Low Frequency Nerve Stimulation of Rat EDL Muscle: Morphology of Myofibers and Neuromuscular Junctions.

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Abstract

Myofibers and neuromuscular junctions (NMJs) of rat EDL muscle undergo structural reorganization following long term continuous stimulation at low frequency. By light and electron microscope morphometry, the effects of imposed increased activity were studied after periods of stimulation ranging from 4 to 30 days. In the absence of muscle damage and regeneration even at early times after the sudden change in activity, the fiber size decreases by thirty percent, the capillary density almost doubles. The mitochondrial content in the myofibers increases up to levels several times higher than those in contralateral unstimulated EDL muscles. Z-bands thick and with a less regular course are common, while the narrow type has almost disappeared. Thus, within a month, the stimulated EDL muscle shows an almost homogeneous population of fibers in which morphological correlates of aerobic oxidative metabolism prevail. This demonstrates that, though the slow twitch myosin isoform is not expressed, the amount and type of imposed activity had profound effects onto fast fatigable EDL of adult rats and changed its ability to withstand fatigue.

Morphological analyses of the NMJ in the same stimulated muscles show that the junction is involved by dynamic not degenerative changes, among which repetitive cycles of sprouting and retraction of terminals prevail. Morphometric evaluations of pre- and post-synaptic structures reveal that several subcellular parameters are significantly changed. The number of synaptic vesicles and the portion of the primary groove associated with axon terminals decrease, while the spacing between openings of secondary folds and the fractional volume of synaptic mitochondria greatly increase. Besides further evidence of mature synapse plasticity, these results demonstrate that in response to long term continuous stimulation at low frequency the NMJ of adult rat EDL undergoes reorganization and adaptive changes to match newly acquired properties of the muscle fiber.

Key words: synapse remodelling; muscle transformation; electrostimulation; morphometry; light-electron microscopy.

BAM 1 (1): 71-81, 1991

Most skeletal muscles contain a mixture of fiber types among which three main types (1, 2A, 2B) can be distinguished on the base of their physiological, biochemical, histochemical and ultrastructural characteristics. In spite of this high degree of specialization, the fibers maintain a remarkable ability for responding specifically to changed pattern of use. It has been shown that fast-twitch muscle fibers are transformed into slow-twitch type when subjected to long term continuous electrostimulation at low frequency [28, 30]. On the other hand, it has been demonstrated that also the mature neuromuscular junction (NMJ) undergoes a life-long remodelling process and is influenced by a large number of factors [15, 36]. However, little attention has been so far paid to modifications occurring in NMJs of muscles subjected to long period of continuous increased use.

Fast-to-slow fiber type complete transformation in response to long term continuous stimulation at low frequency is also shown by the coordinated expression of slow-type contractile proteins in skeletal muscles of different animals [28, 30]. However the expression of slow-type myosin isoforms seems not to be easily induced in innervated fast-twitch muscles of adult rats by the same pattern of stimulation [23]. By light and electron microscopy we have therefore examined the structure of fast rat muscles following continuous (24 hs per day) low frequency (10 Hz) electrostimulation for periods of 4-30 days. Our principal interests have been structural changes occurring in myofibers and NMJs which could be related to metabolic and functional modulations in response to imposed increased activity.

Materials and Methods

Young adult male Wistar rats (60 to 80 days old) were used for experiments. Under thiopental and ether anaesthesia stimulating electrodes were implanted on the right sciatic nerve, near the trochanter. After 4 days pre-conditioning [22], low frequency (10 Hz) electrostimulation was continuously applied (24 hs per day). The animals were killed after 4, 10 and 30 days of continuous stimulation. Stimulated and contralateral unstimulated extensor digitorum longus (EDL) muscles were quickly removed and processed for transmission electron

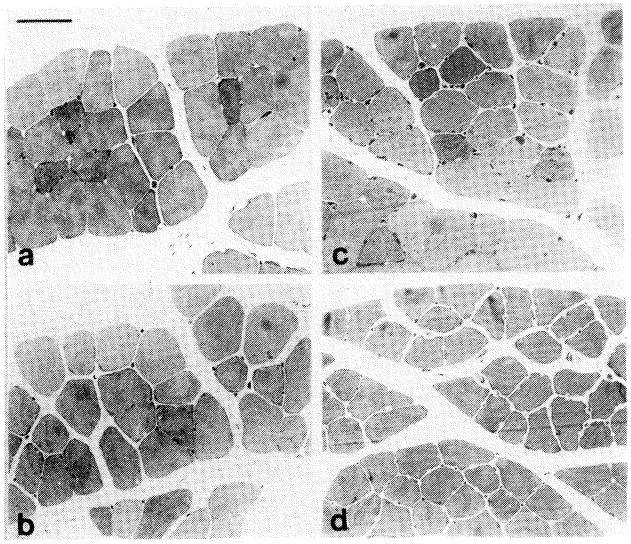

Figure 1 Light micrographs of semithin plastic sections from contralateral unstimulated muscle (a) and from muscles stimulated for 4 (b), 10 (c) and 30 days (d). Four and ten days after stimulation the muscle fibers appear almost unchanged with respect to contralateral unstimulated EDL, while at day 30 the fibers are smaller in diameter than those in control muscle. The increase in capillary density is already evident at day 10 and becomes pronounced at day 30. Scale bar: 50 μm.

