Changes in the Neuromuscular Junction of Rat Soleus Muscle after Hindlimb Unweighting

Yvonne Mounier, Francis Bacou[1] and Maurice Falempin

Laboratoire de Physiologie des Structures Contractiles, Université des Sciences et Technologies de Lille, Villeneuve d'Ascq, France and (1) Laboratoire de Différenciation Cellulaire et Croissance, INRA, Montpellier, France

Abstract

The adaptive properties of the neuromuscular junction to unweighting in simulated microgravity conditions were studied on adult Wistar rats. The animals were submitted to a 15-day hindlimb suspension (HS) protocol. After that, the tibial nerve - soleus muscle preparation was isolated for electrophysiology and the soleus was removed for histochemical studies. The passive electrophysiological properties of the soleus membrane remained unchanged except for an increase in the input resistance associated with the muscle atrophy. The amplitude histogram of the mepps became bimodal, while their frequency of discharge was unaffected by HS. The total AChE activity increased without changes in the relative proportions of its different molecular forms. Therefore, the data suggested that muscles atrophied after simulated microgravity exhibited an adaptation pattern of the transmission process at the neuromuscular junction, while the passive electrophysiological properties of the muscle membrane remained unaffected.

Key words: microgravity, unweighting, soleus muscle, neuromuscular junction, electrical membrane properties, AChE activity.

BAM 5 (2): 163-168, 1995

Unweighting associated with real or simulated microgravity conditions induce limb muscle atrophy and affect several morphological, biochemical and physiological properties of rat skeletal muscles. The most important changes are observed in slow antigravitational muscles such as the soleus, (for review, see [29]) and include decreased muscle mass and fiber diameter associated with loss of force. These effects occur on the whole muscle [8, 30] as well as on isolated fibers [14, 18, 25, 26]. Moreover, the kinetics of the soleus contraction change, approaching those typical of fast-twitch muscles. Therefore, the role of innervation appears to be crucial since it has been largely demonstrated that the pattern of nerve activity determines muscle fiber typing. Hypotrophy of the soleus myelinated nerve fibers [11] and changes in the electromyographic activity of the soleus muscle have been described after real [21] or simulated [1, 5] microgravity conditions. This suggested that under such conditions, the motoneuron impulse activity is affected and consequently, the post synaptic membrane properties might exhibit adaptative characteristics. This led us to examine how weightlessness may affect the properties of the neuromuscular junction. To simulate the effects of weightlessness the hindlimb suspension (HS) model was used [31]. The passive electrophysiological membrane properties, the amplitude and frequency of miniature end-plate potentials (mepps) and the specific activities and molecular forms of acetylcholinesterase (AChE) in the soleus muscles of control and suspended rats were studied.

Materials and Methods

Animals

The experiments were performed on male Wistar rats weighing about 230-250 g, divided into control and HS groups. The animals in the latter group were submitted to 15 days of HS using a classical protocol which permits to reduce both load-bearing and muscle activity [31]. This duration of HS was chosen because it induces maximal effects in the mechanical properties of the soleus muscle [12]. The experiments and protocol as well as the maintenance conditions of the animals received authorization (n° 03805) from both the Agricultural and Forest Ministry and the National Education Ministry.

Preparation and electrophysiological recordings

Control and HS rats were anaesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg body wt, i.p.). Tibial nerve-soleus muscle preparations were removed and mounted in a chamber perfused at a rate of 1-2 ml/min with an oxygenated (95% O_2 + 5% CO_2) solution of the following composition (in mM): NaCl 135; KCl 5; $CaCl_2$ 2; $MgCl_2$ 1; $NaHCO_3$ 12; $NaHPO_4$ 1; glucose 11 (pH: 7.4; temperature 32°C). The connective tissue was removed under high magnification (x 50), especially in atrophied muscles where it appeared more developed.

Conventional glass microelectrodes having resistances of 5-10 MΩ, selected for low noise and filled with 3M KCl were used for the intracellular recordings of electrotonic potentials and mepps. End-plate regions were explored and identified by the presence of the mepps with rise times of 0.5 - 1.0 msec. About 100 mepps were recorded on film and measured either by hand after magnification or using an on-line digital averager. The frequency of mepps was determined for periods of 1 min. For each muscle, three end-plates were generally tested. For the study of the membrane properties, a second current-passing microelectrode was inserted in superficial fibers of the plate region.

