Suppression of Motor Innervation Induces Fiber Diversity in Rat Soleus Muscle

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Abstract

Rat soleus muscle was submitted to 15 days of spinal cord ventral root section (deefferentation) in order to specifically disturb motor innervation. The electrophoretic analysis of the deefferented soleus muscle showed a reduction in myosin heavy chain (MHC) 1 (53% versus 92%) and new expressions in MHC 2D/X (12%) and MHC 2B (22%) relative concentrations, while MHC 2A composition did not evolve significantly. Single skinned fibers from control and deefferented soleus were identified according to their MHC composition and their functional properties (calcium/strontium activations and maximal shortening velocities) were established. Control soleus contained 72% of slow (S) fibers expressing MHC 1 and slow functional properties, and 28% of hybrid fast (HF) fibers, coexpressing MHC 1 with MHC 2A predominantly, and presenting fast functional properties. In deefferented soleus, four fiber types were discriminated: S and HF, and new fast types expressing MHC 2A, and/or MHC 2D/X plus MHC 2B. Moreover, deefferented soleus fibers were very much atrophied and presented alterations in Ca activation properties, the effects being more marked on the fast fibers. All the data support the use, on the rat, of deefferented muscle as a model for motoneuron disease.

Key words: atrophy, calcium and strontium activations, deefferentation, maximal shortening velocities, myosin heavy chains, myosin light chains, slow-to-fast transitions.

Basic Appl Myol 10 (4): 181-190, 2000

The importance of the nervous system in the determination and maintenance of structural and functional properties of skeletal muscles has been extensively studied, using experimental models including denervation, cross-reinnervation and electrical stimulation (for review, see [26]). The muscle adaptation to altered functional demands seems to be very dependent on the level and on the kind of changes in motor activity [6]. One way to investigate specifically the role of the motoneurons on the muscle characteristics is the use of animal models for "motor neuron disease" (MND). The term "MND" is used to designate a variety of neuromuscular disorders, which principal features are attributable to dysfunction of upper or lower motoneurons. Patients with Spinal Muscular Atrophies (SMA), the most common diseases, manifest specific losses of motoneurons that lead to progressive muscle weakness, and muscle fiber atrophy [11, 17]. The animal models are generally obtained in the mouse (for review, see [18]) by mutations, either spontaneous e.g. mnd (motor neuron degenerative), pmn (progressive motor neuropathy) or wobbler mice models, or experimentally-induced e.g., transgenic mice carrying the gene encoding human Cu/Zn superoxyde dismutase, toxic models. Most of SMA diseases are generally studied on the mouse and what is more, on pathological fast-twitch skeletal muscles, which have been found to transform into slower muscles [20, 28, 32]. Here, we were interested in studying the effects of this kind of affections on a slow-twitch skeletal muscle such as the rat soleus, well known to be capable of undergoing slow-to-fast fiber type transitions after disuse atrophy induced, for instance, by hindlimb unloading [19, 29, 31]. Lastly, myofibrillar proteins of skeletal muscles are expressed as multiple isoforms which induce fiber type diversity [25]. This property enables the skeletal muscles to adapt their phenotype in response to exogenous factors such as mechanical load, nervous and hormonal influences. Whether these factors are increased or reduced, the muscles are able to modify the quantity and/or the qual-

ity of protein expression and, thereby, their physiological properties. Among all the contractile proteins, the myosin molecule, existing as different MHC (myosin heavy chain) and MLC (myosin light chain) slow and fast isoforms, seems to be the key marker to assess muscle fiber type [25].

Thus, in the present study, we induced the specific suppression of motor innervation in the slow type soleus muscle of the rat by a deefferentation procedure, i.e. bilateral sections of L4 and L5 spinal cord ventral roots. In these conditions, the motor efferent innervation of the soleus is removed while the sensitive afferent system remained intact and enabled us to establish, in the rat, basal data for a model of motoneuron specific affection. Therefore, we examined after 15 days, the deefferentation-induced transitions in myosin (MHC and MLC) isoform pattern of i) whole soleus muscle and ii) isolated single skinned fibers, in relation to their functional characteristics such as calcium and strontium activation properties and maximum shortening velocity.

