Changes in the Synthesis of Total Proteins Induced by Chronic Electrical Stimulation of Skeletal Muscle

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Abstract

Rates of protein synthesis were measured by constant infusion of ^{3}H-tyrosine in control fast and slow muscles of the rabbit hind limb and in fast muscles subjected to chronic electrical stimulation.

Slow muscle had synthesis rates more than twice those of fast muscle, and its RNA content was 20% higher.

Stimulation of fast muscle brought about a striking phasic increase, lasting several weeks, in protein synthetic rates, RNA content and RNA activity. There was indirect evidence for an accompanying increase in the rate of protein degradation. In the long term, synthesis rates stabilized at levels similar to those found in slow muscle.

These results provide evidence of an overall increase in protein turnover that is significant in relation to both experimental and therapeutic applications of chronic electrical stimulation.

Key words: chronic stimulation, muscle, RNA, protein synthesis.

Basic Appl. Myol. 9 (1): 19-28, 1999

When a mammalian skeletal muscle is subjected to a radical and sustained increase in its normal contractile activity it undergoes adaptive changes that enable it to cope more effectively with the new working conditions. This remarkable capacity is seen to its fullest extent in the response of fast muscles to low-frequency electrical stimulation delivered continuously over a period of weeks [79, 80]. Under these conditions a fast–to–slow fibre type transformation takes place that involves dramatic changes in calcium transport, contractile characteristics, major pathways of energy metabolism, blood supply, and fine structure. Much interest has focused on the underlying quantitative and qualitative changes in the protein composition of the muscle [71], and on the associated regulatory events, many of which appear to occur at a pre-translational level [1, 3, 8-10, 35-39, 42, 46-48, 50, 51, 53, 54, 72-74, 85, 86, 88, 89]. Much less attention has been paid to changes in protein turnover. We show here that the effects of chronic stimulation on rabbit fast muscles include a striking generalised increase in the level of protein synthesis that will have a major influence on the extent and time course of changes in the expression of fibre type-specific proteins.

Materials and methods

Materials

L-(side chain-2,3-^{3}H) tyrosine (specific radioactivity 15-16 Ci/mmol) and ^{51}Cr-EDTA (specific radioactivity 1-2 Ci/mg) were obtained from Amersham International.

All the more specialised chemicals were purchased from Sigma (London) Chemical Co. Routine laboratory chemicals of Analar grade were obtained from BDH Chemicals and Fisons Scientific Equipment.

Stimulation and infusion

Adult New Zealand White rabbits were operated under aseptic conditions for the intra-abdominal implantation of miniature electronic stimulators. Two types of stimulator were employed. For periods of stimulation from 7 d to 11 weeks the stimulator was based on that originally described by Salmons [77]. For shorter periods of stimulation (from 12 h to 7 d) a switchable stimulator was used [5]; this could be activated several days post-operatively, so that the possible effects of anaesthetic or operative trauma could be dissociated from those of stimulation. In both cases the electrodes were led subcutaneously to the left hind limb where they were fixed close to the common peroneal nerve.

Stimulation of this nerve activates muscles of the anterior compartment, in particular, the fast tibialis anterior (TA) and extensor digitorum longus (EDL) muscles.

Stimulation consisted of a continuous train of supramaximal pulses at a frequency of 10 Hz. At the end of the stimulation period, protein synthesis was measured by constant infusion of L-(side chain-2,3-^{3}H) tyrosine via the marginal ear vein at a dose of 0.5 mCi/kg body weight [64]. The infusion rate was set by a motor-driven syringe at 1.5 ml/hour for periods of 6–6.5 h. Throughout infusion, animals were restrained in a cloth jacket fastened with Velcro [11], but had access to food and water. Laboratory rabbits have a sedentary habit, and this degree of restraint caused no apparent distress. The animals were killed by anaesthetic overdose. The TA and EDL muscles of both hind limbs and the slow soleus muscle of the right hind limb were then removed immediately, frozen in liquid nitrogen and stored at -47 C pending analysis.