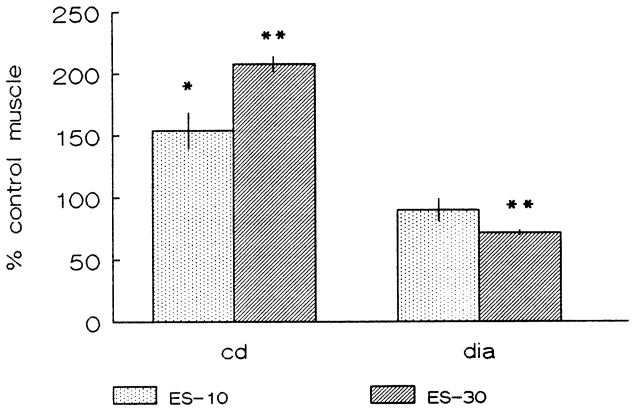

*Figure 2 Quantitative measurements of capillary density and diameter of fibers in continuously stimulated EDL muscle. The bar chart shows mean values for number of intrafascicular capillaries/muscle fiber (cd) and for diameter of fibers (dia). Column heights are expressed as percentage of contralateral unstimulated muscle. Bars represent standard error of the mean. Statistical level of significance, * $p < 0.01$, ** $p < 0.001$.*

microscopy. The procedures followed were essentially as in [24]. In addition, before embedding the middle part of each muscle (which is rich in neuromuscular junctions) was separated and cut in small fragments which all were then embedded in Epon mixture. Semithin sections (0.5-1.0 m thick) were stained with toluidine blue and studied by light microscopy. Ultrathin sections (silver to pale gold interference color) were stained with uranyl acetate and lead citrate and examined using a Philips EM 301 electron microscope. For enlargement calibration of each series of electron micrographs taken carbon grating replicas of cross line spacings were used which have a spacing of 2160 lines/mm.

Morphometry.

Six, five and three EDL muscles continuously stimulated for 4, 10 and 30 days respectively were used for quantitative analyses. Contralateral unstimulated EDL muscles (N= 14) were used as controls. From each experimental batch of samples, that is, from each group of embedded samples representing the complete transverse section of each experimental muscle, five to eight blocks were selected randomly and transversely cut. On light micrographs taken at constant magnification from semithin plastic sections stained with toluidine blue the number of intrafascicular capillaries per muscle fiber and the fiber diameter, that is the diameter of a circle the area of which is equivalent to fiber cross sectional profile, were determined.

For evaluations of mitochondria in the myofiber from each block ultrathin sections were made of a single area conventionally predetermined and corresponding to the upper left corner on the semithin section. Ultrathin sections were collected on single-hole membrane-coated grids. The profile of the fiber roughly lying in the center of the section and those of the four nearest fibers in orthogonal position were recognized at the electron microscope. From each fiber profile two micrographs were systematically taken at constant magnification (9.1 kX) from two different areas, corresponding respectively to the most central portion and to the most peripheral part in the upper left quarter of the fiber. Volume density, specific surface and numerical density of mitochondria were measured on micrographs printed at calibrated magnification. It is known that within a single fiber there are spatial gradients of structures and particularly of mitochondria which are more concentrated at the periphery of the fibers [10, 20, 21]. A region within 1.5 μm of the sarcolemma was then excluded from the remaining core of the fiber. Mitochondria in the outer 1.5 μm annulus were defined as any other one localized in this region. Furthermore, a second order of spatial gradient of structures was considered. In young adult rats the muscle fibers are densely packed with contractile filaments organized into myofibrils. Independently from fiber type, interfibrillar mitochondria are mainly concentrated at I-band level, where they run transversely to the main axis of the fiber and form a variably branched and complex network [21]. Mitochondria at A-band level, on the other hand, are almost parallel to the fiber axis and their concentration is related to the energy metabolism of the fiber and/or to type of fiber [31]. We were mainly interested to find out morphological correlates of changes in oxidative metabolism of muscle fibers. For this reason we decided to analyze by morphometry mitochondria localized at A-band level. From the core of the fiber A-band level mitochondria were defined as any which in transverse sections of muscle fibers were seen