Materials and Methods

Animals and samples

The experiments were performed on eight adult male Wistar rats, weighing about 220 g at the beginning of the experiments. The animals were randomly distributed into two groups: a sham group called Cont group (Cont, n=4), which received the whole surgery, except deefferentation, and another one submitted to deefferentation procedure (Deeff, n=4) described as follows. Animals were deeply anaesthetized by intraperitoneal injection of sodium pentobarbital (35 mg/kg body weight). Under aseptic conditions, the midline dorsal musculature was retracted laterally and a laminectomy was made between L3 and L6. Bilateral ventral roots L4 and L5 were exposed and lidocaine hydrochloride (2%) was applied. L4 and L5 were cut at their point of entry into the spinal cord and 1 cm portion of the roots was removed. The cut ends of the roots retracted into the spinal cord. Gelfoam was packed between the caudal and rostral portions to minimize bleeding. A strip gel film was laid on the spinal cord to prevent adhesion between spinal cord and paravertebral muscles. Dorsal musculature was sutured (3-0 chronic gut) to cover the laminectomized area. Skin incision was sutured (3-0 Ethelon monofilament nylon). Following recovery, the rats were maintained in isolated plastic cages. Consumption of rat chow and water was verified daily. Antibiotics (0.1 ml sterile penicillin G procaine by intramuscular injection) were administered for 6 days following surgery. To prevent skin infection, an antiseptic (Betadine) was applied to the incision area once a day. Reflex testing on the hindlimbs was performed once a day, i.e. withdrawal reflex to verify the reality of the bilateral deefferentation. Throughout the study, there was no response to toe pinching, assuming the absence of motor response. At the end of the deefferentation period, two recording platinium electrodes were placed on the L4 dorsal root. The recording of a positive qualitative neurogram after stretching the hindlimb proves that the afferent activity was unaltered.

After 15 days of deefferentation, both groups of rats were weighed, anaesthetized with an intraperitoneal injection of sodium pentobarbital (35 mg/kg body weight), and the muscles were immediately removed and weighed. In the control rats, both soleus (n=8) and extensor digitorum longus (EDL, n=8) were dissected out, whereas in the Deeff rats, only the soleus muscles (n=8) were excised. Indeed, for some parameters, the EDL muscle fibers were used as fast controls.

The left Cont and Deeff soleus muscles were divided into two parts: one part (~1/3) was used for the analysis of whole muscle MHC and MLC electrophoretic compositions, and the other part was chemically skinned for the study of single fiber functional (contractile) and structural (electrophoretical) properties. The right muscles were kept for other experiments, not presented here. The experimental protocol, animal care and treatment were approved by both the Ministry of Agriculture and Forestry and the Ministry of Education (Veterinary service for health and animal protection; authorization 03805).

Whole muscle electrophoretic analysis

Each 1/3 removed Cont (n=4) and Deeff (n=4) soleus muscle was mounted in embedding medium (TEK ACT Compound), frozen in isopentane precooled to its freezing point by liquid nitrogen, and stored at - 80 °C until analysis. Serial transverse sections (20 mm thick) were cut, treated according to [3] and dissolved in lysis buffer [62.5 mM Tris HCl, l0% glycerol (v/v), 2% SDS (w/v), 5% ß-mercaptoethanol (v/v) and 0.02% Bromophenol blue (w/v)]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 0.75 mm thick separating gels of 7.5% and 12% acrylamide to identify, respectively, MHC [13] and MLC [10] isoform compositions. The gels were silver stained [10] and the relative proportions of each type of MHC and MLC isoforms were determined using a UV scanning densitometer (Quantiscan Microvial Systems, Biosoft, UK).

Single skinned fiber preparation and apparatus

The skinning procedure was performed through the exposition of ~2/3 of the Cont (n=4) and Deeff (n=4) soleus muscles to an EGTA [ethylene glycol-bis (ß-aminoethyl ether) N, N, N', N'-tetraacetic acid] solution for 24 hours at 4°C. Then, the skinned muscle biopsies were stored at -20°C in a glycerol-skinning solution (storage solution) for up to 2 months [24].

The apparatus and the experimental procedure for functional measurements have been described in detail in previous studies [24, 29]. In brief, on the day of an experiment, an individual fiber segment (mean length, 2.52±0.40 mm) was dissected from a skinned bundle, transferred to the experimental chamber that contained relaxing solution (see Solutions section) and glued, with cellulose polyacetate dissolved in acetone, between the hook of an isometric tension transducer (AE 801, AME, Norway) and the arm of a feedback controlled stepping motor (model 6350, Cambridge Technology Inc, USA). Tension and motor position were recorded on a chart recorder (serial 2015, Gould Inc, USA) and on a digital oscilloscope (model 310, Nicolet Inc, USA). A He-Ne laser beam was directed onto the fiber so that the resulting diffraction pattern could be controlled. The fiber was exposed for 2x7 min to the relaxing solution containing 10% Brij-58, which resulted in an irreversibly dysfunctional reticulum sarcoplasmic. After this treatment, the diameter of the fiber segment was determined with a micrometer through a high magnifying binocular (x80). Then, the relaxed fiber was stretched to a sarcomere length of 2.6 mm, which permitted the optimal isometric tension to be elicited [30]. All the experiments were conducted at 17±1°C.

