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Time Dependent Effects on Contractile Properties, Fibre Population, Myosin Light Chains and Enzymes of Energy Metabolism in Intermittently and Continuously Stimulated Fast Twitch Muscles of the Rabbit*

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Summary. Fast-twitch tibialis anterior and extensor digitorum longus rabbit muscles were subjected to long-term intermittent (8 h daily) or continuous (24 h daily) indirect stimulation with a frequency pattern resembling that of a slow motoneuron.

Increases in time to peak of isometric twitch contraction were observed without parallel changes in the pattern of myosin light chains or alterations in the distribution of slow and fast fibres as discernible by the histochemical ATPase reaction. However, changes in the fibre population and in the myosin light chain pattern were observed after intermittent stimulation periods exceeding 40 days or continuous stimulation periods longer than 20 days. Under these conditions even higher increases were found in contraction time. In one animal a complete change in fibre population was observed. In this case myosin light chains of the slow (LC_{S1}, LC_{S2}) and of the fast type (LC_{f1}) were obviously synthetized simultaneously within the same fibre. Early changes in the enzyme activity pattern of energy metabolism indicated a conversion of the fibres including their mitochondrial population. These changes and the earlier reported changes in the sarcoplasmic reticulum are probably responsible for the early changes in contractile properties which occur before the transformation of the myosin.

Key words: Nerve-muscle interaction – Contractile speed – Transformation of fibre types – Myosin light chains – Enzyme activity pattern of energy metabolism – Mitochondrial differentiation.

INTRODUCTION

Long-term stimulation of fast-twitch tibialis anterior and extensor digitorum longus muscles of the rabbit with a frequency pattern resembling that of a slow motoneuron has been shown to result in a profound transformation of their functional and structural properties (Salmons and Vrbová, 1969; Pette et al., 1972, 1973, 1975; Brown et al., 1973; Cotter et al., 1973). An increase of myoglobin concentration as well as increased mitochondrial content indicate a transition of the stimulated fast muscles in the direction of a "red" muscle. These changes are also reflected by changes in the isozyme pattern of lactate dehydrogenase and in the activity pattern of a variety of key enzymes in energy metabolism (Pette et al., 1973). The prominent change in function of the stimulated muscles concerns their contraction velocity. Marked increases in time to peak of isometric contraction and time to half relaxation suggest a transition from a fast to a slow twitch muscle. Similar changes in contractile properties of long-term stimulated fast twitch cat muscles were observed by Al-Amood et al. (1973).

Since fast and slow twitch muscles have been shown to differ in their specific myosin adenosine triphosphatase (ATPase) activities (Bárány et al., 1965; Bárány, 1967; Sréter et al., 1966; Samaha et al., 1970b) and are also characterized by specific patterns of myosin light chains (Locker and Hagyard, 1968; Perrie and Perry, 1970; Sarkar et al., 1971; Sréter et al., 1972; Lowey and Risby, 1971; Weeds and Pope, 1971) it appeared conceivable that changes in contractile parameters as induced by long-term stimulation might be parallelled by alterations in myosin ATPase and myosin light and heavy chains. Such changes are well known to occur in muscles transformed by cross-innervation (Buller et al., 1969; Mommaerts et al., 1969; Samaha et al., 1970a; Bárány and Close, 1971; Jean et al., 1973; Sréter et al., 1974; Hoh, 1975; Weeds et al., 1974, 1975). Sréter and coworkers (1973)

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have reported a significant decrease in Ca^{2+}-activated as well as in K^+-EDTA-activated myosin ATPase activity in long-term stimulated tibialis anterior and extensor digitorum longus muscles of the rabbit and have observed the concomitant appearance of light chains characteristic of slow muscle. These changes were interpreted as reflecting either an increase of slow muscle fibres or as synthesis of new myofibrillar material within the fast fibres. It was shown in a preceding study that intermittent long-term stimulation had no influence on the distribution of fast and slow fibres as discernible by histochemical staining for myofibrillar ATPase (Pette et al., 1975). Since morphometric analyses had nevertheless suggested a change in the population of muscle fibres, it appeared worthwhile to perform a study on the pattern of myosin light chains in the stimulated muscles. This study appeared also necessary because Sréter et al. (1973) had observed in their experiments that a period of at least three weeks of continuous stimulation was needed in order to obtain changes in the myosin light chain pattern. The first changes in contractile characteristics had been recorded in our experiments as early as 4 days after the onset of stimulation (Pette et al., 1973). This discrepancy could mean that the early changes in contractile properties are not related to changes in the myosin light chain pattern and depend on alterations of other constituents. A decrease in Ca^{2+} stimulated ATPase activity and concomitant changes in the electrophoretic pattern of proteins of the sarcoplasmic reticulum were found already after one day of stimulation in our laboratory (Ramirez and Pette, 1974). Alternatively, different experimental conditions might have caused the various findings. In our experiments an intermittent long-term stimulation was applied in which the animals were stimulated 8 h daily, whereas Sréter et al. (1973) used a continuous long-term stimulation with 24 h of stimulation daily.

In the present study the effects of intermittent and continuous long-term stimulation on fast rabbit skeletal muscles were compared. Contractile properties, activity levels of selected enzymes of energy metabolism, patterns of some structural proteins in SDS electrophoresis as well as the populations of fast and slow muscle fibres were examined. The results of these studies suggest that the two stimulation programs induced different effects and that significant changes may occur in contractile properties without parallel changes in muscle fibre population and the myosin light chain pattern respectively.