Cable properties

The cable properties were determined by depolarizing or hyperpolarizing potential changes (V), that resulted from passage of square current pulses (50 ms duration) across the cell membrane at different distances, x, from the recording electrode. The analysis depends on the steady-state solution of the cable equation [17],

$$V(\infty, x) = 1/2\sqrt{(r_m r_i)}\, I \exp(-x/\sqrt{r_m/r_i})$$

where I is the applied current, r_m (ohm.cm) is the transmembrane resistance of a unit length of fiber, r_i (ohm/cm) i s the resistance of the myoplasm per unit length of fiber, $\sqrt{r_m/r_i} = \lambda$ is the fiber space constant and $1/2\sqrt{r_m r_i} = R_{in}$ is the input resistance at the current source. The two electrodes were inserted in the nerve-muscle junction and were initially separated by a distance x equal to 100 - 200 µn. Then the current passing electrode was withdrawn and reinserted at, at least, three other distances. Fibers which, during this procedure, depolarized by more than 5 mV were rejected. Current intensity (I) was measured by recording the voltage drop across a 10 kΩ resistance in the current passing circuit. R_{in} and λ were estimated from the slope and intercept, respectively, of a straight line obtained by plotting log (V/I) versus x. The data were accepted only if the plot was a straight line. The values of r_m and r_i were calculated from λ and R_{in} using the following equations: $r_m = 2 R_{in}\lambda$ and $r_i = 2R_{in}/\lambda$.

The resistance of 1 cm^2 of membrane or specific membrane resistance (R_m) and the resistivity of myoplasm (R_i) were related to r_m and r_i respectively by: $R_m = r_m \pi d$ and $R_i = r_i (\pi d^2/4)$ where d is the fiber diameter. Therefore, R_m was estimated from r_m, r_i and R_i using these two equations without measuring the cell diameter. We assumed a value of 180 ohm.cm for R_i [6, 13] for normal and atrophied muscles since it is already known that R_i does not differ appreciably in normal and atrophied dystrophic muscle fibers [20].

Extraction and assay of AChE molecular forms

To extract total AChE, whole soleus muscles were homogenized in a glass-glass Potter homogenizer in 10 volumes of a "HST" detergent-saline buffer (0.01 M Tris-HCl, pH 7.2; 1 M NaCl; 1 mM EGTA; 1% Triton X-100; 5 TIU/l Aprotinin; 1 mM Benzamidin) and centrifuged at 25,000 g, 4°C for 30 min. The supernatant was used for assays of AChE activity and distribution of AChE molecular forms.

AChE molecular forms were analyzed by velocity sedimentation in 5-20% sucrose gradients in extraction buffer and run at 4°C, 40,000 rev/min for 18hr, in a SW-41 Beckman or a TST-41 Kontron rotor. Horse liver alcohol dehydrogenase (4.8S) and Escherichia coli β-D-galactosidase (16S) were used as internal sedimentation standards. Each of the fraction was assayed for AChE activity by a slightly modified version of the colorimetric method [10] in the presence of 0.1 mM tetraisopropyl pyrophosphoramide (Iso OMPA), as an inhibitor of butyrylcholinesterase.

Data analysis

Values are expressed as means ± SE. All the results were analyzed using the Student's t test.

Results

Passive electrophysiological properties of the postsynaptic membrane

The passive membrane cable properties of normal and 15-day suspended soleus muscles are summarized in table 1. The mean resting potential (RP) equalled - 77.3 ± 1.4 (n = 70) and - 78.6 ± 2.8 (n = 52) in control and HS prepara-

Table 1. Passive electrophysiological properties of the postsynaptic membrane of control and atrophied soleus muscles after 15-day hindlimb suspension (HS). Values are means ± SE. Numbers in parenthesis indicate the number of experimental fibers/number of muscle.