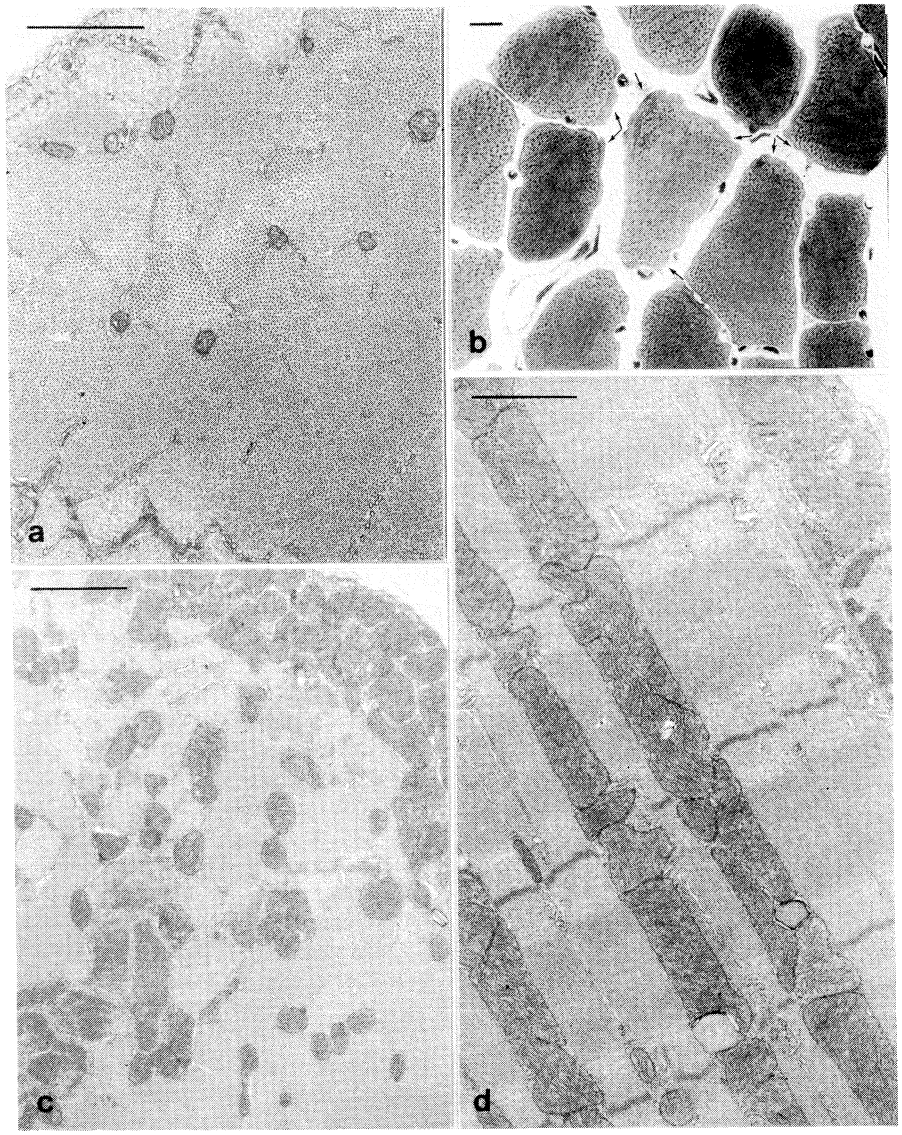

Figure 3 Light and electron micrographs of transverse and longitudinal sections from control (a) and 30 days stimulated muscle fibers (b, c, d). After stimulation the myofibers are homogeneously rich in mitochondria (b) also forming peripheral accumulations (arrows). By electron microscope mitochondria appear enlarged in size and number (c) and arranged in longitudinal rows (d). Scale bar: 1 μm (a, c, d) and 10 μm (b).

at A-band level surrounded for at least the two thirds of their sectional profile by transversely cut myosin thick filaments.

The same blocks which had been selected for measurements of mitochondria in the myofiber, each one containing several tens of muscle fibers, were used to analyze neuromuscular junctions. Each block was thoroughly examined, by cutting semithin and ultrathin sections at pre-determined different levels, thus realizing a sort of stratified random sampling [35]. Knowing the mean length of the synaptic region in rat EDL muscle [23], cutting levels were spaced 80-100 μm from each other. On each level ultrathin sections were cut of NMJs recognized on the semithin section by light microscopy and collected on single-hole membrane-coated grids. At the electron microscope, all nerve terminals present in the thinnest section were recorded at constant magnification (15 kX). Ultrastructural parameters were measured on electron micrographs printed at calibrated magnification and included: diameter of axon terminals (diameter of the circle of equivalent area), portion of the synaptic gutter covered by the terminal (percent length of the primary gutter in close unobstructed contact with the pre-synaptic membrane), interfold length (distance between openings of junctional folds), appositional membrane length (length of the pre-synaptic membrane as percent of the terminal circumference left uncovered by Schwann cell profiles), number of synaptic vesicles per unit area of the terminal. Furthermore, volume density, specific surface and numerical density of mitochondria contained in the nerve terminals were estimated in each nerve terminal observed.

Measurements were done using a semiautomatic image analyzer consisting of a computer-assisted tracing device equipped also with stereological functions [35]. Functions for evaluating

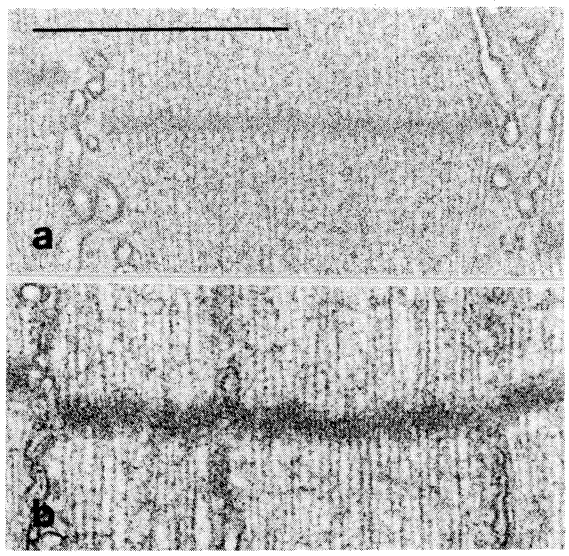

Figure 4 Electron micrographs showing Z-bands from unstimulated (a) and 30 days stimulated (b) fibers. Following long term stimulation the narrow type Z-band (a) typical of fast glycolytic EDL fibers is only rarely observed. The great majority of myofibers show thick and electron dense Z-bands. Scale bar: 1 μm.

volume and numerical densities and specific surface of particles in the test area were used in this study. The volume density is defined as the volume fraction of test objects per unit reference volume; similarly numerical density is defined as the number of test objects in the reference volume [35]. Measurements from both functions give dimensionless values and throughout this paper are also referred to as volume and number of test objects. Specific surface is given by the relation of surface of particles and volume of particles and therefore is inversely related to the radius of particles. For ease of representation the reciprocal values of measurements were computed and expressed in $\mu m 10^{-2}$. Due to the fact that measured objects were almost round shaped, specific surface is also referred to as size of test objects.

Data are expressed as mean ± SEM and/or percent frequency distribution. To test the significance of differences between control and stimulated muscle with regard to structural parameters measured, analysis of variance (ANOVA) was used.

Results

MICROSTRUCTURE OF STIMULATED MUSCLE FIBERS.

Overall changes.

EDL muscles of young adult rats were studied by light and electron microscope following continuous (24 hs per day) nerve stimulation at low frequency (10 Hz) for 4, 10 and 30 days. No evident degenerative changes were found at any stimulation time considered, though 30 days after stimulation the fibers appear atrophic (Fig. 1). By day 10 the increase in capillary density is obvious and becomes pronounced 20 days later (Fig.1c and d). Morphometric analyses demonstrate that the number of intrafascicular capillaries per muscle fiber has almost doubled (from 1.3 ± 0.1 in the control, up to 2.8 ± 0.1 at day 30) while the mean diameter of fibers is significantly reduced by 30% (Fig . 2). In parallel in the stimulated muscles there is a remarkable increment in mitochondria which can be well appreciated even by light microscopy (Fig. 3b). At the ultrastructural level mitochondria are numerous both in the core and at the periphery of stimulated fibers where they form large accumulations (Fig. 3c). In longitudinal sections interfibrillar mitochondria are mainly arranged in long and parallel chains (Fig. 3d). In addition, rare if any fibers are observed with narrow Z-bands similar to those present in the majority of contralateral unstimulated fibers (Fig.4a). After 30 days of continuous stimulation (Fig. 4b) Z-bands appear thick and with a less regular course. When determined on longitudinal sections as the length of the overlap of thin filaments from adjacent sarcomeres [29], the Z-band width was found to range between about 60 and 100 nm (80.8 ± 4.3 nm, the mean value).