METHODS

Experiments were performed on male rabbits of the strain "Weisse Wiener". Animals of about 2 kg body weight were anaesthetised with pentobarbitone sodium, and electrodes were implanted in the left hind limb using a similar technique as described by Salmons and Vrbová (1969). Stimulating electrodes (coiled multistrand stainless steel wires) were fixed under aseptic precautions on either side of the lateral popliteal nerve. The wires were connected to an insulated lead and led subcutaneously to the back of the animals. They were then connected to a stimulator delivering impulses at a frequency of 10/s, duration 0.15 ms. Stimulation was performed at a frequency of 10 Hz. The intensity (2–9 V) was adjusted so that it did not cause any noticeable discomfort to the rabbits. The conditions were different for each animal and were controlled regularly several times during the day. The animal could move freely in its cage during the period of stimulation. The animals were fed with a standard diet (Altromin, Fa. Altrogge, Lage/Lippe, Germany) and in the average gained weight between 200 and 500 g during the period of stimulation.

Intermittent Long-Term Stimulation. The animals were stimulated 8 h daily. Stimulation was performed between 8 a.m. and 4 p.m.

Continuous Long-Term Stimulation. The animals were stimulated 24 h a day. Stimulation lasted for various periods between 3 and 62 days. The animals were disconnected from the stimulator the evening before the day at which the measurements were performed. In few cases, however, stimulation lasted until 2 h before starting the measurements.

Measurement of Contractile Parameters. The animals were anesthetized by intravenous injection of pentorbarbital (50 mg/kg body weight). The peroneal nerves and the tendons of the tibialis anterior and extensor digitorum longus muscles in both legs were freed. The legs were fixed to steel holders by drills through the head of the tibia and the ankle. Contractions were elicited by stimulating the cut end of the peroneal nerve with square-wave pulses of 0.2 ms duration and supramaximal intensity, using bipolar electrodes connected to a Grass S-9 stimulator. Isometric contractions were recorded using two strain gauges (Universal transducing cells, model UC2, Statham Instruments, Inc., Oxnard, Cal., U.S.A.) and displayed onto a Tektronix 5103 N oscilloscope. They were photographed by polaroid camera (Tektronix Inc., Beaverton, Oregon, U.S.A.). The length of the muscle was adjusted so as to produce the maximal tension on stimulation. To produce maximal tetanic tension muscles were stimulated at 80 Hz for 400 ms. The body temperature was kept constant using a heating pad. Dissection and physiological measurements did not last longer than 2 h leaving the vascular supply intact, so that the muscles could be used for morphological and biochemical studies immediately thereafter.

Histochemical Studies. In order to analyse strictly comparable samples, control and stimulated tibialis anterior muscles were quickly frozen in stretched position by immersion into melting isopentane (−160° C). Whole cross-sections of the central part were cut in a cryostat at −20° C. Typing of the fibres was done on serial sections being alternately stained for succinate dehydrogenase (Nolte and Pette, 1972) and for myofibrillar ATPase after acid (pH 4.1) and alkaline (pH 10.4) preincubation according to the method described (Müller, 1974). Microphotographs were taken at low magnification (8×) and the respective paper prints were assembled to give montages of the whole cross sections. Classification of the fibres was performed in the cross-sections stained for ATPase after acid preincubation. The light and dark fibres obtained in this staining were then marked in the montages of the parallel section stained for ATPase after alkaline preincubation. In this way the same fibres stained for myofibrillar ATPase at two different pH values could be examined.

Measurement of Enzyme Activities. Samples of control and stimulated muscles were frozen immediately after dissection in liquid nitrogen and were stored until further treatment at −79° C. Extraction of total activities of glyceraldehydephosphate dehydrogenase (EC 1.2.1.12), citrate synthase (EC 4.1.3.7) and 3-hydroxyacyl-CoA

dehydrogenase (EC 1.1.1.35) was performed in two steps in 100 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.2 as has recently been described (Pette et al., 1973). The activities of these soluble enzymes were determined in the combined supernatants of the two extractions. Mercaptoethanol was added to the combined supernatants to give a final concentration of 5 mM. Activity determinations for glyceraldehydephosphate dehydrogenase, citrate synthase and 3-hydroxyacyl-CoA dehydrogenase were performed according to methods previously described (Bücher et al., 1964; Bass et al., 1969). Extraction and measurement of total activity of hexokinase (EC 2.7.1.1) were performed according to Kubišta et al. (1971). The total activities of structure-bound mitochondrial glycerolphosphate dehydrogenase (EC 1.1.99.5) and succinate dehydrogenase (EC 1.2.99.1) were determined in the resuspended sediment fractions of the centrifuged homogenates. These sediments which were free of soluble enzyme activities, were resuspended in 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.2 to a 10% concentration as referred to the original weight of the extracted muscle. The activities of the two enzymes were measured spectrophotometrically by using the previously described assay system (Brdiczka et al., 1968; Kubišta et al., 1971) in which the phenazine methosulfate mediated reduction of cytochrome *c* is followed.