PARAMETERS	CONTROL	HS
RP (mV)	-77.3 ± 1.4 (70/10)	-78.6 ± 2.8 (52/9)
R_{input} (MΩ)	0.23 ± 0.08 (15/5)	0.76 ± 0.09 * (15/5)
λ (µm)	517 ± 106 (15/5)	371 ± 61 (15/5)
R_m (Ω.cm^2)	614 ± 96 (15/5)	820 ± 176 (15/5)
τm (ms)	1.78 ± 0.36 (2V6)	1.26 ± 0.29 (21/7)
C_m (µF/cm^2)	4.12 ± O.52 (15/5)	2.85 ± 0.70 (15/5)

* indicates a statistical difference (P = 0.05).

tions, respectively. A significant increase in the R_{in} was observed in HS conditions while R_m did not appear significantly modified. Similarly, the space constant (λ), the membrane time constant (τm) and the specific membrane capacitance (Cm) were not significantly altered by HS although they appeared slightly decreased.

mepp characteristics

Spontaneous miniature end-plate potentials were detected about midway along the fiber. The different characteristics of the mepps are given in table 2. The amplitude histogram of mepps in the control soleus muscle was unimodal with a peak at 280-300 µV. In disuse HS conditions, the amplitudes of the mepps varied considerably and the amplitude histogram became bimodal, with two peaks at 160-180 and 360-380 µV (Fig. 1). These two types of mepps were recorded simultaneously during the same impalement in HS muscle fibers. The duration of the different mepps recorded from control or HS muscles (small and large amplitudes) did not differ. The mean frequency of discharge of the mepp in the control soleus was 1.18 ± 0.12 Hz and remained unchanged and equal to 1.53 ± 0.22 Hz after 15-day HS conditions.

Specific activities and molecular forms of AChE

The total AChE activity in the soleus muscle was significantly increased after HS, compared to control values, although the proportion of activity of the different molecular forms of AChE was similar in the two groups (Table 3). This was confirmed by the comparison of the sedimentary profiles presented in figure 2.

Discussion

After hindlimb suspension, the soleus muscle was atrophied but its passive electrophysiological properties such as RP, λ, R_m, C_m remained unchanged suggesting that the intrinsec excitability of the muscle was not modified. Thus, HS-induced muscle atrophy differs from other conditions which also led to atrophy, such as denervation [28] or tetrodotoxin-induced disuse [7] in which there is significant decreases in RP.

Table 2. Characteristics of mepps from control and 15-day suspended rats (HS).

	CONTROL	HS
potential number	1490 (14 plates)	1850 (17 plates)
peak amplitude (µV)	280-300	160-180 360-380
duration (ms)	4.55 ± 0.30	3.86 ± 0.29
frequency (H_Z)	1.18 ± 0.12	1.53 ± 0.22

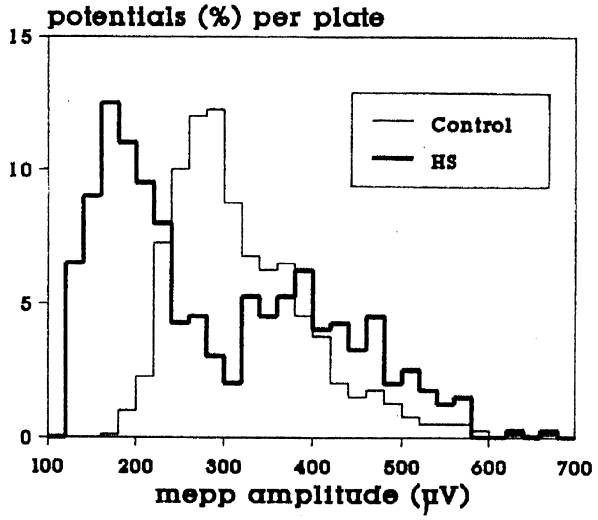

Figure 1. Amplitude histogram of mepps in soleus muscle fibers of control (—) and 15-day suspended (—) rats. Measurements were obtained from 100 potentials for each plate at the beginning of the microelectrode insertion. Control rats: 14 plates / 4 muscles; 15-day suspended rats: 17 plates / 5 muscles.

The large increase in R_{in} without changes in R_m observed after HS might be explained by the atrophy of the disused muscle. Indeed, since R_{in} of the muscle membrane is dependent on the fiber diameter [19] it might be anticipated that the decrease by about 40% of the soleus fiber diameter after 15 days of HS [25] would lead to increased R_{in}.