Morphometry of mitochondria in stimulated muscles.

Changes in mitochondria following continuous stimulation have been analyzed by morphometry. On electron micrographs, volume and numerical density and specific surface of mitochondria were measured at A-band level in the core (Fig. 5A to D) and in the outer 1.5 μm annulus of fibers (Fig. 5A' to D') from contralateral unstimulated and 10- and 30-day stimulated muscles. As shown in Fig. 5, continuous nerve stimulation at low frequency remarkably increases the volume fraction of mitochondria in EDL muscle. In particular, 10 days after stimulation mitochondria localized at A-band level in the core of fibers increase to the same extent both in size and number (Fig. 5B and C) then they further increase in number 20 days later (Fig. 5C) when their mean fractional volume is more than three times higher than that in contralateral unstimulated muscle (Fig. 5D). Mitochondria localized at the periphery of fibers show a similar increment (Fig. 5A' to D') though this becomes evident at the longest stimulation time. In addition, a small fraction (about 15%) of the fiber population which in unstimulated muscles is distinguished by accumulations of large mitochondria in the outer annulus (Fig. 5A' to C') seems not to be particularly influenced by continuous stimulation.

MICROSTRUCTURE OF NMJs IN STIMULATED MUSCLES.

Overall changes.

A normal NMJ from a contralateral unstimulated EDL muscle is shown in Fig. 6a. The axon terminal is deeply lodged in the synaptic groove and contains a large number of clear vesicles and small rounded mitochondria [27]. In the synaptic space the openings of secondary folds are regularly spaced. As already reported for muscle fibers (see above), no evident degenerative changes were observed involving NMJs at any stimulation time considered. By electron microscopy, however, several abnormalities were evidenced. At day 10, small terminal profiles are mainly associated with large boutons or arranged in groups to fill up well developed post-synaptic sites. However by this time terminals covering only in part large primary gutters are found (Figs. 6b, c). At the longest stimulation time studied, the most common ultrastructural features are a widespread disproportion between the size of terminals and the width of gutters and a large variability in shape, size,

*Table 1. Ultrastructural parameters measured in NMJ profiles from unstimulated and from 10- and 30-day stimulated EDL muscles of adult rats. Values given are mean ± SEM. In brackets: number of muscles examined. Statistical level of significance, *p < 0.025 ; ⊕p < 0.01 ; **p < 0.001; [a]) see Materials and Methods for details*

Type of muscle	Nb of profiles	Profile [a] diameter (μm)	Part of groove associated with axon terminal (% lenght)	Junctional folds spacing (μm)	Appositional membrane (% length)	Number of clear vesicles (per μm^2)
Control	25 (14)	1.94 ± 0.2	90.8 ± 1.9	0.38 ± 0.03	59.3 ± 2.1	46.8 ±4.8
ES-10	52 (5)	1.48 ± 0.1*	83.8 ± 2.5*	0.47 ± 0.04*	52.2 ± 2.3	29.4 ± 3.4⊕
ES-30	78 (3)	1.42 ± 0.1*	70.8 ± 3.8**	0.61 ± 0.05**	58.7 ± 1.9	31.2 ± 3.3*

orientation and number of secondary folds (Figs. 6d, e, 7). Furthermore, a multilayered basal lamina is frequently observed surrounding redundant Schwann cell profiles (Fig. 7).

Morphometry of end plate parameters.

By morphometry, more subtle modifications were evidenced in stimulated NMJs. Several parameters were evaluated on pre- and post-synaptic components. As shown in Tab.1, continuous stimulation greatly influences NMJ morphology. All morphometric parameters, except the appositional membrane length, are significantly changed in respect to control muscles. In particular, the diameter of terminals and the number of clear vesicles contained are decreased by day 10 and do not recover thereafter. Changes of the portion of synaptic groove associated with the axon terminal and of the spacing between openings of junctional folds into the synaptic space increase with the increasing time from the onset of stimulation. In Tab. 2 are reported the results from the stereological analysis of mitochondria in the synapse. The volume density of mitochondria dramatically decreases by 50% at day 10 and then fully recovers, the decrease being related to the decrease in the specific surface (size/shape) of mitochondria which then undergo pronounced proliferation so their numerical density on area increases and accounts for the recovery in volume density.

Discussion

Myofibers transformation occurs in the absence of fiber necrosis and regeneration.

Our results show that continuous (24 hs per day) low frequency (10 Hz) nerve stimulation of rat EDL muscle transforms myofibers from fast fatigable to fatigue resistant and that such a transformation which is not supported by degeneration-regeneration events is paralleled by a pronounced remodelling of neuromuscular junctions.