Electrophoretic Analyses of Myofibrillar Proteins. Extraction of myofibrillar structure proteins followed the procedure of Weeds et al. (1974) as originally described by Báraný and Close (1971). The muscles were freed from connective tissue and minced in a special micro-meat grinder (Mikrofleischwolf, Fa. B. Braun, Melsungen, Germany). Homogenization was performed in a 20-fold volume of wash solution (30 mM KCl, 5 mM potassium phosphate, pH 6.7). The tissue suspension was cooled with ice and homogenized 3 times 30 s in a Sorvall Omni-Mixer. The homogenate was then centrifuged at 10000 × g. The sediment was resuspended in fresh wash solution and recentrifuged. This washing procedure was repeated once more. The final pellet was then extracted 30 min at 4° C in a 10fold volume (referred to the original muscle weight of the following medium: 0.3 M KCl, 0.15 M potassium phosphate, 5 mM MgATP, 0.1 mM dithiothreitol, pH 6.6. The supernatant which was obtained after 15 min at 27000 × g was dialysed overnight at 4° C against 10 l of 30 mM KCl. The preparation was centrifuged at 10000 × g and the sediment was washed with 30 mM KCl. Afterwards it was solved in 0.6 M KCl, 20 mM potassium phosphate at pH 6.7. The solution was cleared by 30 min centrifugation at 40000 × g and the resulting supernatant was dialysed against 30 mM KCl, 2 mM potassium phosphate, pH 6.7. The precipitated protein was sedimented by centrifugation in a microcentrifuge. The pellet was solved in 0.6 M KCl, 20 mM potassium phosphate, pH 6.7. Protein concentration was determined by means of the biuret reaction. Before application to electrophoresis in presence of sodium dodecylsulfate (SDS), protein concentration was adjusted to about 3 mg/ml and potassium ions were removed. This was done by precipitating the proteins by dilution of the sample with a 100fold volume of quartz distilled water. The precipitate was centrifuged in a microcentrifuge and was dissolved in a medium of the following composition: 6% SDS, 5% mercaptoethanol, 62.5 mM Tris-HCl buffer, pH 6.8, 27% sucrose with pyronin G. Polyacrylamide (PAA) disc gel electrophoresis in the presence of SDS was performed according to Laemmli (1970). 0.1% SDS gels contained 12.5% and 3% PAA in separating and stacking gels respectively. Stacking gels were prepared in 125 mM Tris buffer, pH 6.8. Separating gels were prepared in 375 mM Tris buffer, pH 8.8. Electrophoresis was peformed during 2–3 h at 40 mA per gel in 25 mM Tris/192 mM glycine buffer, pH 8.3 with 0.1% SDS. The gels were stained with 0.05% Coomassic Blue (R-250) in 25% isopropanol and 10% acetic acid. Destaining was performed in 10% acetic acid. The gels were calibrated using cytochrome *c* (mol. wt. 14000), adenylate kinase (mol. wt. 21000), lactate dehydrogenase (mol. wt. 35000), aldolase (mol. wt. 40000) and phosphofructokinase (mol. wt. 93000).

RESULTS

Changes in Myosin Light Chains Following Long-Term Stimulation

In 17 rabbits the tibialis anterior and extensor digitorum longus muscles were stimulated for 8 h a day at 10 Hz during 3 weeks. The pattern of myosin light chains of control and stimulated muscles was then compared. No changes were observed in the pattern of myosin light chains. In a second series of experiments measurement of contractile properties was performed on the same muscles which were also analysed for changes in myosin light chains. Although, as Table 1 clearly shows, the contraction time of the stimulated muscles increased, no change in the pattern of myosin light chains could be detected in these muscles. Intermittent stimulations for longer periods were then performed, but no changes could be detected in the myosin light chains after stimulation up to 6 weeks. In these animals, nevertheless, increases were found in time to peak of isometric twitch contraction which amounted in average to 1.6 fold in the tibialis anterior and to 1.4 fold in the extensor digitorum muscle. An example from such an experiment is illustrated in Figure 1. The characteristic pattern of the three light chains of the fast muscle (LC_{f1}, LC_{f2}, LC_{f3}) was preserved and no increase of the faint bands of the slow type light chains ($LC_{s1\,a}$, $LC_{s1\,b}$, LC_{s2}) could be detected. The faint bands of the slow type light chains are regularly present in these two rabbit muscles and may be due to a small percentage of slow fibres. Only after 60 days of stimulation for 8 h a day has a change in the light chain pattern occurred (Fig. 2f and g).

Experiments in which muscles were stimulated continuously for periods of 13–60 days (10 animals) revealed more pronounced changes in contractile speeds than intermittently stimulated muscles (compare Table 1). This type of stimulation induced changes in the pattern of light chains of myosin after much shorter time. Changes were observed already after 3 weeks of stimulation. An electrophoresis prepared from muscles stimulated continuously for 21 days is shown in Figure 2. There is an increase in the intensity of the bands representing the slow type light chains (i.e. LC_{s1} and LC_{s2}). The pattern of light chains typical of fast muscles is altered only in that there is a slight decrease in the intensity of LC_{f3}. It is interesting that the changes in light chain pattern found after intermittent stimulation for a period of 60 days are similar to those found after 21 days of continuous stimulation. As is seen in Figure 2g, the changes are less pronounced in the 60 days intermittently stimulated extensor digitorum muscle. No changes were seen in the myosin light chains of the stimulated tibialis

Table 1. Time to peak of isometric twitch contraction in control and intermittently (8 h daily) and continuously (24 h daily) stimulated tibialis anterior and extensor digitorum longus muscles after various periods of time. Weights of control (c) and stimulated (st) extensor digitorum longus are given in the last columns