All procedures were conducted in strict accordance with the legislation governing the experimental use of animals in the United Kingdom.

RNA content

Ribonucleic acid (RNA) was extracted and measured according to Munro and Fleck [62]. Muscle samples were homogenized in distilled water, and proteins and nucleic acids precipitated by the addition of 0.6N perchloric acid. RNA was extracted from the precipitate by hydrolysis in 0.3M sodium hydroxide at 37 C for 1 h. Protein was again precipitated by the addition of 1.2N perchloric acid, the RNA remaining in the supernatant. The precipitate was washed twice with 0.2N perchloric acid for complete recovery of RNA, and the washings were combined with the earlier supernatant to form the total RNA extract. The optical density (O.D.) of RNA was read at 260 nm and its concentration determined from the equivalence: 1 absorbance unit at 260 nm = 32 µg/ml RNA, pathlength l cm [52]. Protein contamination was determined using the procedure of Lowry, Rosebrough, Farr & Randall [57]. The correction was based on the equivalence: 1 mg/ml protein = 1 absorbance unit at 260 nm [52].

Specific radioactivity of free and protein-bound tyrosine

TA and EDL muscles were combined for this analysis. Samples were homogenised in distilled water, and protein was precipitated by the addition of 2.5 N perchloric acid. After centrifugation, the precipitate was resuspended in 0.5 N perchloric acid, centrifuged again and the two supernatants combined to form the free amino acid extract. The protein precipitate was washed in acetone, alcohol, and ether, and air dried. The free amino acid extract was adjusted to pH 4.0 by addition of 5M potassium hydroxide in order to precipitate potassium perchlorate. The supernatant obtained on centrifugation was freeze-dried. L–tyrosine was converted to L–tyramine by the specific enzyme L–tyrosine decarboxylase, a procedure that excludes from measurement any D–tyrosine contained in the infusion medium [23]. L–tyramine was then assayed by the nitrosonaphthol fluorometric method [84] as modified by Sigma, and the radioactivity of duplicate samples determined in a Searle Mark III (6880) liquid scintillation counter equipped with external standardization.

A small amount (20 mg) of the dried muscle protein was hydrolysed in 6 M hydrochloric acid for 24 h at 110 C in a sealed glass tube. The resulting hydrolysate was diluted with water to a volume of 20 ml and assayed for tyrosine content by the nitrosonaphthol fluorometric method (Sigma). Radioactivity was measured in triplicate samples of diluted hydrolysate. The fractional synthesis rate was calculated from the following equation [22]:

$$\frac{S_B}{S_h} = \frac{R}{(R-1)} x \left\{ \frac{\left(1-e^{-K_s t}\right)}{\left(1-e^{-RK_s t}\right)} - \frac{1}{(R-1)} \right\} \quad \text{… Eq. (1)}$$

where

t = time of infusion (d)

R = ratio of pool sizes of protein–bound to free tyrosine in muscle, determined as 700 for the animals in this study

S_B = specific activity of protein–bound tyrosine in muscle (dpm/nmole)

S_h = specific activity of free tyrosine in muscle (dpm/nmole)

K_s = fractional rate of protein synthesis (d^{-1})

Extracellular space

Assay of extracellular space was performed by the method described by Poole-Wilson & Cameron [76], utilising ^{51}Cr-EDTA as the extracellular marker.

Results

RNA content

Stimulated animals showed no obvious postural or locomotor asymmetry, and our previous studies suggest that the unstimulated hind limb is an appropriate source of control tissue [6, 17]. Certainly control values obtained in this way for the total RNA content of the muscles (see legend to figure 1) were in good agreement with those published by earlier investigators [49, 63]. As further evidence of the absence of any contralateral effect of stimulation on these control measurements, it was observed that they failed to show any significant trend during experimental periods up to 10 weeks, a linear regression against time having a correlation coefficient of 0.04 (n = 44; P = 0.8).