A high increase in activity is imposed to fast muscles when 10 Hz continuous stimulation is applied which induces about 800,000 twitches per day, compared with the few thousands of contractions occurring in freely moving adult rats [16]. Nevertheless, light microscope examinations of EDL stimulated muscles did reveal neither a massive necrosis (myofibrils' coagulation and lysis, macrophage infiltration, etc.) nor the regenerative changes (myoblast proliferation and fusion, for-

*Table 2. Stereological analysis of mitochondria in nerve terminals from unstimulated and from 10- and 30-day stimulated EDL muscles of adult rats. Values given are mean ± SEM. Statistical level of significance: *p < 0.025 ; #p < 0.005 ; **p < 0.001. [a]) see Materials and Methods for details.*

Type of muscle	Number of profiles	Volume [a] density (per μm^2)	Specific surface ($\mu m 10^{-2}$)	Numerical density (Nb/μm^2)
Control	25	0.28 ± 0.03	6.7 ± 0.4	3.0 ± 0.5
ES-10	52	0.14 ± 0.03#	5.0 ± 0.5*	2.8 ± 0.6
ES-30	78	0.26 ± 0.03	4.6 ± 0.2**	5.2 ± 0.7*

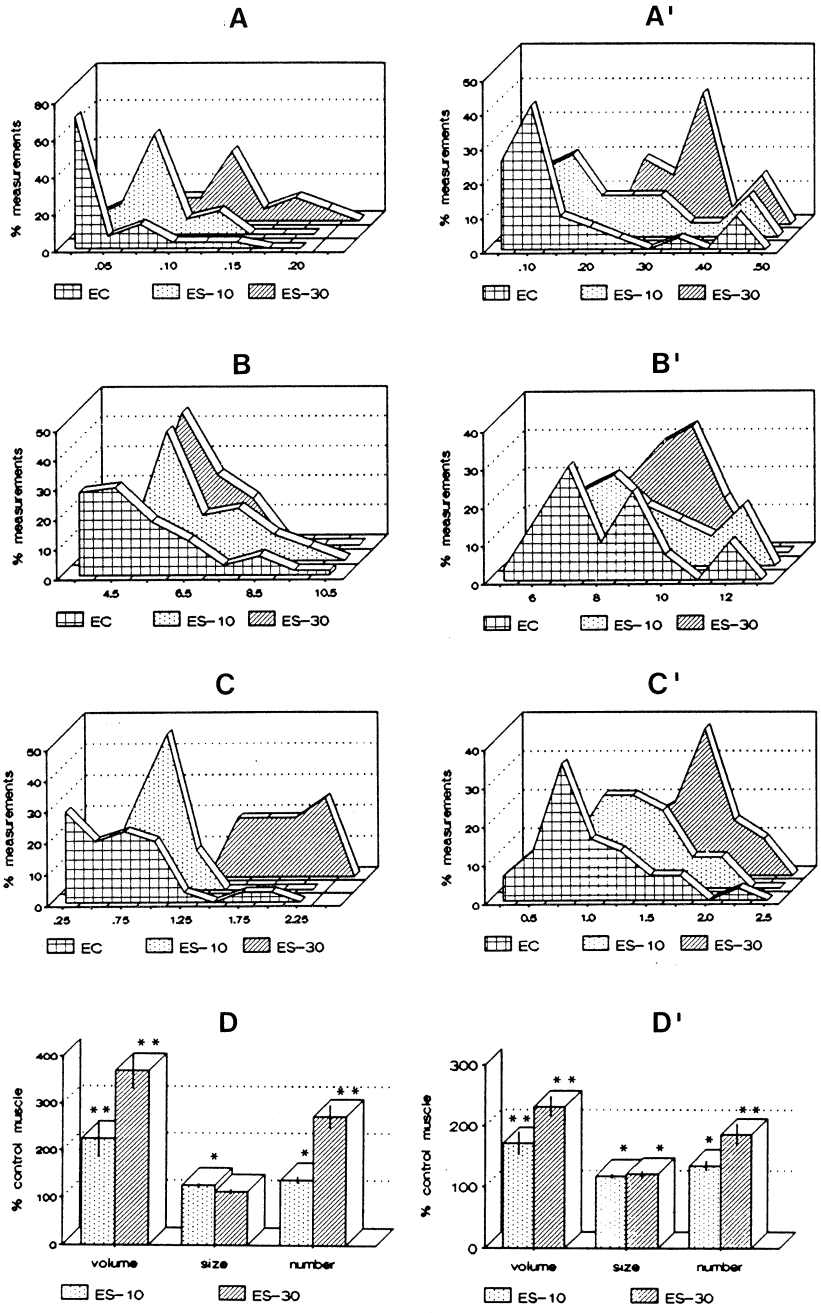

*Figure 5 Morphometric analysis of mitochondria in the core (A-D) and in the outer 1.5 μm annulus (A'-D') of fibers from control and stimulated muscles. Percent distribution of volume density (A, A'), specific surface (B, B') and numerical density (C, C') measurements obtained from contralateral unstimulated muscles and from muscles continuously stimulated at low frequency for 10 and 30 days. Bar charts in D and D' show mean values for volume, size (specific surface) and number of mitochondria in the core and in the outer annulus of fibers respectively. Column heights are expressed as percentage of contralateral unstimulated muscle. Bars represent standard error of the mean. Statistical level of significance, * $p < 0.01$, ** $p < 0.001$.*

mation of myotubes) which would be expected if modulations described depended upon loss of type 2B fibers and proliferation of type 2A. Furthermore, careful electron microscope examinations of stimulated myofibers did not reveal duplications of their basal lamina. A double layer of basal lamina around myofibers has been proved to be a reliable marker for cell replacement during nerve and muscle degeneration and regeneration [24, 26]. The newly formed cellular elements develop and grow inside the original basal lamina sheet. This one persists long after a new sheet has formed in close contact with the regenerated myofiber. The old basal lamina is easily recognizable as an outermost and redundant layer often decorated by peculiar membranous inclusions and it is commonly present after a single [24] as well as after repetitive episodes of injury and regeneration (personal observations). Thus it seems evident that the widespread structural modulations and se-