Rabbit No.	Days of stimulation	Time to peak of isometric twitch contraction						Weights of extensor digitorum longus		
		tibialis anterior			extensor digitorum longus					
		c	st	st/c	c	st	st/c	c	st	st/c
		[ms]			[ms]			[g]		
	8 h daily									
2984	8	18	20	1.1	18	21	1.2	1.62	1.77	1.1
2975	21	16	26	1.6	22	31	1.4	1.52	1.30	0.9
2973	23	19	29	1.5	23	31	1.4	1.48	1.48	1.0
2964	23	18	30	1.7	20	29	1.4	1.74	1.61	0.9
2963	31	18	28	1.6	22	31	1.4	1.61	1.71	1.1
2960	39	18	30	1.7	19	30	1.6	1.73	1.21	0.7
2962	43	20	29	1.5	22	28	1.3	1.69	1.87	1.1
2961	60	23	36	1.6	25	33	1.3	2.32	1.77	0.8
	24 h daily									
3013	3	20	23	1.2	26	30	1.2	–	–	–
2982	13	20	31	1.6	24	34	1.4	1.54	1.52	1.0
2976	21	20	44	2.2	24	42	1.8	1.99	1.10	0.6
2977	39	21	46	2.2	23	42	1.8	1.85	1.02	0.6
3007	56	19	84	4.4	22	78	3.6	3.88	3.39	0.9
3002	62	24	62	2.6	26	60	2.5	3.40	2.19	0.6

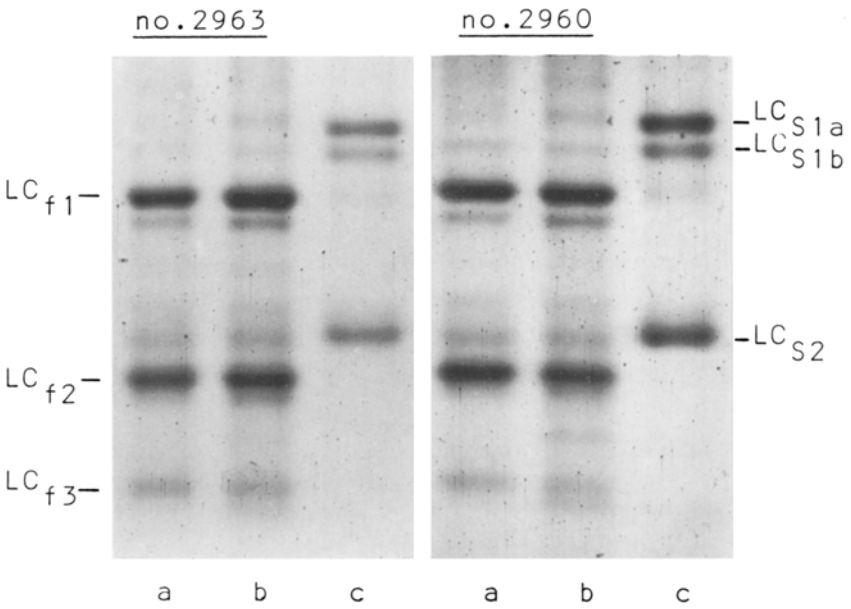

Fig. 1
Polyacrylamide gel electrophoresis of myofibrillar proteins extracted from control (a) and intermittently (8 h daily) stimulated (b) tibialis anterior muscles and soleus muscle (c) of rabbit no. 2693 (stimulated during 31 days) and rabbit no. 2960 (stimulated during 39 days). LC_{f1}, LC_{f2}, LC_{f3} denote the myosin light chains of the fast type, LC_{S1a}, LC_{S1b} and LC_{S2} those of the slow type. Increase in isometric twitch contraction time of stimulated tibialis anterior muscles was 1.7 fold in rabbit no. 2960 and 1.6 fold in rabbit no. 2963

anterior muscle from the same animals (not shown) although the increase in contraction time was of the same order (compare Table 1, no. 2961).

It is possible that the initial change in time to peak of the isometric twitch contraction is not associated with a decrease in velocity of shortening, but with a longer duration of the "active state" during a single contraction. If this was the case, then the tension produced during a single contraction relative to the maximal tetanic tension ought to be greater. To test this, tension during a single contraction (twitch tension) was related to maximal tetanic tension which was produced by stimulating the muscle at 80 Hz. This ratio was consistently higher in the stimulated muscle, confirming earlier findings (Salmons and Vrbová, 1969). Thus in the stimulated muscles a larger proportion of the total tetanic tension is produced during a single twitch and this may be reflected in the increase of time to peak. In some experiments however the increase of the time to peak was greater than the increase

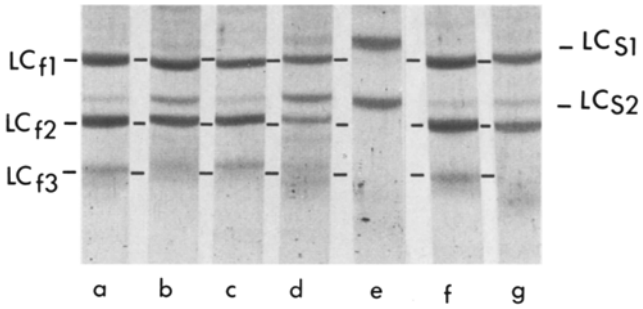

Fig. 2a–g. Polyacrylamide gel electrophoresis of myofibrillar proteins extracted from control and stimulated tibialis anterior and extensor digitorum longus muscles from a 21 days continuously stimulated rabbit (no. 2976) and a 60 days intermittently stimulated rabbit (no. 2961). (a) control tibialis anterior (no. 2976); (b) 21 days continuously stimulated tibialis anterior (no. 2976); (c) control extensor digitorum longus (no. 2976); (d) 21 days continuously stimulated extensor digitorum longus (no. 2976); (e) normal rabbit soleus; (f) control extensor digitorum longus (no. 2961); (g) 60 days intermittently stimulated extensor digitorum longus (no. 2961). LC_f and LC_S denote the different myosin light chains of the fast and slow type