HS affected the amplitude of the mepps in two opposite ways since the histogram of amplitudes changed from a unimodal form on the control preparations to a bimodal one after atrophy. This suggested complex modifications including the increase in R_{in} and structural modifications. Indeed, it is admitted that amplitude of mepp is dependent on the size of the synaptic space and that the motor end plates undergo ultrastructural changes after 7 days of HS both at the presynaptic and postsynaptic levels [9]. The innervation exhibited thickenings, swellings or spherical enlargements along the preterminal segments while the spacing between subneural structures appeared decreased and a longitudinal expansion of the end plates was observed. Moreover a significant diminution of the mean number and size of synaptic vesicles has been described after real microgravity (biosputnik 782, 20-day duration) [4]. Thus, ultrastructural changes might explain modifications in the spontaneous transmitter release and consequently a decreased amplitude of the mepps. Finally, the bimodal characteristic of the mepp amplitude histogram after HS might reflect the existence of different types of synaptic vesicles whose changes in size might be not homogeneous. The mepp frequency was not modified after 15 days of HS. It is that resting Ca^{2+} activity contributes to the mepp frequency; activity of the nerve terminals and the

Table 3. Specific activities and percentage of the different molecular forms of Acetylcholinesterase (AChE) of control and suspended rat soleus muscles. Each result corresponds to mean ± SE calculated from 6 to 13 (n) different muscle samples. Although the minor asymmetric A4 form represents a significant component in soleus muscles (see sedimentation profiles), its contribution is generally negligible and is not listed.

	ACbE activity (mU/mg prot.)	% A12	% A8	% A	% G4	% G2+G1	% G
Control(n=6)	1.58 ± 0.09	9.7 ± 1	15.4 ± 1.4	25.1 ± 2	18.2 ± 3.9	56.7 ± 4.9	74.9 ± 2
HS(n=13)	2.30 ± 0.07*	8 ± 0.6	14.3 ± 1	22.4 ±1.5	15.6 ± 1	62 ± 1	77.6 ± 1.5

* indicates significant difference (P = 0.05).

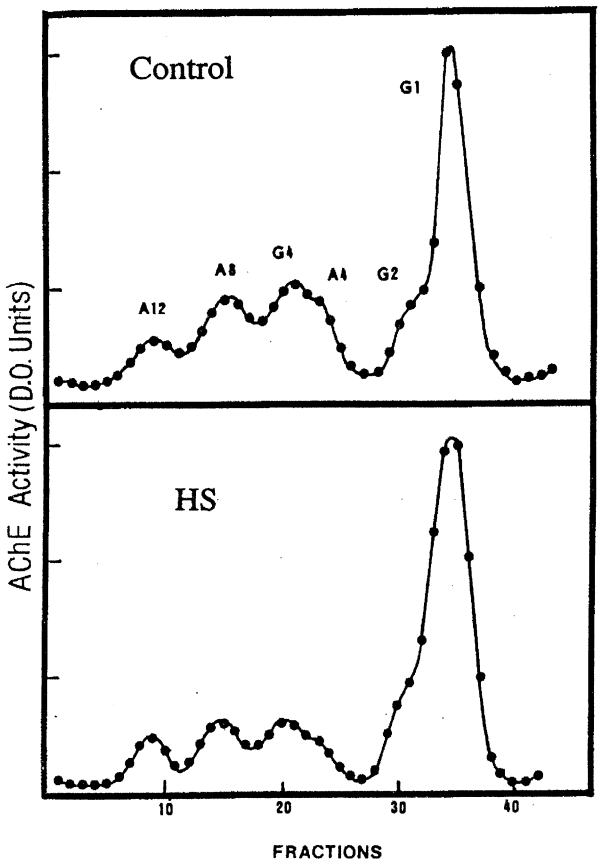

Figure 2. Sedimentation profiles of AChE molecular forms in control and suspended soleus rat muscle. AChE activity is plotted on an arbitrary scale but the profiles corresponding to the control and HS muscles have been normalized so that the activities of each form are directly comparable.

relative extent of motoneuron recruitment or the pattern of motor activity might also play an important role in determining the synaptic responses to disuse. At the present time we have no direct data concerning electrophysiology of the nerve in HS animals. Even if it were modified, as suggested by some EMG results, [1, 5] the electrical activity of the nerve terminals after 15 days of HS, must have remained appropriate to preserve the basal function of the neuromuscular junction.