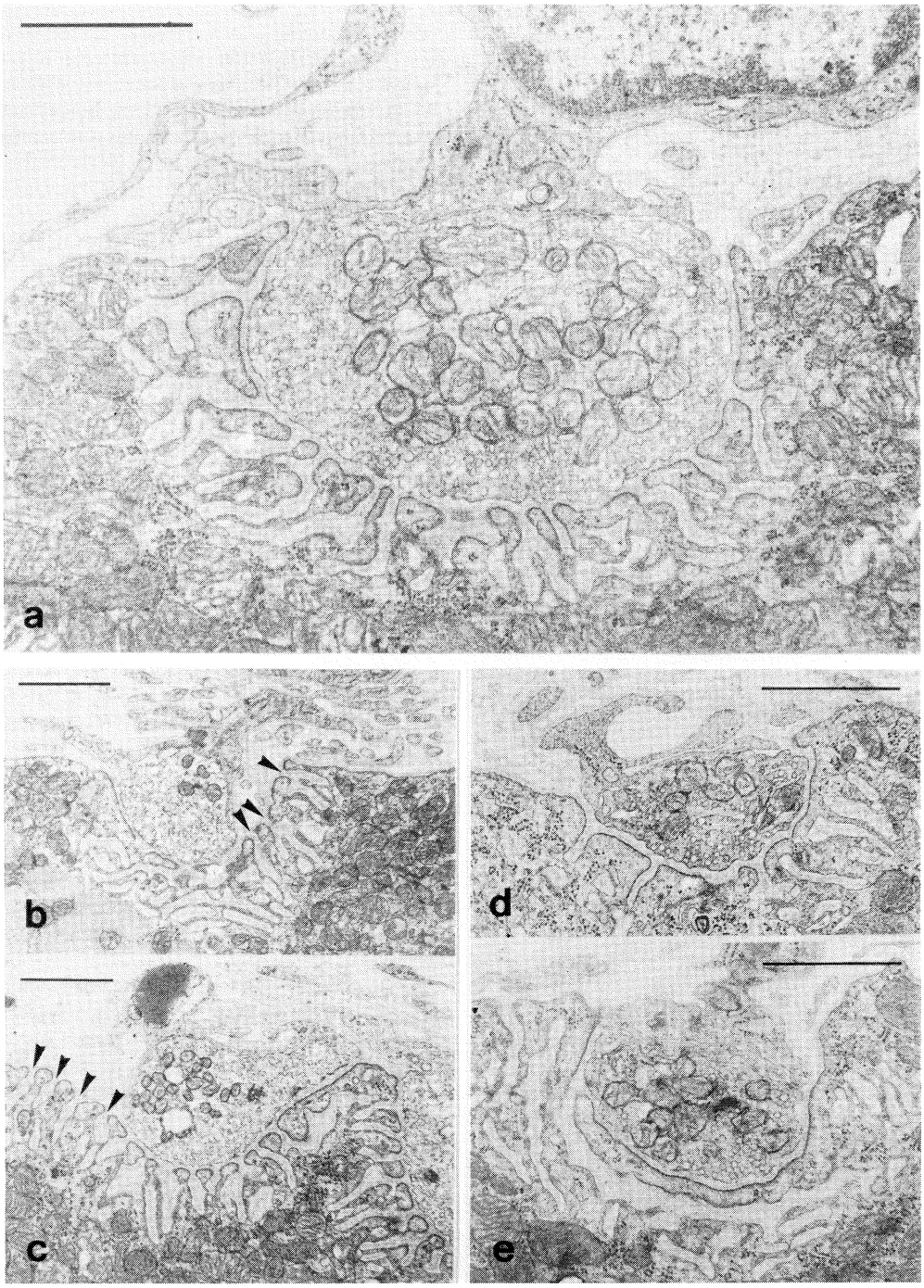

Figure 6 Transverse sections through NMJs from unstimulated (a) and from 10 (b, c) and 30 days (d, e) stimulated EDL muscles. In control muscles (a) the axon terminals completely fill deep synaptic grooves; the junctional folds are numerous and regularly spaced. After stimulation, the axon terminals cover only in part the synaptic gutter (b,c) and areas of junctional folds are not associated with axon terminals (arrow heads). Note in d) and e) the great variability in shape, size, orientation and number of junctional folds. Scale bar: 1 µm.

quence of changes here reported occurred in pre-existing cellular elements and are related to continuous stimulation of the motor nerve.

Transformation of EDL myofibers from fast fatigable to fatigue resistant type.

Rat EDL is a fast twitch muscle which contains a mixture of type 2 muscle fibers, besides a very minor proportion of type 1 fibers. When judged on the base of myosin heavy chains (MHC) identified by gel electrophoretic analyses [5] or by

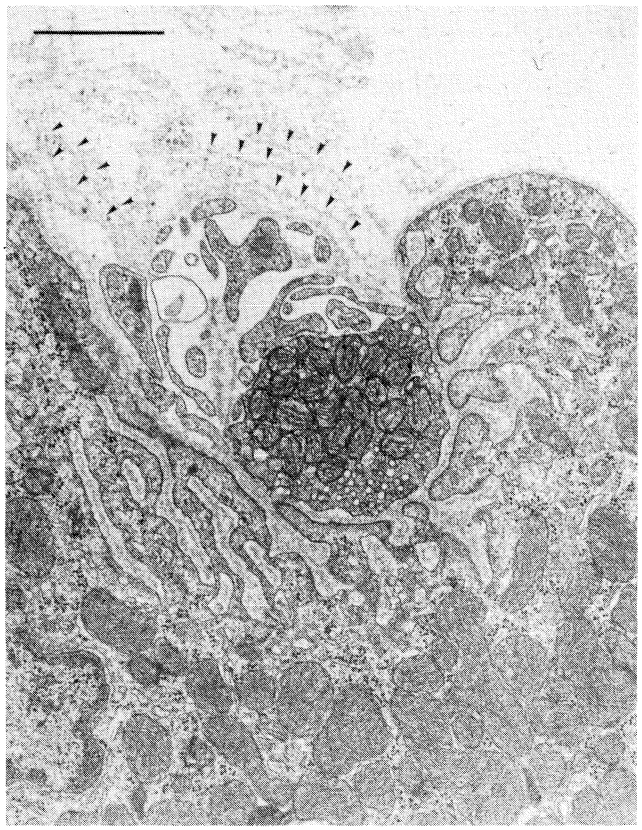

Figure 7 Transverse section of a NMJ from a 30-day stimulated EDL muscle. The synaptic groove is only partially occupied by the nerve terminal. Note the fragmented Schwann cell cytoplasm and the surrounding multilayered basal lamina (arrow heads). Scale bar: 1 μm.

