hormones clearly have a role in the regulation of myosin synthesis, but an altered thyroid status is evidently not prerequisite for the induction of slow myosin expression by chronic stimulation.

Fibres that are regenerating under conditions of chronic stimulation may be more susceptible to transformation [41]. We therefore examined the extent of stimulation-induced damage in a separate preliminary experiment. This showed that less than 1% of the muscle fibres was damaged under the conditions of stimulation employed in this study. Even if damage were an ongoing process, the contribution of slow myosin isoforms from a regenerating fibre population of this size could not have influenced our results significantly. We conclude that the slow isoforms were synthesized within pre-existing fibres in these muscles. Such a mechanisms is well established in the rabbit [12,20,45–47].

The effects of stimulation in the rat are more variable, and the time course of conversion is much more extended, than that seen in the rabbit. This may reflect a genuine species difference, or it could indicate residual technical difficulties in maintaining consistent stimulation over prolonged periods in these highly active animals. None of the muscles in this study showed complete conversion to a slow isoform profile, and it is possible that such conversion would require stimulation to be maintained at a higher frequency or for a longer period.

In conclusion, this study provides clear evidence that, contrary to previous reports, fast-to-slow transformation can take place in rat hind limb muscles that are subjected to continuous activity by chronic stimulation. The observation removes a puzzling anomaly, and suggests that the skeletal muscle of all mammalian species may now be expected to share the same adaptive behaviour in response to an increase in use. In view of the wealth of molecular biological tools available for studies in the rat, this finding has the particular value of showing that stimulation-induced mRNA changes in muscle, and the associated regulatory mechanisms, are accessible to study in this species.

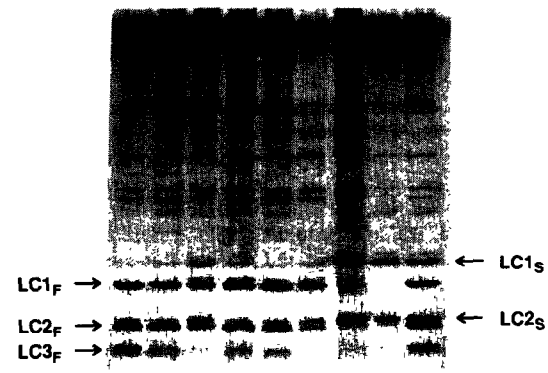

Fig. 2. SDS-PAGE analysis of MLCs in extracts from rat hind limb muscles. Lanes 1, 8 and 9 correspond, respectively, to control EDL, control SOL and a mixture of control EDL and control SOL. Lanes 2–7 are myosin extracts from EDL muscles of individual rats that were stimulated continuously at 10 or 20 Hz for 55–61 days. There is clear evidence in the EDL muscles of stimulated rats of an increase in the proportion of MLCs characteristic of slow muscle. (Lanes 2, 3: 10 Hz; lanes 4–7: 20 Hz.)

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