An increase by 45% in the soleus AChE activity was observed after 15 days of HS, which is in agreement with previous results [16, 23], although the relative contribution of each AChE molecular form, asymmetric and globular, to the total enzyme activity was not different from that of control muscles. The total increase in AChE activity after HS is further supported by AChE histochemical staining of the neuromuscular junction which appeared darker (Falempin: personal communication). This might be related to HS muscle atrophy and/or to the conversion of the slow-to fast-twitch speed of contraction which is well known to occur after HS [14, 24, 25, 27, 30]. These results are in agreement with data from the literature which reported a higher AChE activity in fast-twitch muscles compared to that of slow ones, such as control soleus [3, 15]. However, these results are in contradiction with those observed on the EDL in which AChE activity decreased significantly by 18% after HS and do present a different AChE polymorphism between control and HS muscles [2]. The discrepancy in AChE characteristics in slow and fast HS muscles attests a different regulation in the biosynthesis of AChE and its molecular forms in both muscles. It can be further related to physiological behaviour of different types of muscles under microgravity conditions, as it is well known that fast-twitch muscles are much less affected than slow-twitch ones after hindlimb unweighting [22, 26, 30].

In conclusion, these results obtained on slow-twitch muscles suggest a different regulation of the neuromuscular junction characteristics under microgravity conditions.

Acknowledgments

The authors are grateful to Mrs Montel and Lefevre for their technical assistance. This work was supported by grants from the Centre National d'Etudes Spatiales.

Address correspondence to:

Professeur Yvonne Mounier, Laboratoire de Physiologie des Structures Contractiles, Bâtiment SN4, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq Cedex, France, tel. 20 43 40 88, fax 20 43 68 88.

References

[1] Alford EK, Roy RR, Hodgson JA, Edgerton VR: Electromyography of rat soleus, medial gastrocnemius and tibialis anterior during hindlimb suspension. *Exp Neurol* 1987; 96: 635-649.

[2] Bacou F, Falempin M, Mounier Y: Changes in the neuromuscular junction of rat soleus muscle induced by disuse atrophy, in Massoulie J, Bacou F, Barnard EA, Chatonnet A, Doctor BP, Quinn DM (eds): *Cholinesterases: Structure, Function, Mechanism, Genetics, and Cell Biology* (Proceedings of the 3rd International Meeting on Cholinesterases, La Grande-Motte, May 12-16,1990). Washington DC, American Chemical Society, 1991, pp 111.

[3] Bacou F, Vigneron P, Massoulié J: Acetylcholinesterase forms in fast and slow rabbit muscle. *Nature* 1982; 296: 661-664.

[4] Baranski S, Marciniak M: Stereological ultrastructural analysis of the axonal endings in the neuromuscular junction of rats after a flight on Biosputnik 782. *Aviat Space Environ Med* 1979; 14-17.

[5] Blewett C, Helder GCB: Quantitative EMG analysis in soleus and plantaris during hindlimb suspension and recovery. *J Appl Physiol* 1993; 74: 2057-2066.

[6] Boyd IA, Martin AR: Membrane constants of mammalian muscle fibers. *J Physiol London* 1959; 147: 450-457.

[7] Bray JJ, Hubbard JI, Mills RG: The trophic influence of tetrodotoxin-inactive nerves on normal and reinnervated rat skeletal muscles. *J Physiol London* 1979; 297: 479-491.

[8] Caiozzo VJ, Baker MJ, Herrick RE, Tao M, Baldwin KM: Effect of spaceflight on skeletal muscle: mechanical properties and myosin isoform content of a slow muscle. *J Appl Physiol* 1994; 76: 1764-1773.

[9] D'Amelio F., Daunton NG, Fast T, Grindeland R: Preliminary findings in the neuromuscular junctions of the soleus muscle of adult rats subjected to simulated weightlessness. Light and electron macroscopy. *Proceeding of Symposium on Neuroscience* 1988; 204.