Determination of Tension/pCa and pSr relationships

The Tension/pCa and pSr relationships were established as previously described [29] by recording tensions obtained with various pCa and pSr solutions. First, a maximal tension (P0) was elicited by applying a fully activating solution with a pCa (-log[Ca]) of 4.2. Then, each tension (P) obtained at various pCa (range 6.4-4.2) was followed by a maximal tension (P_0). The corresponding ratio (P/P_0) was calculated and related to the Ca concentration (in pCa) to obtain relative Tension/pCa curves. The Tension/pCa experimental data were fitted to the Hill equation: $P/P_0=([Ca]/K)^{n_H}/[1+([Ca]/K)^{n_H}]$, where P/P_0 was the normalized tension, n_H the Hill coefficient (steepness of the curve) and K the apparent dissociation constant ($-\log K=pK=pCa_{50}$, the pCa for 50% maximal tension). The same procedure was performed to obtain the Tension/pSr relationships, except that the fiber was activated in a range of pSr 5.8-3.4. We used the following parameters deduced from the Tension/pCa (Sr) curves: the pCa threshold defined as the lowest Ca concentration required to obtain the development of a tension; the pCa_{50} (pSr_{50}) value which corresponded to the affinity of the fiber for Ca (Sr); the Hill coefficient n_H which reflected the degree of cooperativity of the regulatory contractile system in the activation of tension. To better fit the experimental data, two separate straight lines were calculated using the Hill plot linearization [23]: one above 0.50 P/P_0 (Hill coefficient n_1) and another one below 0.50 P/P_0 (Hill coefficient n_2). Moreover, as described by [7] and used in our previous studies [30], the individual fibers were classified as slow or fast type on the basis of their relative affinity to Ca and Sr ions (pCa_{50}-pSr_{50} difference, or D criteria). According to a statistical test of normality (anamorphosis law, data not shown), we established that fibers showing values in the range 0.10-0.40 were identified as slow, whereas fast fibers were characterized by values in the range 1.00-1.30.

Determination of maximal shortening velocity

The maximal shortening velocity was measured using the slack test method [5]. During maximal activation (in pCa 4.2 solution), the fiber was subjected during 100 ms to a rapid (1 ms) reduction in length, so that force fell to zero. The fiber shortened under zero load, took up the slack and redeveloped force. The fiber was then returned to its original length, so that force and sarcomere length recovered completely, and relaxed in R solution. A series of five or six shortening steps was imposed upon the fiber. For each length change (DL), the time for the fiber to shorten and just redevelop force was measured (Dt). The graph of DL versus Dt was fitted with a straight line in the range of 20 to 90 ms by least square regression ($r > 0.98$) and Vmax was taken as the slope of this line. For each fiber, this value was standardized to the stretched length of the fiber segment (L_0) and expressed in fiber length per second ($FL.s^{-1}$).

Single fiber electrophoretic analysis

Each fiber, for which all the functional properties (Tension/pCa (pSr) curves and Vmax) had been determined, was also identified following its electrophoretic profile. Single fibers were placed in capillary tubes containing 20 ml of lysis buffer, boiled 2 minutes at 95°C, stored at -80°C and MHC electrophoretic analysis was performed using the same protocol as that described above for whole muscle study.

Solutions

The solutions were prepared according to [24]. The following solutions were used for the experimental procedure: a washing solution to eliminate EGTA traces, composed of adenosine triphosphate (ATP, 2.5 mM), morpholinopropane sulphonic acid (MOPS, 20 mM), potassium propionate (185 mM), and magnesium acetate (Mg Ac_2, 2.5 mM). The relaxing (or skinning) solution was composed of ATP (2.5 mM), MOPS (20 mM), potassium propionate (170 mM), Mg Ac_2 (2.5 mM) and K_2 EGTA (5 mM). The pCa and pSr activating solutions consisted in washing solution plus various concentrations of free Ca (Sr) buffered with EGTA and added in determined proportions to obtain the different pCa (pSr) values.

Statistical analysis

All the results were presented as means±S.E.M. The data were studied statistically using a one-way analysis of variance (ANOVA) and Student's *t* test was used as a post hoc test to estimate differences among means. Differences were considered significant at $P < 0.05$.

Results

Whole muscle analysis

The body weight of 15 days-deefferented rats was not different from that of controls. However, the soleus mean weights expressed in absolute value or related to the body weight were significantly decreased by, respectively, 58% (from 145±5 mg, n=8, to 61±5 mg, n=8) and 56% (from 0.46±0.02 mg/g body wt, n=8, to 0.20±0.02 mg, n=8). MHC and MLC electrophoretic profiles of Cont and Deeff soleus muscles are illustrated in Fig. 1 (A and B) and Fig. 2 (A and B) quantifies the gel analyses. The control soleus muscle expressed predominantly the slow isoform MHC 1 (92%), with only a small amount of fast MHC 2A (8%). The MLC profile of the Cont soleus consisted in MLC ls and MLC 2s isoforms predominantly expressed with small amounts of MLC 1 f and MLC 2f. No type 2D/X or 2B MHC, or MLC 3, was detected in any of the control rat soleus muscles. Fifteen days after deefferentation, the MHC composition of the soleus was changed so that MHC 2D/X and, more particularly, MHC 2B isoforms were newly expressed and their relative amounts attained 12 and 22%, respectively. This surexpression of the MHC fast isoforms in the Deeff soleus was concomitant with a 2-fold decrease in the amount of the slow MHC 1. The composition in MHC 2A isoforms did not evolve significantly.