in twitch/tetanus ratio. This can be seen in Figure 3 where the increase in time to peak of the stimulated muscles is plotted against the twitch/tetanus ratio. In muscles where time to peak increased more than twitch/tetanus ratio (above interrupted line in Fig. 3) a change in force-velocity relationship may have occurred. It is interesting that with the exception of 2 muscles. a change in myosin light chains was found only in the muscles where the increase in time to peak exceeded the increase in twitch/tetanus ratio. It could be that a change of myosin light chains occurs only when the force-velocity relationship is altered. However in the absence of direct measurements this is only a suggestion.

Figure 4b shows the electrophoretic analysis of myosin light chains in a muscle which was stimulated continuously for 62 days. It is evident that in this muscle high concentrations of the slow type light chains LC_{S1} and LC_{S2} are present and a decrease in the fast type light chain LC_{f3} is also noticeable. No qualitative changes are, however, seen in the bands of fast type light chains LC_{f1} and LC_{f2} although it appears that the intensity of the LC_{f2} band has slightly decreased. In this case the myosin light chain pattern resembles that of a muscle with a mixed population of fast and slow fibres. This is also supported by histochemical findings obtained from the same muscles (cf. Table 2, rabbit no. 3002).

Only in one case, a nearly complete transformation of the fibre population was observed (cf. Fig. 6, Table 2, rabbit no. 3007). In this animal, which was stimulated continuously for 56 days, increases in time to peak of isometric twitch contraction amounted to 4.4 and 3.6 fold in tibialis anterior and extensor

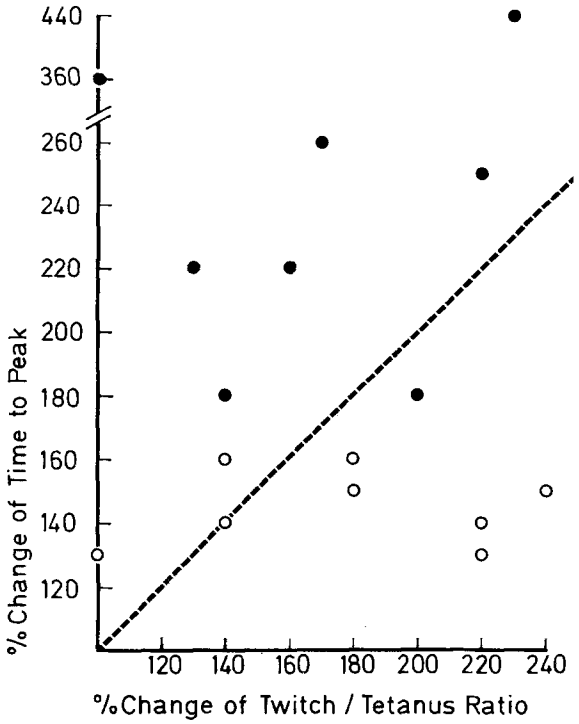

Fig. 3. The percentage increase in time to peak of stimulated muscles is plotted against the percentage increase of twitch/tetanus ratio. (●) continuously stimulated muscles, (○) intermittently (8 h a day) stimulated muscles. The interrupted line indicates the values at which the increase in time to peak would be the same as that of the twitch/tetanus ratio

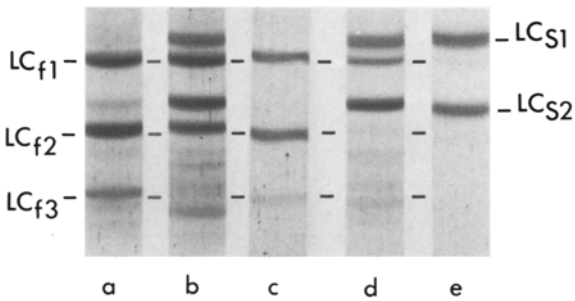

Fig. 4a–e. Polyacrylamide gel electrophoresis of myofibrillar proteins extracted from control and continuously stimulated tibialis anterior muscles of two rabbits with different degree of fast to slow type fibre transformation (cf. Table 2 and Fig. 6) after comparable periods of stimulation. Rabbit no. 3002 was stimulated for 62 days and rabbit no. 3007 for 56 days. (a) control tibialis anterior (no. 3002); (b) 62 days continuously stimulated tibialis anterior (no. 3002); (c) control tibialis anterior (no. 3007); (d) 56 days continuously stimulated tibialis anterior (no. 3007); (e) normal rabbit soleus muscle. LC_f and LC_S denote the different myosin light chains of the fast and slow type

digitorum longus respectively. In this case the myosin light chain pattern can be distinguished from that of a soleus muscle only by the persistence of the slowest moving fast type light chain LC_{f1}. The two other light chains LC_{f2} and LC_{f3} can no longer be detected in this stimulated muscle.