immunologic approach [2] the great majority of EDL fibers are found to belong to type 2B, while a minority (about 25%) are of type 2A. On a morphological point of view, besides by well developed sarcoplasmic reticulum and T-tubules, type 2A muscle fibers of rat EDL muscle (as well as those in other fast or mixed muscles like tibialis anterior and diaphragm muscle) are characterized by thick and broad Z-bands and a rich complement of mitochondria which form accumulations at the periphery of fibers and longitudinal rows among the fibrils. In other words, type 2A fibers in fast twitch rat muscles have an oxidative-glycolytic metabolism and, though fast contracting and relaxing, are able to sustain a continuous exercise. In control EDL muscles these fibers have a smaller diameter and are well recognizable among larger ones even on semithin plastic sections viewed by light microscope (Fig. 1a). At least in part they participate to the formation of frequency peaks relative to higher values for mitochondrial parameters (fractional volume, size and number) measured in unstimulated EDL muscle and shown in Fig. 5. Following 30 days of continuous stimulation at low frequency the morphology of muscle fibers appears modified at either light and electron microscopy. The increased homogeneity of fiber population (Fig. 1d) is due partly to the nearly 30% decrease in diameter and in part to the great mitochondrial increment (Fig. 3b) which levels off morphological differences between mitochondria-rich and mitochondria-poor muscle fibers [31]. Indeed, morphometry on electron micrographs demonstrates that changes of fractional volume of A-band level mitochondria in the core of fibers attain levels more than three times higher than in contralateral unstimulated muscles (Fig. 5D). When other stereological parameters are estimated it becomes evident that the enlarged fractional volume at day 10 is related to an increase in size (specific surface) and in number (numerical density). During the following 20 days of stimulation, mitochondria enormously proliferate, without changing their size. In the same time, the fractional volume and number as well as the size of mitochondria increase also at the periphery of fibers. All parameters change significantly both at day 10 and 30 (Fig. 5D'), however the extent level of changes attained at the longest stimulation time is lower than that found in the core of fibers. In addition, proliferation seems to prevail. This could indicate that there are migration currents of mitochondria from the periphery to the core of the fiber.

Undoubtedly, long term continuous stimulation at low frequency produced great effects on the morphology of rat EDL muscle fibers which at day 30 have acquired a mitochondrial complement comparable or even larger than the one shown for slow twitch muscle fibers [10-13]. Taking into account that the oxidative capacity of a muscle is in direct proportion to the total volume of mitochondria contained [32] it becomes obvious that stimulated rat EDL fibers, having enormously increased their energy metabolism, had undergone transformation into fatigue resistant fibers. The parallel vascular development (Fig. 2) is in keeping with these events as well as with the modification in Z-band width (Fig. 3). Though an extensive analysis of it has still to be performed, the range of the Z-band width we have measured in 30-day stimulated fibers of rat EDL is similar to that in red vastus muscle of guinea pig [10, 12] and almost coincides with that measured in rabbit tibialis anterior muscle after fast-to-slow transformation by low frequency electrostimulation [29]. Furthermore, when our qualitative and quantitative morphological changes are compared to those reported for transformed rabbit muscle [13, 29] striking similarities are found particularly in the increment of mitochondrial volume and Z-band width.

It has been conclusively demonstrated that continuous low frequency nerve stimulation transforms a fast into a slow muscle in chicken [19], rabbit [30, 33], dog [1], goat [9], sheep [7] and man [9]. This has been proved not only by morphological, histochemical and physiological parameters, but also by molecular analysis of contractile proteins [28, 30]. By gel electrophoretic analyses of myosin heavy chains it has been shown that after 30 days of continuous stimulation at 10 Hz in both EDL and tibialis anterior of the rat there is a dramatic disappearance of MHC 2B which is fully substituted by MHC 2A, but not by MHC 1 [23]. This demonstrates that fast rat muscles following stimulation have changed their pattern of myosin expression, but in a way which contradicts the basic hypothesis of the fundamental importance played by the extent of imposed activity in the modulation of slow type myofiber characteristics [28]. A tentative explanation is that the rat shows a peculiar species specific inability of fast type myofibers to express type 1 myosin [2]. On the other hand, it has been demonstrated that MHC 1 accumulates in denervated muscle

fibers of adult rat EDL in response to long term direct stimulation at low frequency [6]. A massive necrosis and regeneration of myofibers were observed in these experiments [22]. It remains therefore an open question if muscle regeneration is responsible for the controversial effects of low frequency continuous electrostimulation on myosin expression in rat fast muscles.

Low frequency continuous stimulation alters the remodelling process of synapse in rat EDL muscle.

Nerve terminal sprouting and new synapse formation as well as terminal retraction are typical signs of synapse remodelling which occurs in vertebrate muscles under physiological conditions, without direct functional consequences [36]. In rat soleus muscle ultrastructural evidences of NMJ reorganization are reported as widespread during the growth period from three to five months of age [4]. During this same period synapse remodelling is less frequently observed in gastrocnemius and is not evident in diaphragm and EDL muscles of rats. The different occurrence of the process is very likely related to rising workload imposed to postural muscles by continuous body growth [4]. One or more features of synapse remodelling were seen in nearly 90% of NMJs from both 10- and 30-day stimulated rat EDL muscles. This demonstrates that continuous low frequency nerve stimulation greatly influences the "normal physiological" process and that the modifications we reported are neither transient ones nor only related to the sudden change in activity.

Signs of synaptic remodelling in stimulated NMJs.