The changes in MLC composition followed those observed at the MHC level, since both MLC 1s and 2s amounts decreased in favour of an increase in MLC lf and 2f. MLC 3 was also present in Deeff muscles (see the gels in Fig. lB) but in little amount, not detected by the densitometric analysis system.

Fiber type identification

The single fibers were identified according to both their relative affinity to Ca and Sr (D functional criterion) and their MHC composition (structural criterion) (Table 1). With the use of these criteria, we were able to identify two populations of fibers in the Cont rat soleus

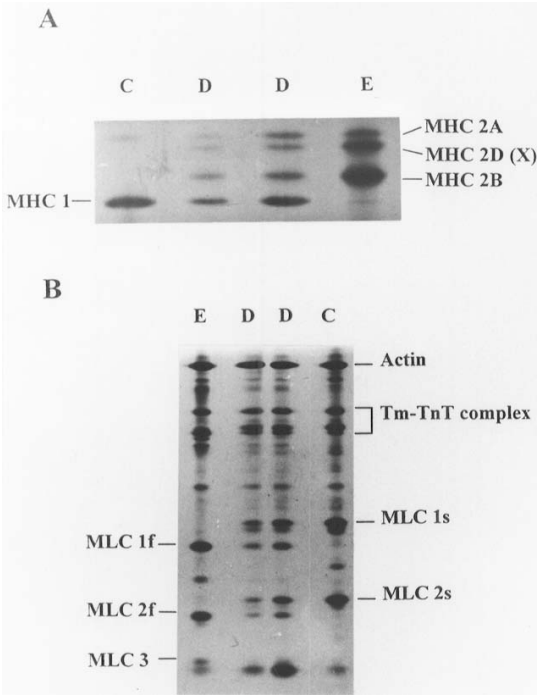

Figure 1. Gels illustrating myosin heavy chain (MHC) (A) and myosin light chain (MLC) (B) isoform compositions of control and deefferented soleus muscles. MHC and MLC typical expression patterns of control soleus muscle are indicated by lanes C and those of deefferented soleus muscles by lanes D. Lane E corresponds to MHC and MLC patterns of EDL muscle, acting as fast control. Tm-TnT: Tropomyosin-Troponin T.

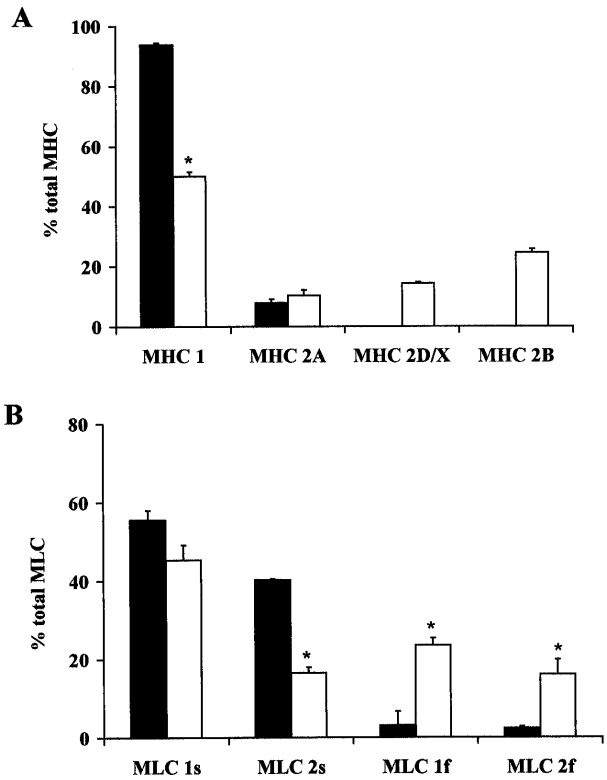

*Figure 2. Effects of 15 day-de-efferentation on whole muscle MHC (A) and MLC (B) compositions. Each amount of MHC or MLC isoform is expressed in% of total MHC or MLC content. Values are means ~ SEM. Solid bars: Cont data; open bars: Deeff data. * Significantly different from Cont data, $P < 0.05$.*

Table 1. Functional and electrophoretic identifications of single skinned fibers.