Table 2. Quantitative evaluation of fibre typing according to histochemical staining of myofibrillar ATPase after acid (pH 4.1) preincubation of whole cross sections of control (c) and stimulated (st) tibialis anterior muscles in long-term continuous (24 h daily) stimulation experiments of various periods of time. Dark (n_D), intermediate (n_I) and light (n_L) fibres were distinguished. In each case parallel sections were also stained for ATPase after alkaline (pH 10.4) incubation

Rabbit No.	Time to peak [ms]	n_D	%	n_I	%	n_L	%	$n_{total} = 100\%$
2976	c 20	782	2.6	78	0.3	28832	97.1	29692
	st 40	1262	4.4	2804	9.8	24710	85.9	28776
2977[a]	c 21	793	2.9	32	0.1	26605	97.0	27430
	st 46	2232	8.1	3407	12.3	21990	79.6	27629
3002	c 24	893	2.9	86	0.3	29645	96.8	30624
	st 62	2563	8.7	2604	8.8	24296	82.5	29463
3007[b]	c 19	394	1.5	115	0.4	26695	98.5	27104
	st 84	0[c]		25327	98.1	482	1.9	25809

[a] cf. Figure 5
[b] cf. Figure 6
[c] no truly dark fibres as in the control muscle were observed

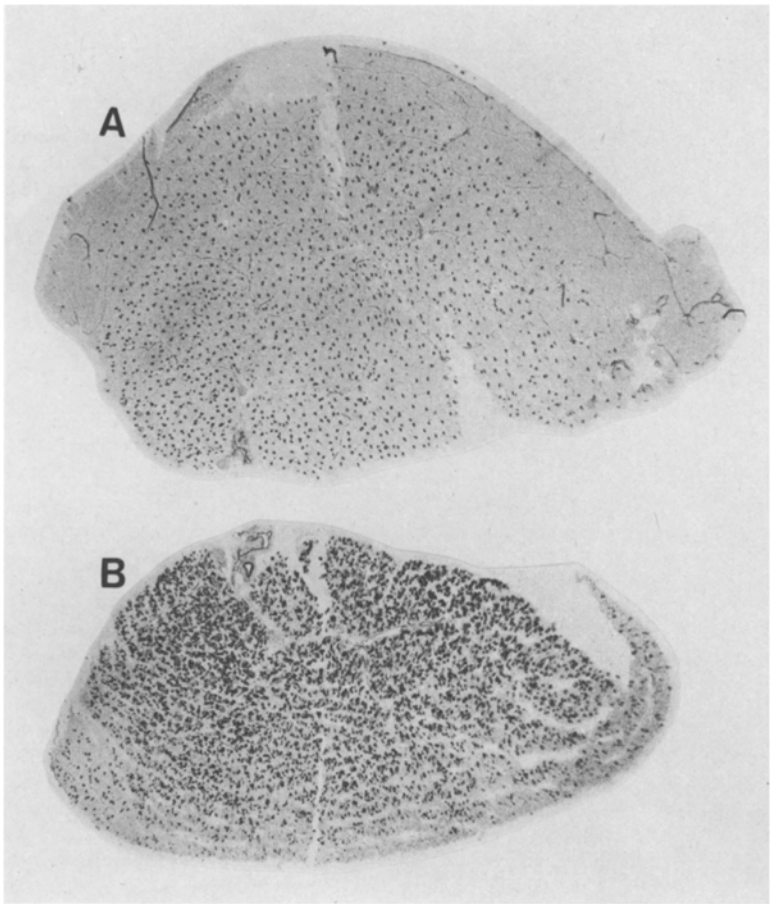

Fig. 5A and B
Cross-sections of control (A) and 39 days continuously (24 h daily) stimulated (B) tibialis anterior muscles (rabbit no. 2977). Sections were stained for myofibrillar ATPase after acid preincubation (× 7). Number and percentage of different fibres are given in Table 2

Histochemical Evaluation of Whole Cross-Sections

Whole cross-sections of control and 39 days continuously stimulated tibialis anterior muscle are shown in Figure 5. The sections were stained for myofibrillar ATPase after acid preincubation. The number of dark stained fibres has increased significantly in the stimulated muscle. This finding is substantiated by the quantitative data given in Table 2. Evaluation of the histochemical changes in the tibialis anterior muscles of

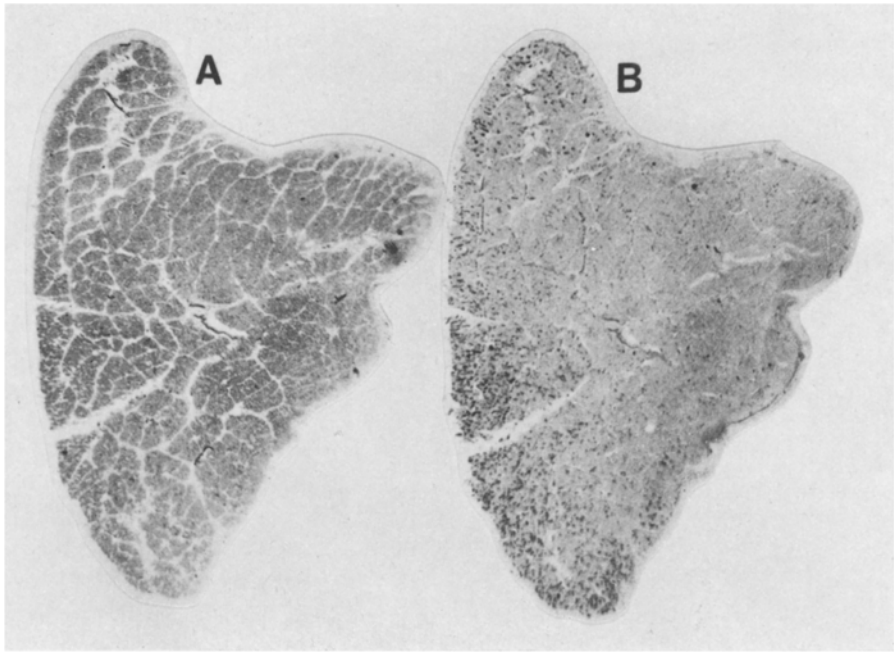

Fig. 6A and B
Serial cross-sections of 56 days continuously (24 h daily) stimulated tibialis anterior muscle (rabbit no. 3007). Sections of whole muscle were stained for myofibrillar ATPase after acid (A) or alkaline (B) preincubation (×7). Number and percentage of different fibres are given in Table 2