Stimulation of fast muscles induced a substantial rise in RNA concentration which was already apparent after only 2 d. The RNA concentration reached a maximum

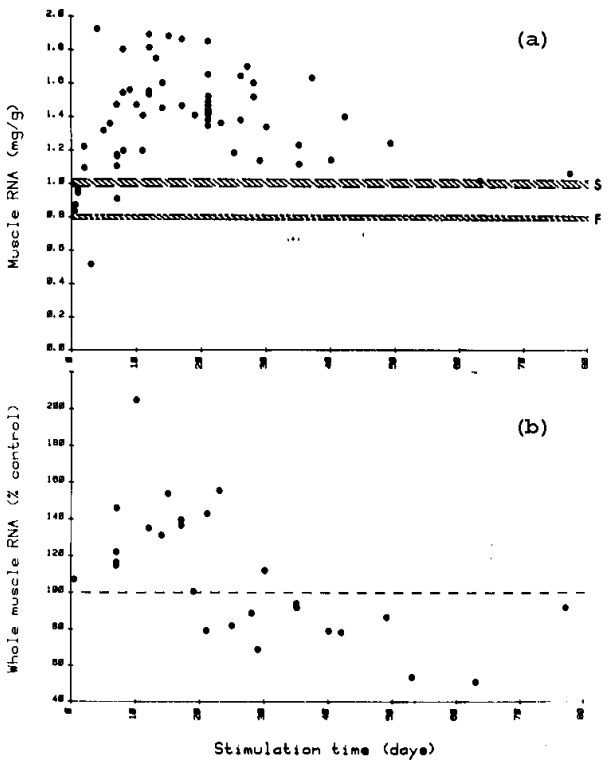

Figure 1. RNA levels in chronically stimulated rabbit fast muscle (TA). Each point represents a single determination. In this and the following figure the bands marked F and S represent the mean ± S.E.M. of control values obtained from fast and slow muscle, respectively. (a) Concentration of total RNA. Control values were 0.80 ± 0.01 mg/g for fast muscle (TA, n = 49), and 1.01 ± 0.02 mg/g for slow muscle (soleus, n = 41); the difference was significant at $P < 2 \times 10^{-11}$ (Student's t-test applied to the unpaired data). (b) Whole muscle content of RNA, presented as a percentage of the value for the contralateral muscle in each case. Thus the initial increase in mg/g of RNA was not due to a decrease in the weight of the muscle.

of 1.55 ± 0.09 mg/g (mean ± S.E.M., n = 7) between 10 and 12 d; this was very much higher than both control fast and slow muscle levels ($P < 0.0001$, $P < 0.0007$, respectively). It then declined slowly to values typical of slow muscle by 9 weeks of stimulation (figure 1a). During this period the muscles showed a reduction in wet weight. However, the increase in total RNA per gram wet weight could not be ascribed to a mere concentration effect, for the rise in total RNA content during the first 3 weeks of stimulation was still evident if the results were expressed on a whole muscle basis (figure 1b). After 3 weeks, total RNA fell below the values for the contralateral muscle as a result of further reduction in the weight of the stimulated muscle (figure 1b).

Protein content

In agreement with earlier work, the stimulated muscles showed a significant reduction in wet weight, to 57% of control values in fast muscles stimulated for 4 to 6 weeks. However, there was no significant difference between the mean protein content per gram of muscle for the 4– to 6–week–stimulated fast muscles (222 ± 16 mg/g, n = 7), and that of the control fast muscles (239 ± 4 mg/g, n = 22).

Protein synthesis

The specific activity of ^{3}H-tyrosine in the total protein fraction of the muscle provided a measure of protein synthesis (figure 2). Again the control values showed no significant trend with the period of stimulation of the contralateral limb. Stimulated fast muscles had begun to show specific activities significantly greater than those of their control counterparts by 7 – 12 d (mean for this period: 28.6 ± 4.4 dpm/nmole, n = 7; cf. mean for control fast muscles: 11.8 ± 0.7, n = 33; difference significant at $P < 0.008$). With further stimulation the specific activity continued to increase, reaching levels as much as five times the control fast muscle level by 3 weeks. The specific activity at this stage was significantly greater than that of control slow muscle ($P < 3 \times 10^{-6}$) but declined over the subsequent 6 weeks of stimulation towards the slow muscle level.