Group	Fiber type	n	pCa_{50}-pSr_{50}	Relative percentage of MHC isoforms			
			(D)	MHC 1	MHC 2A	MHC 2D/X	MHC 2B
Cont	S	10	0.22±0.02	100±0.0	-	-	-
	HF	4	1.31±0.09	18.2±3.4	82.7±4.1	-	-
Deeff	S	12	0.18±0.03	100±0.0	-	-	-
	HF	12	1.20±0.03	14.8±4.5	86.5±5.5	-	-
	F(2A)	6	1.32±0.07	-	100±0.0	-	-
	F(2D/X+2B)	7	1.29±0.02	-	-	66.8±8.3	34.2±6.5

Values are means±SEM. n, number of fibers. Cont: control soleus fibers; Deeff: deefferented soleus fibers. S: Slow fiber, HF: Hybrid Fast fiber and F (2A) and F (2D/X-2B): Fast fiber populations. pCa_{50}-pSr_{50}: relative affinity to Ca and Sr ions (or D criterion). MHC: Myosin Heavy Chains. The amount of each MHC isoform is expressed relative to total MHC content for a given fiber.

muscle. On a total of 14 Cont fibers, we characterized 10 fibers expressing only the MHC 1 slow isoform and showing a low D value, thus called S (slow) fibers, and 4 fibers called HF (Hybrid Fast). These latter corresponded to fast fibers exhibiting a high D value and expressing both MHC 2A (predominantly, ~82%) and MHC 1 (~18%). It should be noticed that these fiber type proportions were established in order to characterize a sufficient number of fibers of both Cont slow and fast types, and not to represent the whole muscle typing. This was especially difficult for fast (HF) fiber types, present in small amounts in the Cont soleus. After deefferentation, four fiber populations were identified in the soleus muscle: a slow population, identical to the one found in Cont soleus, and three multiple fast type ones. Among the fast groups, we found i) fibers presenting high D values and coexpressing MHC 2A (~86%) and MHC 1 (~14%), not different from the hybrid (HF) population already found in the Cont soleus. However, this HF group was more represented in Deeff than in Cont soleus since we got this fiber type within the biopsy more easily, the fibers being taken randomly; ii) fibers exhibiting also high D values similar to those obtained for the HF populations and containing either MHC 2A alone, called F (2A) population, or coexpressing MHC 2D/X and 2B isoforms and named F (2D/X-2B) population. In this latter population, MHC 2D/X isoform was always expressed predominantly (~67%) when compared to MHC 2B.

Fiber atrophy

Table 2 summarizes the different values of diameter, maximal tension P_0 expressed in absolute value (N) and per CSA (cross sectional area, kN/m^2). Mean diameters of S and HF types of Deeff fibers were significantly decreased by 26 and 35%, when compared to their respective S and HF Cont values. Compared to HF Cont values, F (2A) and F (2D/X-2B) Deeff mean diameters were reduced by 33 and 30%, respectively. P_0 in absolute values was also decreased after deefferentation: decline of 10 and 37% for Deeff S and HF fibers (compared to S and HF Cont values) and decline of 34 and 45% for F (2A) and F (2D/X-2B) fibers (compared to HF Cont fibers), respectively. This decrease in P_0 was

Table 2. Effects of deefferentation on soleus fiber diameters and tensions.

Group	Fiber type	Diameter (mm)	P_0, x 10^{-4} N	P_0, kN/m^2
Cont	S	71.88±2.05	2.73±0. 17	70.63±7.44
	HF	71.83±3.13	2.79±0.20	72.56±6.67
Deeff	S	53.25±2.79*	2.46±0.12	79.25±6.22
	HF	46.88±2.08*§	1.76±0.15*≅§	93.58±9.18
	F(2A)	47.92±3.61*§	1.83±0.13*≅§	98.53±13.17
	F(2D/X-2B)	50.25±4.72*§	1.54±0.17*≅§	71.29±11.65

Values are means±SEM. The number of fibers in each population is the same as that described in Table 1. Cont: control soleus fibers; Deeff: deefferented soleus fibers. S: Slow fibers; HF: Hybrid Fast fibers; F (2A) and F (2D/X-2B): Fast fibers. P_0: maximal tension. * Significantly different from Cont data of the same fiber type, $p<0.05$. ≅ Fast (HF, F (2A) or F (2D/X-2B)) significantly different from S data within the same group, $P<0.05$. § Fast (HF, F (2A) or F (2D/X-2B)) Deeff significantly different from S Cont, $P<0.05$.

only significant in the case of fast type fibers.

Tension/pCa parameters

T/pCa relationships of slow and fast soleus fibers from Cont and Deeff groups are shown in Fig. 3A and B. As previously described [30], the Ca threshold for activation, the Ca affinity and the Hill coefficients differed between slow and fast fibers: HF Cont fibers, containing predominantly MHC 2A, had higher Ca threshold (lower pCa) and steeper T/pCa relationship (higher n_H, n_1, and n_2 values); no significant difference was found between S and HF pCa_{50} values (Table 3). After deefferentation, fibers containing MHC 1 (S group) exhibited a slight (but non significant) pCa unit-rightward shift of the T/pCa curve when compared with Cont fibers. Since no pure F fibers were found in the Cont soleus fibers, the T/pCa curves of both HF, F (2A) and F (2D/X-2B) Deeff populations were compared to the HF Cont fiber groups. Thus, the rightward shift of the T/pCa curve from HF Deeff fibers was very important (0.14 pCa units) when compared to HF Cont values. The pCa_{50} values of Deeff F (2A) and F (2D/X-2B) were decreased to a lesser extent, but significantly, by 0.10 and 0.09 pCa units, respectively. Moreover, the F (2D/X-2B) Deeff population was found to have the largest Hill coefficient values (especially n_H and n_1) among the other fast groups.