[10] Ellman GL, Courtney KD, Andres V, Featherstone RM: A new and rapid colorimetric determination of acetylcholinesterase. *Biochem Pharmacol* 1961; 7: 88-95.

[11] Falempin M, Delorme P, Holy X, Leterme D, Mounier Y: Suspension hypokinesia-induced morphological changes in rat soleus nerve. *14th annual meeting IUPS on gravitational physiology Berlin* 1992.

[12] Falempin M, Leclercq T, Leterme D, Mounier Y: Time course of muscle - change in and - recovery from disuse. *The Physiologist* 1990; 33: 88-89.

[13] Fischbach GD, Robbins N: Effect of chronic disuse of rat soleus neuromuscular junctions on postsynaptic membrane. *J Neurophysiol* 1971; 34: 562-569.

[14] Gardetto PR, Schluter JM, Fitts RH: Contractile function of single muscle fibers after hindlimb suspension. *J Appl Physiol* 1989; 66: 2739-2749.

[15] Groswald DE, Dettbarn WD: Nerve crush induced changes in molecular forms of acetylcholinesterase in soleus and extensor digitorum longus. *Exp Neurol* 1983; 79: 519-527.

[16] Gupta RC, Misulis KE, Dettbarn WD: Changes in the cholinergic system of rat sciatic nerve and skeletal muscle following suspension-induced disuse. *Exp Neurol* 1985; 89: 622-633.

[17] Hodgkin AL, Rushton WAH: The electrical constants of a crustacean nerve fiber. *Proc Roy Soc B* 1946; 133: 444-479.

[18] Holy X, Mounier Y: Effects of short spaceflights on mechanical characteristics of rat muscles. *Muscle Nerve* 1991; 14: 70-78.

[19] Katz B, Thesleff S: On the factors which determine the amplitude of the miniature end-plate potential. *J Physiol London* 1957; 137: 267-278.

[20] Kerr LM, Sperelakis N: Membrane alterations in skeletal muscle fibers of dystrophic mice. *Muscle Nerve* 1983; 6: 3-13.

[21] Leterme D, Falempin M: Changes in muscle electrical activity in response to unloading conditions. *International Union of Physiological Sciences Glasgow* 1993.

[22] Oganov VS, Skuratova SA, Potapov AN, Shirvinskaya MA: Physiological mechanisms of adaptation of rat skeletal muscles to weightlessness and similar functional requirements. *The Physiologist* 1980; 23: 16-21.

[23] Patterson GT, Dettbarn WD: Changes in skeletal muscle following hindlimb suspension. *The Physiologist* 1985; 28: 133-134.

[24] Reiser PJ, Kasper CE, Moss RL: Myosin subunits and contractile properties of single fibers from hypokinetic rat muscles. *J Appl Physiol* 1987; 63: 2293-2300.

[25] Stevens L, Mounier Y, Holy X, Falempin M: Contractile properties of rat soleus muscle after 15 days of hindlimb suspension. *J Appl Physiol* 1990, 68: 334-340.

[26] Stevens L, Mounier Y, Holy X: Functional adaptation of different rat skeletal muscles to weightlessness. *Am J Physiol* 1993; 264: 770-776.

[27] Templeton GH, Padalino M, Manton J, Glasberg M, Silver CJ, Silver P, De Martino G, Leconey T, Klug G, Hagler H, Sutko JL: Influence of suspension hypokinesia on rat soleus muscle. *J Appl Physiol* 1984; 56: 278-286.

[28] Thesleff S: Physiological effects of denervation of muscle. *Ann NY Acad Sci* 1974; 288: 89-104.

[29] Thomason DB, Booth FW: Atrophy of the soleus muscle by hindlimb unweighting. *J Appl Physiol* 1990; 68: 1-12.

[30] Winiarski AM, Roy RR, Alford PC, Chiang PC, Edgerton VR: Mechanical properties of rat skeletal muscle after hindlimb suspension. *Exp Neurol* 1987; 96: 650-660.

[31] Wronski TJ, Morey-Holton ER: Skeletal response to simulated weightlessness: a comparison of suspension techniques. *Aviat Space Environ Med* 1987; 58: 63-68.