The fractional synthesis rate for control slow muscle was more than twice that determined for control fast muscle (7.94 ± 0.91, n = 10 and 3.61 ± 0.29, n = 11, respectively; difference significant at $P < 2 \times 10^{-4}$). These results confirm previous reports in which slow muscle has consistently shown a higher rate of protein turnover than fast muscle [14, 25, 44, 45]. Measurements of fractional synthesis rate required precise estimation of the free tyrosine. Since instrumentation of the requisite sensitivity only became

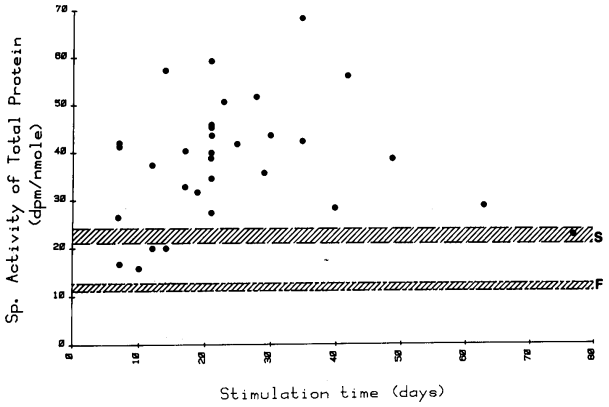

Figure 2. The specific activity of protein–bound tyrosine in total cellular protein of chronically stimulated fast muscle (TA and EDL combined). Control values were 11.8 ± 0.7 dpm/nmole for fast muscle (TA and EDL combined, n = 33) and 22.6 ± 1.5 dpm/nmole for slow muscle (soleus, n = 17). This difference was significant at $P < 10^{-6}$.

available at a late stage of the study there are fewer data that can be presented in this way. These measurements did, however, serve to confirm that the specific activity of free tyrosine in the muscle homogenate (S_h) was similar in both control and stimulated muscles throughout the course of stimulation (Table 1). Therefore the changes in specific activity, S_B (figure 2), also reflect changes in the fractional synthesis rate, K_s (see Eq. 1). The available measurements for the latter do, in fact, show a similar time course (figure 3).

The results illustrated in figures 1 and 3 could also be expressed in terms of the efficiency of translation, defined as the rate of peptide bond formation per unit of translational apparatus [60]. This increased over a time course similar to that illustrated in figure 3 for fractional synthesis rate. The translational efficiency, in grams of protein synthesized per gram RNA per day, was 10.9 ± 0.9 (n = 11) for the control fast muscles. Fast muscles stimulated for 25 – 53 days all showed values exceeding the 95% confidence limits for the control population (Table 2).