Maximal shortening velocities

Vmax values related to MHC composition are plotted in histograms of Fig. 4 for each fiber from Cont (n=14, part A) and Deeff (n=35, part B) muscles. The two types

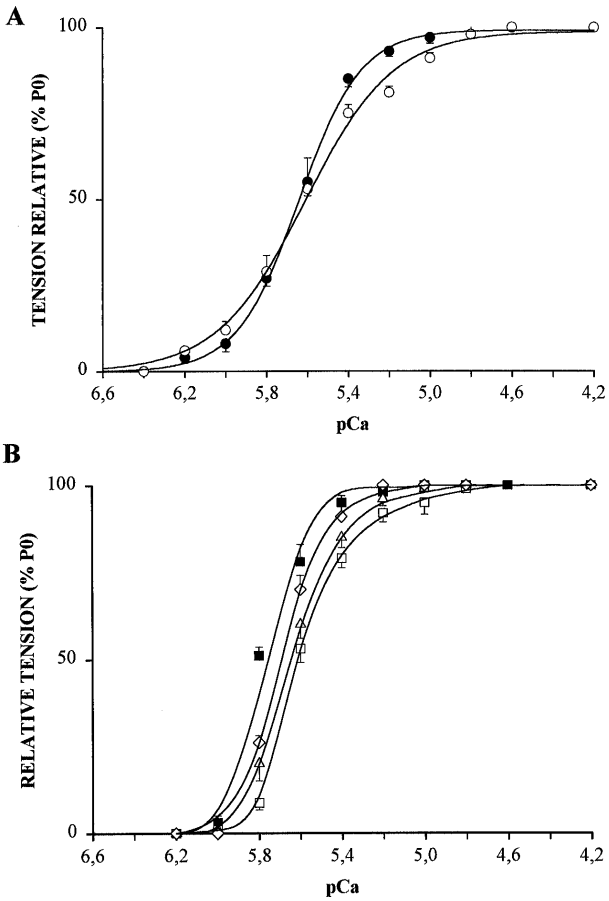

Figure 3. Tension/pCa relationships of single fibers after de-efferentation. A: S Cont (●) and S Deeff (○) populations. B: *HF Cont (■) and HF (□), F (2A) (△) and F (2D/X-2B) (◇) Deeff populations. Values are means±SEM.*

Table 3. Effects of deefferentation on Tension/pCa parameters.

Group	Fiber type	pCa_{th}	pCa_{50}	n_H	n_1	n_2
Cont	S	6.41	5.66	2.22	1.77	2.30
		±0.02	±0.02	±0.20	±0.25	±0.34
	HF	6. 15≅	5.73	3.32≅	2.54	4.95≅
		±0.06	±0.03	±0.46	±0.52	±0.54
Deeff	S		5.60	2.29	1.70	2.60
		±0.05	±0.03	±0.18	±0.27	±0.24
	HF	6.02*≅§	5.59*	3.13≅§	2.29	4.95≅§
		±0.01	±0,01	±0.18	±0.52	±0.30
	F(2A)	6.02 *≅§	5.64*	3.69≅§	3.22≅§	4.58≅§
		±0.01	±0.02	±0.40	±0.60	±0.49
	F(2D/X-2B)	6 12≅§	5.68	4.74*≅§	4.80*≅§	5.44≅§
		±0.01	±0.03	±0.38	±0.95	±0.52

Values are means±SEM. The number of fibers in each population is the same as that described in Table 1. pCa_{th}: pCa threshold for activation; pCa_{50}: half-maximal tension or Ca affinity parameter; n_H, n_1, n_2: Hill coefficients or slopes of the curves. Cont: control soleus fibers; Deeff: deefferented soleus fibers. S: Slow fibers; HF: Hybrid Fast fibers; F (2A) and F (2D/X-2B): Fast fibers. P_0: maximal tension. * Significantly different from Cont data of the same type, $P<0.05$. ≅ Fast (HF, F (2A) or F (2D/X-2B)) significantly different from S data within the same group, $P<0.05$. § Fast (HF, F (2A) or F (2D/X-2B)) Deeff significantly different from S Cont, $P<0.05$.