In the absence of evident degeneration long term continuous stimulation induces in NMJs of rat EDL morphological modifications which are typical as signs of synapse remodelling and might have functional consequences also because of their intensity and widespread occurrence. One of the most striking features of the long term (30 days) stimulated NMJ is the disproportion between size of the terminal and width of the primary gutter. In other words, the majority of axon terminals are small and associated with large synaptic grooves. This is shown with morphometry as a significant decrease in the mean diameter of profiles and in the portion of the groove covered by the nerve terminal (Tab.1). The extent of change of the last parameter is much greater at 30 than at 10 days after stimulation. Taking into account that the diameter of terminals does not change significantly between 10 and 30 days, this difference could indicate that the synaptic contact is progressively reduced and the terminal is going to be withdrawn from the junction. Such a regressive phenomenon is obvious during aging. In this case the Schwann cell cytoplasm progressively envelopes the terminal, protruding into the synaptic cleft which, in parallel, widens [14]. As a consequence, there is an increasing reduction of the neuronal appositional membrane, that is of the pre-synaptic membrane in close unobstructed contact with the post-synaptic membrane. This process leads to complete withdrawal of the terminal from the gutter. Nerve terminals at different stages of detachment from the junction have been described during aging both in mammalian [3] and in avian [14] muscles in which a parallel and increasing impairment of cholinergic functions has been demonstrated [14, 18].

As shown in Tab.1 the appositional membrane measured as percentage of terminal circumference does not change following stimulation though the nerve terminals are reduced in diameter at the same extent 10 and 30 days after stimulation. Actually, at day 10 the majority of small terminal profiles resemble newly formed sprouts, being either associated with large and almost normal boutons or arranged in groups to fill up synaptic sites. Such a frequent sprouting is in keeping with changes in the dimensions of ChE-stained junctional sites we have described in rat EDL muscle as an early sign of modulation in response to unusual imposed activity [23, 25]. The end-plates become more compact and less branched within the first ten days of stimulation, that is, when in our experimental conditions muscle fiber atrophy is very mild and not yet significant (Figs. 1b, 2).

Small terminals in 30 days stimulated NMJs are nearly always associated with redundant Schwann cell profiles which, though not infiltrating the synaptic cleft are surrounded by multiple layers of basal lamina (Fig. 7). Because of the absence of morphological evidence of degeneration in nerve terminals the presence of the multilayered basal lamina around the Schwann cells suggests that repetitive cycles of sprouting and retraction of terminals have occurred. This supports the hypothesis that in mammalian junctions nerves retract rather than degenerate [36] and indicates that in low frequency stimulated EDL NMJs undergo continual reorganization.

The results reported here also show that both vesicles and mitochondria contained in the synapse change following continuous stimulation. In particular, by day 10 the number of clear vesicles on area is significantly decreased by about 35%. A similar extent of decrease is found twenty days later (Tab.1). Concomitantly, mitochondria in the synapse undergo a pronounced reduction in size followed by a remarkable increase in number (Tab.2). This indicates that the decrease is a transient phenomenon probably due to sustained unusual activity imposed to the synapse and which is then counterbalanced by proliferation.

Prolonged nerve stimulation at low frequency has been demonstrated to reduce the number of vesicles per unit area of terminal and to change transmitter release characteristics in frog NMJs [8, 17]. By in vitro experiments Ceccarelli *et al.* [8] found that following 20 min of *in vitro* stimulation at 10 Hz there is a fall in the number of vesicles in the terminal due to the fact that the initial rate of fusion is much greater than the rate of reformation of vesicles. If the sustained nerve activity is maintained a new balance is achieved between fusion and reformation of vesicles which may remain constant for long periods of time. On the other hand, Hinz and Wernig [17] have shown that a fast tonic pattern (10 Hz) of *in vivo* stimulation imposed for several days brings about a profound depression of transmitter release in the cutaneous pectoris muscle of the frog. They suggested that this might have as morphological correlate a decline in the contact length and/or contact area of the presynaptic axon due to retraction or withdrawal of the terminal from the junctional site and formation of abandoned synaptic gutters. It seems, therefore very likely that changes described in chronically stimulated NMJs from rat EDL are long lasting modifications related to continuous and sustained imposed activity. In other words, they can be seen as morphological correlates of the ability of the mature synapse to adapt to new functional demands.

Changes of post-synaptic junctional sites are in keeping with this interpretation. Tortuous, variably deep and enlarged secondary folds are almost found with 30-day stimulated NMJs (Fig. 6d and e, and Fig. 7). Morphometry shows a progressive

increase of the mean distance between junctional openings into the synaptic cleft (Tab. 1). Wernig and Herrera [36] proposed that development and maintenance of secondary folds are related and perhaps depend on synaptic current. More recently, Vautrin and Mambrini [34] have developed an electrical model to analyze the quantal current in the interfold (as a unit, this is the portion of post-synaptic membrane defined by two consecutive openings of junctional folds). They demonstrated the existence of an interfold resistance which differs in relation to muscle and myofibers types, age and pathology and which mainly depends upon the extent length of the junctional interfold. By quantitative measurements we here report that following stimulation the spacing between junctional folds becomes similar to that found for slow twitch muscle fibers [34]. This variation in the interfold length may indicate that long term continuous electrostimulation has changed the normal physiology of the synapse, modulating it towards a fatigue resistant type.

In conclusion, it appears that a coordinated sequence of modulative events occurs in EDL muscle of adult rats in response to continuous low frequency stimulation which without associated damage involves nerve and muscle structural components. Furthermore, independently from the expression of slow type myosin isoforms, it seems that fast fatigable rat EDL is able to acquire upon request characteristics which are consistent with an extended resistance to fatigue.

Acknowledgements

This work was supported by funds from Italian C.N.R. to the Unit for Muscle Biology and Physiopathology and to Progetto Finalizzato: Tecnologie Biomediche e Sanitarie (TBMS). The authors wish to thank Dr. U. Carraro for his advice and discussion of the data, V. Gobbo and S. Belluco for their skillful technical assistance.

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