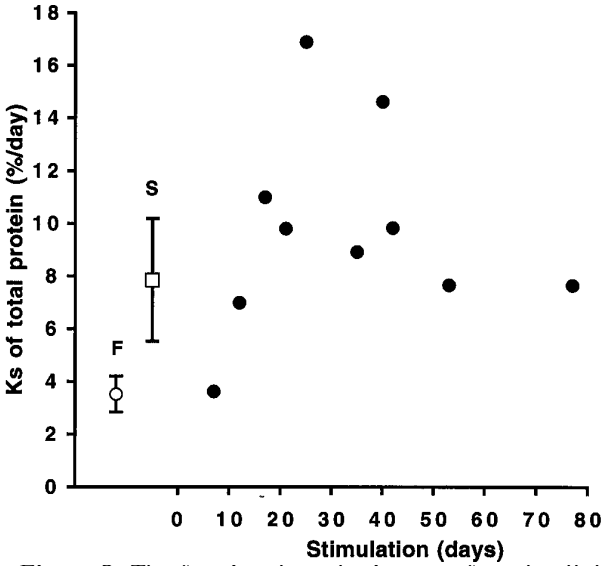

Figure 3. The fractional synthesis rate of total cellular proteins in rabbit skeletal muscle. Unfilled circle, F, control fast muscle (TA and EDL combined); unfilled square, S, control slow muscle (soleus); the error bars represent 95% confidence limits in each case. Filled circles, fast muscle stimulated for the period indicated on the abcissa.

Extracellular space

We calculated the rate of protein synthesis on the basis that total free amino acids in the muscle homogenate formed the precursor pool for protein synthesis. Whether there is a need to correct for the contribution of extracellular amino acids to this total depends on one's view of the relative accessibility to the protein synthetic apparatus of amino acids in the intracellular and extracellular compartments [56, 83]. In order to see whether such a correction would have a significant influence on the result we determined, in separate experiments, the extracellular space (ECS) for control and stimulated muscles using the ^{51}Cr–EDTA technique [76]. The mean values (± S.E.M.) for the ECS in control fast and slow muscles were, respectively, 7.90 ± 0.50 (n

Table 1. Specific activity of free and bound tyrosine in muscle during chronic stimulation. There was no significant difference between the free tyrosine levels (s_h) of the stimulated and control muscles (paired t-test, P = 0.15). In addition to the results shown in this Table, values for protein-bound tyrosine (s_B) were also available from the earlier series of measurements, for which there were no corresponding values of s_h. For the control muscles in that larger series, s_B was 12.8 ± 4.2 (mean ± standard deviation, n=22). We therefore attach no significance to the apparent rise in the tabulated control values for s_B of individual animals between 21 and 35 d.

Duration of stimulation	Specific activity of tyrosine (dpm/nmole)			
(days)	S_h (free)		S_B (protein-bound)	
	Control	Stimulated	Control	Stimulated
7	1328	2178	6.9	16.7
12	1187	1245	6.0	20.0
17	1410	1240	6.5	32.9
21	1100	1187	10.9	27.6
25	1310	1063	13.8	41.9
29	1504	1502	14.8	35.9
35	1366	2059	14.3	42.6
40	788	802	6.5	28.4
42	1484	1675	12.4	20.5
53	1097	1231	7.8	28.2
77	1329	1439	6.6	23.1
Mean ± S.E.M.	1264 ± 63	1420 ± 125	9.7 ± 1.1	28.9 ± 2.6

Table 2. Changes in the efficiency of translation of muscle RNA during chronic stimulation. RNA translational activity in the stimulated muscles was greater than that in the corresponding control muscles of the contralateral limb ($P < 0.002$; paired t–test), the differences being most significant between 25 and 53 days of stimulation.

Duration of stimulation (days)	RNA activity (g protein synthesized/g RNA/day)	
	Control	Stimulated
7	8.4	7.0
12	7.1	10.2
17	6.8	13.3
21	11.4	16.0
25	16.1	32.1
29	12.0	20.2
35	15.4	18.1
40	11.0	28.9
42	11.6	18.3
53	11.1	17.5
77	9.2	16.1
Mean ± S.E.M.	10.9 ± 0.9	18.0 ± 2.2

= 18) and 11.10 ± 0.74 (n = 19) ml/100g, and these values were significantly different (P = 0.001; Student's unpaired t-test). In stimulated muscles, values for ECS were more variable, ranging from 9.57 to 32.9 ml/100g, although always greater than in the unstimulated contralateral muscles ($P < 2 \times 10^{-6}$, Student's paired t-test, n = 16). If free amino acids in the extracellular space were not available for protein synthesis we would have to make a correction for the extracellular contribution to total free amino acids that would, in the worst case, reduce the highest fractional synthesis rate observed by only 6.5%. Conversely, if free amino acids in the ECS were the only precursors available to protein synthesis, no correction would be needed, because the specific activity of free tyrosine in the ECS (i.e., plasma) did not differ significantly from that in the homogenates (homogenate: plasma = 0.80 ± 0.25, n = 5). Thus the nature of the precursor pool had no significant influence on the results.