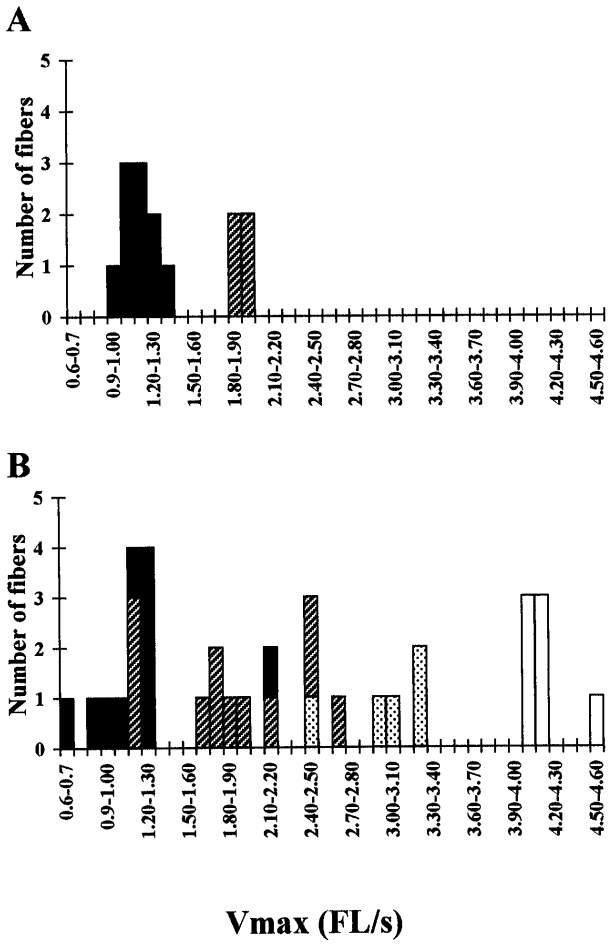

Figure 4. Histogram distribution of Vmax measurements from single fibers of Cont (A) and Deeff (B) *muscles in relation to their MHC composition. Vmax was measured by the slack test method and MHC composition was determined by single fiber SDS-PAGE analysis. S (solid bars), HF (hatched bars), F (2A) (dotted bars) and F (2D/X-2B) (open bars) fibers.*

of fibers described in Cont soleus showed different values of Vmax (Fig. 4) (in FL/s): for S fibers (expressing only MHC 1), 1.14±0.05 (n=l0); and for HF fibers (with MHC 2A>1), 1.90±0.01 (n=4). Thus, in Cont soleus, type (2A> 1) fibers were significantly faster than type 1 fibers. After deefferentation, Vmax values were found to increase in the following order: S, 1.20±0.12 (n=10) < HF, 1.84±0.13 (n=12) < F (2A), 3.52±0.36 (n=6) < F (2D/X-2B), 4.06±0.02 (n=7), the mean Vmax values being significantly different between the four fiber types. Moreover, the whole distribution range of Vmax values was greater after deefferentation (from 0.65 to 4.52 FL/s) (Fig. 4B) than in Cont conditions (from 0.91 to 1.93 FL/s) (Fig. 4A). Finally, it should be noticed that the mean Vmax values of S and HF fibers from the Cont soleus were not significantly different from those of S and HF fibers from Deeff soleus.

Discussion

The major finding of this study relative to the soleus adaptation to deefferentation conditions was the slow-to-fast transition of this muscle. It consisted in a spectacular expression of relative amounts of MHC 2D/X (15%) and 2B (26%) isoforms, while MHC 2A composition did not evolve significantly. Since neither of the MHC 2D/X and 2B isoforms have been observed in control soleus, it can be supposed that these two proteins were newly synthesized during deefferentation. Such an increase in fast MHC isoforms has been extensively described in the soleus after denervation (suppression of both afferent and efferent messages) but the results of these studies were very conflictual and seemed to depend on the length of the denervation period [8, 12, 15, 16, 22]. After 2 weeks of denervation, the authors described changes, on the soleus, generally restricted to a shift of MHC 1 to MHC 2A isoform. In most of these studies, the fast types 2A and 2D/X comigrated because of the methodology used to separate these two isoforms. Hence it is possible that, in these studies, the increase also concerned the MHC 2D/X. However, Huey and Bodine [15] performed a time course of soleus denervation (2, 4, 7, 10 and 30 days) and showed, after 30 days only, a measurable expression of MHC 2D/X (9.4% of total protein) isoform. The expression of the MHC 2B protein in the denervated soleus was even less described. For instance, Huey and Bodine [15] did not show the appearance of MHC 2B, neither at the protein nor at the mRNA level and whatever the duration of denervation. After spinal cord transection, Talmadge et al. [33] demonstrated in soleus the relative increase by 8% of MHC 2B. Thus, as changes in myosin expression were more marked in deefferentation than in denervation conditions, we could suggest that specific suppression of motor innervation appeared as a key factor to trigger off marked transitions in normal soleus myosin pattern. The fact that MHC 2A composition was unchanged would suggest that the step of transition from MHC1 to MHC2A isoform was already achieved after deefferentation while that from MHC 2A to MHC 2D and 2B was in process.

At the single fiber level, the increase in the relative amount of fast type MHC isoforms was reflected by a significant multiplicity of “hybrid” fast type fiber populations coexpressing MHC 1 and MHC 2A, and especially, the emergence of pure fast fibers expressing only 2A or coexpressing 2D/X and 2B isoforms. Thus the scheme for MHC conversion should occur in the order MHC 1 MHC 2A MHC 2D/X MHC 2B, already suggested in rat soleus muscle after unloading conditions [31]. However, we never observed fibers with MHC 2A and MHC 2D/X combinations, or fibers

expressing MHC 2B alone. This could possibly be related to the lack of significant modification in MHC 2A relative amount in whole muscle study, suggesting that some fibers had the opportunity to " jump" certain steps of transformation, as previously proposed after soleus spinal cord transection [33].