four rabbits revealed a significant increase in the number of dark fibres. This increase was 2–3 fold in 3 of the animals studied (no. 2976, 2977, 3002). In these animals an even more pronounced increase was seen in the number of fibres which stained intermediately. Only a few of these fibres are found in control muscles, whereas up to 10% of these fibres could be found in the stimulated muscles. The transformation of the fibre population is most obvious in rabbit no. 3007, in which a nearly complete transformation of the fibre population has been induced. The percentage of intermediately staining fibres has increased to 98% (Fig. 6a).

Table 1 shows that the continuously stimulated muscles lose much weight. In view of this it is remarkable that the total number of fibres has remained constant in these continuously stimulated muscles. This finding suggests that the loss of weight of the stimulated muscles is due to a decrease in the mean fibre diameter. The smaller cross-sectional area of the stimulated muscles found in the present experiments (cf. Fig. 5) is also a reflexion of the decrease in fibre diameter.

The loss of weight was negligible in the intermittently stimulated muscles; the weight of the stimulated muscle being on average 0.95 ± 0.15 that of the control muscle (see Table 1). After 3 weeks of intermittent stimulation it was found that the fibres tended to become smaller and more uniform in size (Pette et al., 1973, 1975). After long-term continuous stimulation, on the other hand, a heterogeneity of the fibre population was found in some cases. Not only was the fibre size different, but also the mitochondrial content of individual fibres. Most fibres had a high activity of succinate dehydrogenase, but among them pale fibres were also seen, and these were characterized by the ATPase reactions as fast.

Changes of Enzymes Involved in Energy Metabolism

Changes in the activity levels of a few selected enzymes in energy metabolism are summarized in Table 3. It appears that stimulation induced similar changes of the enzyme activity pattern no matter whether the muscles were stimulated intermittently or continuously. Changes in the enzyme activity pattern occur already after 4 days of intermittent stimulation and reach their maximum after 2–3 weeks (Pette et al., 1973). Thus changes of enzymes involved in energy metabolism are induced much earlier than changes in myosin light chains and may not depend on a change of force-velocity relationship.

The present experiments suggest that prolonged periods of intermittent stimulation do not lead to essentially greater changes than observed already after 3 weeks (Table 3). Continuous stimulation, on the other hand, induced changes which increase to some extent with time. This is suggested for the decrease in glyceraldehydephosphate dehydrogenase and mitochondrial glycerolphosphate dehydrogenase. The ratio of these two enzymes remains thus rather unchanged which supports the concept of this constant proportion group (Pette and Bücher, 1963; Pette, 1966).

The metabolic transformation of the stimulated muscles is mirrored even more clearly by the changes in the activity ratio of glyceraldehydephosphate de-

Table 3. Relative activity levels of selected cytosolic and mitochondrial enzymes of energy metabolism in intermittently (8 h daily) and continuously (24 h daily) stimulated tibialis anterior muscle after various periods of time. Enzyme activities of the stimulated muscle have been referred for each animal to the respective enzyme activity in the contralateral control muscle

Rabbit No.	Days of stimulation	Glyceraldehyde-P dehydrogenase	m-Glycerol-P dehydrogenase	Succinate dehydrogenase	Citrate synthase	3-Hydroxyacyl-CoA dehydrogenase	Hexokinase
	8 h daily						
2975	21	0.77	0.79	3.1	3.3	3.6	–
2964	23	0.81	1.20	3.2	3.5	4.5	4.5
2973	23	0.68	1.00	3.6	3.8	4.9	5.7
2963	31	0.78	0.75	3.1	4.1	4.3	4.8
2960	39	0.81	0.77	1.8	5.1	4.2	3.2
2962	43	0.83	–	2.8	3.3	4.1	2.4
2961	60	0.73	0.46	3.0	6.4	6.6	5.2
	24 h daily						
2982	13	0.84	0.77	1.4	1.8	3.2	8.1
2976	21	0.34	0.38	4.1	4.3	5.3	8.2
2977	39	0.41	0.83	3.1	2.9	4.5	4.0
3005	52	0.29	0.50	2.6	3.7	5.8	8.9

Table 4. Enzyme activity ratios in control and stimulated tibialis anterior and extensor digitorum longus muscles. Data are given for intermittent (8 h daily) and continuous (24 h daily) stimulation at various periods of time