Discussion

We present here evidence that protein synthesis increases markedly in mammalian fast muscles when they are subjected to low–frequency stimulation. Protein synthesis is normally higher in slow than in fast muscle [14, 25]. In the present experiments the rate of protein synthesis in stimulated fast muscle eventually converged on levels typical of slow muscle, but only after a rise of several weeks' duration that was too large to be attributed to mere differences between fast and slow levels of synthetic activity. An increase in protein synthesis has been observed previously in a study of chick muscle cells stimulated *in vitro* [4]. However, we believe that this is the first time such a phenomenon has been reported for a mammalian muscle stimulated electrically *in vivo*.

An elevation in the RNA content of muscles that had been subjected to chronic stimulation was noted by Heilig and Pette [33] and by Pluskal and Sréter [75], but the yields of total RNA were low and the results were interpreted as a mere transition from typically fast to typically slow muscle levels. Subsequent results from one of these laboratories [67], based on an intermittent pattern of stimulation, revealed a phasic increase in total RNA of somewhat similar time course to that demonstrated in the present study. Clearly the stimulation–induced increase in total RNA is very much greater during the first few weeks than would be expected from fibre type transformation alone.

Changes in messenger RNA (mRNA) composition occur in the early stages of the response to chronic stimulation (see [9], for example), but this would not be expected to contribute appreciably to changes in total RNA, of which mRNA represents only a small proportion. Ribosomal RNA (rRNA), on the other hand, constitutes approximately 80% of the total RNA [90], and is probably responsible for most of the increase. This assumption is supported by ultrastructural evidence of an increase in ribosomes and polyribosomes in stimulated muscle [17, 78], and of enlargement and elaboration of nucleoli [43], all of which is consistent with an increase in ribosomal synthesis.

The stimulation-induced increase in RNA content confers on the muscle an enhanced capacity for protein synthesis, but we also found evidence for a change in the efficiency of translation [60]. Millward and his colleagues [61] showed that RNA activity in control fast muscles of the rat could vary over a wide range (9.2 – 18.2 g protein synthesized/g RNA/day), the higher values being seen in young, rapidly growing animals. This would suggest that there is some room for modulation of the efficiency of translation, a proposition supported by the present results.

Non–muscle cells may have made a small contribution to the observed increase in total protein synthesis, but such cells represent a minority population in skeletal muscle. Collagen, one of the major proteins produced by non-muscle cells, constitutes only 2.5% of the total protein in rabbit muscle [66]. Its fractional synthesis rate in the rabbit is similar to that of total mixed muscle protein [66], and changes commensurately with that of total protein under conditions of intermittent stretch [65]. The contribution of collagen to the observed changes in protein synthesis is therefore unlikely to have been substantial. The rapid growth in capillaries that takes place in response to stimulation [81] may have contributed to the increase in protein synthesis

during the first 14 days but could not be responsible for the bulk of the changes, which occurred at a later stage.

Changes in protein turnover are known to occur with ageing in the rat [55]. Ageing effects, if present, should have manifested in unstimulated muscles of the contralateral limb, yet control values derived from these muscles did not differ significantly between experiments of longer and shorter duration. The probable explanation is that the lifespan of the rabbit is much longer than that of the rat and is not, therefore, a significant factor in experiments of the duration considered here. It is, nevertheless, possible that some of the inter-animal variation in our data may have been the result of non–systematic variation in age at the termination of each experiment.