The study of soleus MHC isoforms was completed by the analysis of MLC isoforms. As already described, MLC followed the changes obtained at MHC level [4]. This suggested that deefferentation could induce a multiplicity of fiber types at the isomyosin level, with a possible coexistence of fast and slow MLC isoforms in combination with one or more MHC ones.

Another effect of deefferentation was the specific atrophy of some fiber types. Indeed, fast fibers exhibited the greatest changes: significant decreases in diameter and absolute maximal tension while slow fibers only showed a significant smaller diameter. These fast fibers that were surexpressed after deefferentation conditions, might be a mixed population of initially fast fibers affected by deefferentation (all fast type fibers were atrophied, had their T/pCa relationship shifted to the right and an elevated Vmax), and/or of initially slow fibers that had transformed into fast fibers. Similarly, the fibers that were slow (S) after deefferentation might be a mixing of initially S fibers that had not transformed and/or fibers that were just undergoing changes.

Deefferentation resulted in a reduction in Ca affinity of both slow and fast fibers, which has already been found after denervation [21] or unloading [9, 30] conditions. The exact mechanism for this rightward shift in T/pCa relationships remains unknown. One possibility is the expression of protein fast isoforms in Deeff fibers [19]. However, S Cont and S Deeff fibers expressed the same MHC 1 profile but a different value of Ca affinity. Even if it is generally described that all the myofibrillar protein isoforms tend to be coexpressed with those of the MHC [25], we could not rule out the hypothesis that the regulatory proteins, such as Troponin C, particularly responsible for Ca affinity (pCa_{50} parameter) [23] did not follow the same change course as MHC. Then it might be possible that S Cont and Deeff fibers did not express the same TnC profiles and therefore had different T/pCa positions.

The analysis of the T/pCa relationships of fast Deeff fiber populations was more complicated. We found that, for the fast Deeff fibers, the pCa_{50} values evolved in the following order: HF (2A>1) < F (2A) < F (2D/X-2B). This had already been described by Bottinelli et al. [2] in fibers with increasing fast protein isoforms and would suggest that the decrease in Ca affinity was related to the MHC isoform transitions. Thus, the molecular mechanisms underlying the changes in Ca affinity seemed to be complex, and other hypotheses than isoform changes could be put forward after deefferentation, such as a modification of the phosphorylation state of the myofilaments [14] or an altered interfilament lattice spacing [19].

Deefferentation induced a global increase of Vmax. This effect had to be related to the fact that i) deefferentation was found to enlarge the repartition of Vmax values from S and HF populations, and ii) two new populations of fibers appeared: pure fast fibers expressing MHC 2A and MHC 2D/X + MHC 2B isoforms. A good correlation has generally been described between Vmax and MHC isoforms [27]. Here, the distribution of Vmax values appeared more continuous (from 0.67 to 3.30 FL/s) when the Deeff fibers expressed MHC 1, MHC 2A>1, or MHC 2A alone, than when fibers expressed MHC 2D/X and MHC 2B. According to Bottinelli et al. [1], the existence of multiple hybrid fibers (here, in Deeff muscles) could provide an explanation for the overlapping velocities.

Finally, transitions in fiber types were also described in the case of neuromuscular diseases, even if these have generally been described in the mouse model. Indeed, experiments carried out on the mouse soleus, expressing normally a fast phenotype, showed in pathological conditions the induction of an atrophy, and a transition in the fast-to-slow order [20, 28, 32]. In deefferentation conditions, our results showed that the slow-type soleus muscle of the rat undergoes fiber type transitions in the slow-to-fast direction. Therefore, a deefferentation procedure applied to a rat slow muscle can be used to induce graded transformations in a large range of protein isoform expression, which might be helpful to yield important insights in the pathogenesis of motoneuron diseases, and thus aid in the development of eventual new treatment strategies for this kind of diseases.

Conclusion

The present work permitted not only to bring for the first time basal data for a rat model of motoneuron deficiency, but also to underline the important role of the motor nervous message in the regulation of muscle phenotype and in the mechanisms underlying muscle plasticity. Furthermore, since MHC (as well as other contractile proteins) phenotype was very much affected and exerted a profound impact on the contractile properties, future work would be necessary to focus on the process whereby protein turnover is regulated, and more particularly, on the transcriptional or translational factors involved in muscle protein gene regulation.

Acknowledgements

The authors wish to thank V. Montel for technical assistance. This work was supported by the Centre National d'Etudes Spatiales (grant no. 99-3027), the Fonds Europeen de Développement Régional (grant no. F007)

and the Association Francaise contre les Myopathies (grant no. 7109).

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