Rabbit No.	Days of stimulation	Glyceraldehyde-P dehydrogenase / Citrate synthase				Succinate dehydrogenase / m-Glycerol-P dehydrogenase			
		Tibialis anterior		Extensor digitorum longus		Tibialis anterior		Extensor digitorum longus	
		control	stimulated	control	stimulated	control	stimulated	control	stimulated
	8 h daily								
2975	21	115	27	111	35	1.96	7.68	2.69	6.19
2964	23	126	29	180	39	2.51	4.50	1.62	3.44
2973	23	125	23	134	27	2.29	8.33	4.46	6.79
2963	31	109	21	99	22	2.57	10.67	2.44	7.12
2960	39	170	28	162	31	–	–	–	–
2962	43	129	32	117	38	–	10.97	1.35	4.30
2961	60	150	17	138	20	3.23	20.67	3.33	14.00
	24 h daily								
2982	13	121	55	146	82	3.00	5.24	2.23	3.46
2976	21	103	8	151	16	1.85	15.97	1.04	4.78
2977	39	173	25	157	20	2.74	10.23	2.46	15.49
3005	52	120	9	211	20	3.52	18.44	2.99	13.78
Mean ± S.D.		131 ± 24		146 ± 32		2.6 ± 0.6		2.5 ± 1.0	

hydrogenase/citrate synthase (Table 4) reflecting the ratio of anaerobic to aerobic capacities as well as that of the two structure bound mitochondrial dehydrogenases of succinate and glycerolphosphate. These represent a measure of mitochondrial differentiation (Pette, 1966; Golisch et al., 1970). The changes in the latter ratio are comparable in intermittently and continuously stimulated muscles suggesting a similar transformation of the mitochondria. In 2 of the 4 animals studied the shift in the ratio glyceraldehyde-phosphate dehydrogenase/citrate synthase is greater in the continuously than in the intermittently stimulated muscles.

DISCUSSION

The results of this study and a preceding one (Pette et al., 1975) show that pronounced increases in time to peak of isometric twitch contraction can be induced in fast muscles without demonstrable changes in myosin light chain pattern or in the distribution of histochemically "fast" and "slow" fibres. Transformation of the fibre population as well as of the myosin light chain pattern was observed only if the muscles were subjected to prolonged periods of intermittent stimulation. Continuous stimulation, however, produced these effects much earlier.

During the early period of stimulation the twitch to tetanus ratio increased together or even more than the time to peak of isometric twitch contraction. The increase in time to peak in those experiments can therefore be explained by a slower reaccumulation of Ca^{2+}. Consistent with this are the early changes in the protein pattern and ATPase activity of the sarcoplasmic reticulum in the stimulated muscles (Ramirez and Pette, 1974). These are apparent soon after stimulation started and occur long before the transformation of the myosin light chain pattern. It remains an open question whether the changes of the sarcoplasmic reticulum may explain sufficiently the early changes in contractile properties. The same applies when the relatively early changes in mitochondria are considered (Pette et al., 1972, 1973). The role of mitochondria in red muscle and in heart in participating in segregation and release of Ca^{2+} has been emphasized recently (Lehninger, 1974). Since there is a pronounced increase in mitochondria in the stimulated muscle it appears probable that this change influences the regulation of free Ca^{2+} in a similar fashion as that proposed for mitochondria in slow twitch ("red") muscle. Since the enzymatic equipment of the mitochondria in the stimulated fast muscle becomes to resemble that of a slow ("red") muscle (Table 3 and 4) it is suggested that there is in addition to the quantitative change of mitochondrial content also a qualitative change.

Muscles where the pattern of myosin light chains was altered had also an increased number of fibres which could be classified histochemically as slow. The increase of the dark fibres as well as the appearance of fibres with an intermediate intensity reaction for myofibrillar ATPase suggest that synthesis of slow type myofibrillar proteins has been induced. This synthesis was also shown by the change in the myosin light chains. The histochemical data suggest that the synthesis is confined to certain fibres. Only in animal no. 3007, there was a nearly complete (98%) transformation of the fibre type (Fig. 6). In this case the pattern of myosin light chains also appeared to be completely transformed as judged by the marked increase in the slow type light chains LC_{S1} and LC_{S2} (Fig. 4d). This transformation is of special interest: The staining of the fibres was intermediate rather than dark and moreover there was a persistence of the fast type light chain LC_{f1}. The intensity of this band is so strong that it cannot be accounted for by the persistence of fast fibres. This finding suggests that myofibrillar proteins of the slow and fast type are synthetized simultaneously in the transformed fibres.

It appears that genes which are responsible for the synthesis of the slow type of myosin have been activated atypically by the stimulation procedure within fibres that already synthetize the fast type of myosin. These fibres thus represent a true intermediate type of fibres which are known to occur in a small percentage also in the respective control muscles and have not yet been characterized with regard to their myosin light chains because of their small number.

The extent of the transformation of the fibre population in the different animals varied. The reasons for such variations are not clear. It may be that the position of the implanted electrodes was such that not all the fibres were stimulated all the time. The variation may also be caused by the time needed in different muscle fibres to induce a change. Finally, the question arises why the change of myosin light chains occur only after the muscle had been stimulated for longer, or more vigorously. The present results do not provide an answer. It may be that the relatively slow turnover rates of the myofibrillar proteins (Millward, 1970; Kimata and Morkin, 1971; Low and Goldberg, 1973; Koizumi, 1974) as compared to sarcoplasmic proteins (Dölken and Pette, 1974; Dölken et al., 1974; Pette and Dölken, 1975) are the limiting factor. This, however, cannot be the only reason, for the same degree of transformation of myosin light chains was found to take place in 21 days of continuous stimulation as after 60 days intermittent stimulation. It is possible that the change of the "internal environment" of the muscle cell must reach a certain point before the gene controlling the synthesis of structural proteins is affected.

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