During the course of stimulation the protein content per gram of muscle remained unchanged. The number of fibres in the muscle has been shown not to change over a similar period of stimulation [68, 79]. It is known, however, that stimulation brings about a reduction in the diameter of individual fibres [69, 79]. This is a well-documented effect of chronic stimulation at 10 Hz, and is independent of any overt histopathological changes in the muscle fibres. The present experiments were therefore typical in showing a significant reduction in the wet weight of fast muscles in the 4– to 6–week–stimulated group. The size of a muscle reflects an equilibrium between protein synthesis, which operates to increase muscle bulk, and protein degradation, which tends to reduce it. Thus the reduction in muscle mass, when taken together with the increase in protein synthesis already described, implies that the rate of protein degradation must also have increased substantially in response to chronic stimulation of this type. This would have the effect of accelerating the turnover of existing proteins.

These events occur at a time when the proteins of major molecular systems of the muscle are known to be in the process of adaptive transition (see [7, 21, 34], for example). They therefore make an important, and thus far neglected, contribution to the dynamics of the transformation process, accelerating both the production of new protein and the removal of pre-existing protein. Under less demanding conditions, such as cross–reinnervation [12], stimulation with intermittent régimes [68], or recovery following cessation of stimulation [6, 16], the changes in protein turnover may be less marked, and the time course of transformation correspondingly prolonged. Under the extreme conditions of disuse that result from denervation, protein synthesis is down-regulated [26, 59], and in this case gene–switching could even be dissociated from phenotypic change.

This last point may be illustrated by considering the differences that have been reported between the effects of phasic, high–frequency and tonic, low–frequency patterns of stimulation on the denervated soleus (slow–twitch) muscle of the rat [2, 18, 28-32, 87]. Denervation alone produces a shift towards fast muscle characteristics, but this is of limited extent. A much greater transformation of type is seen if the denervated muscle is stimulated with a phasic pattern. These observations, which are exemplified by the associated changes in myosin heavy chain isoforms [32], may be interpreted as follows. The disuse that results from denervation triggers regulatory changes, including activation of fast muscle genes, but the changes remain largely confined to the pretranslational stage. A small increase in use, brought about by a phasic pattern of stimulation, is sufficient to activate translational and post-translational processes, enabling the fast muscle proteins to be expressed. A larger increase in use, brought about by a tonic pattern of stimulation, up-regulates protein synthesis and also triggers regulatory changes leading to the expression of the slow muscle phenotype. This reasoning explains how differences in the amount, rather than the frequency, of stimulation can produce different outcomes, and is in good agreement with the results of such studies, reviewed recently by Gundersen [29]. Similar arguments can be used to explain differences in the extent of protein and mRNA changes in another experimental model of decreased use, that of hind limb suspension [13, 19].

Although the explicit increase in protein synthesis and implicit increase in breakdown occurred together in these experiments there is no reason to suppose that the synthetic and degradative pathways are responding to the same intracellular signals. It should therefore be possible to influence the two processes differentially by altering the amount or nature of the stimulation employed. We have in fact shown that fast rabbit muscles that are stimulated continuously at a constant frequency of 2.5 Hz undergo a smaller reduction in muscle mass than those stimulated at 10 Hz [41, 58, 82]. This could reflect the higher energy demands of contraction at 10 Hz, in response to which protein, as well as fat and carbohydrate, is recruited as a substrate for the tricarboxylic acid cycle [34, 70]; the activity of 3-oxoacid CoA-transferase, a key enzyme in this pathway, is elevated much more for stimulation at 10 Hz than at 2.5 Hz [58, 82]. On the other hand, muscles that are stimulated with the same amount of aggregate impulse activity lose less mass if the pattern contains bursts of higher frequency [15, 20, 40]. Such patterns generate more force, which is known to be a powerful stimulus for protein synthesis [24, 26, 27, 65]. The muscle hypertrophy that results from intermittent bouts of intensive exercise presumably results from a long-lasting elevation of protein synthesis that is not countered by the degradative effects of continuous metabolic challenge. An appropriate balance between the synthetic and degradative responses to increased muscle activity should be an important design objective in the development of clinical uses of chronic electrical stimulation.

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