Early changes of type 2B fibers after denervation of rat EDL skeletal muscle

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Germinario, Elena, Alessandra Esposito, Aram Megighian, Menotti Midrio, Donatella Biral, Romeo Betto, and Daniela Danieli-Betto. Early changes of type 2B fibers after denervation of rat EDL skeletal muscle. J Appl Physiol 92: 2045–2052, 2002. First published December 21, 2001; 10.1152/japplphysiol.00673.2001.—Skeletal muscle type 2B fibers normally receive a moderate level of motoneuron discharge. As a consequence, we hypothesize that type 2B fiber properties should be less sensitive to the absence of the nerve. Therefore, we have investigated the response of sarcoplasmic reticulum and myofibrillar proteins of type 2B fibers isolated from rat extensor digitorum longus muscle after denervation (2 and 7 days). Single fibers were identified by SDS-PAGE of myosin heavy chain isoforms. Electrophysiological and isometric contractile properties of the whole muscle were also analyzed. The pCa-tension relationship of type 2B single fibers was shifted to the left at 2 days and to right at 7 days after denervation, with significant differences in the Hill coefficients and pCa threshold values in 2- vs. 7-day-denervated fibers. The sarcoplasmic reticulum Ca2+ uptake capacity and rate significantly decreased after 2 days of denervation, whereas both increased at 7 days. Caffeine sensitivity of sarcoplasmic reticulum Ca2+ release was transitory and markedly increased in 2-day-denervated fibers. Our results indicate that type 2B fiber functional properties are highly sensitive to the interruption of nerve supply. Moreover, most of 2-day-denervated changes were reverted at 7 days.

calcium sensitivity; chemically skinned muscle fibers; sarcoplasmic reticulum; extensor digitorum longus

SKELETAL MUSCLES ARE COMPOSED of a variety of fiber types with different morphological, biochemical, and functional characteristics (36, 42). During embryonic and postnatal developments, differentiation into the different fiber types is mainly controlled by innervation, with the pattern and amount of neuromotor impulses playing a major role (21, 35, 42), although the weight of neurotrophic factors cannot be excluded. Therefore, it is not surprising that denervation causes profound changes to skeletal muscle properties. Some alterations, related to sarcoplasmic reticulum properties, occur very early after denervation, whereas alterations of the myofibrillar proteins profiles need, in general, more time. We have, in fact, previously described changes in sarcoplasmic reticulum properties of the slow-twitch type 1 fibers from denervated soleus muscle showing that the maximum Ca2+ uptake capacity and rate were reduced after 2 days of denervation and increased at 7 days (33). Moreover, the initial Ca2+ release rate was reduced in 2-day-denervated type 1 fibers. These changes in sarcoplasmic reticulum properties were related to mechanical changes of the denervated muscle.

In this study, we examined whether also the sarcoplasmic reticulum properties of fast-twitch fibers are nerve dependent and, therefore, need an intact innervation. We focused our attention to type 2B fibers from the extensor digitorum longus (EDL) muscle, i.e., to fibers that, within the continuum of fiber types (1→2A→2X→2B), represent the phenotype with the lower functional demand and thus less stimulation by the nervous system, as opposed to type 1 fibers that are continuously recruited (21). The low level of neuromotor activity discharged over type 2B fibers could suggest that these fibers are less dependent on innervation than type 1 fibers. However, data in the literature on type 2B fiber nerve dependence are very divergent. According to some authors, the expression of type 2B myosin heavy chain (MHC) occurs both in the absence of mechanical load (47) and of neural stimulation (8, 32, 37), leading to the conclusion of type 2B fibers as a default phenotype (42). Diversely, other authors demonstrated that the presence of the nerve is essential for postnatal differentiation of fibers from type 1 to type 2B, especially in rat fast-twitch muscles (1). Moreover, TTX paralysis of mouse fast-twitch muscles causes the reduction of fibers expressing type 2B MHC isoforms (29), although this result was not completely confirmed by others (9). The presence of the nerve appears to be necessary for the de novo expression of type 2B MHC isoform in rat soleus, as induced by a combination of hindlimb suspension and hyperthyroidism (15). Moreover, denervation causes the reduction of fibers expressing type 2B MHC isoform in rat diaphragm (19) and in rat plantaris muscle (20).

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The main goal of the study was to investigate whether and how sarcoplasmic reticulum properties of type 2B fibers from the fast-twitch EDL muscle are altered by denervation. We also studied, in denervated single fiber, the Ca\(^{2+}\) sensitivity of myofibrillar proteins. To evaluate any possible relationships with the properties of denervated muscle, we extended our study at the whole muscle level to examine mechanical and electrophysiological properties.

Our results indicate that denervation causes significant modifications of sarcoplasmic reticulum and myofibrillar properties of type 2B fibers even during the first week of denervation. Moreover, most of the alterations observed at 2 days are reverted at 7 days after denervation. Therefore, these data support the idea of the dependence of type 2B phenotype on nerve activity and/or trophic factors.

**METHODS**

The study was approved by the Ethics Committee of the Medical Faculty of the University of Padova. The experiments were carried out on Wistar adult rats (3–6 mo old). The sciatic nerve was cut unilaterally at the level of the trochanter, while the animals were under ether anesthesia, and ~1.0 cm of the peripheral nerve stump was removed to avoid reinnervation. Two or seven days later, the animals were killed, always under ether anesthesia, and the EDL muscle of both limbs was then excised and utilized for the experimental analyses.

**Single-Fiber Preparation**

Muscle fibers were chemically skinned as previously described (33, 40). Briefly, muscles were tied to a wooden stick and quickly immersed in an ice-cold skinning solution containing (mM): 170 potassium propionate, 2.5 magnesium propionate, 2.5 Na\(_2\)K\(_2\)ATP, 5 K\(_2\)EGTA, and 10 imidazole buffer, pH 7.0. The skinned fibers were stored at ~20°C for no more than 4 wk in a skinning solution supplemented with 50% (vol/vol) glycerol, and, to minimize differences in mechanical and biochemical fiber properties as a result of unpredictable effects of the experimental procedure, attention was paid to store control and denervated preparations for the same period.

**Single-Fiber Functional Studies**

Ca\(^{2+}\) sensitivity of tension development. pCa-tension curves (pCa is \(-\log\) Ca\(^{2+}\) concentration) were obtained by exposing the fiber sequentially to solutions with different free Ca\(^{2+}\) concentration (33), calculated by using apparent association constants described by Orentlicher et al. (34). Single-fiber segments (~1.5 mm length) were inserted between two clamps, one fixed and the other connected to a tension transducer (model 801, AK Sensonor, Horten, Norway). Fibers were immersed in a relaxing solution, at room temperature (22–24°C), with the following composition (mM): 170 potassium propionate, 2.5 magnesium propionate, 5 Na\(_2\)K\(_2\)ATP, 5 K\(_2\)EGTA, and 10 imidazole buffer, pH 7.0. Fibers were stretched manually to a sarcomere length of ~3 μm as measured by light diffraction with a helium-neon laser lamp (33). The tension generated in each pCa solution was continuously recorded, and the baseline tension was established as the steady-state voltage output recorded with the fiber in the relaxing solution. The minimum tension accepted to identify pCa threshold level was 5% of the maximum tension (pCa 5).

Specific tension for each single fiber was calculated by normalizing the maximum tension measured at pCa 5 to the fiber cross-sectional area, as calculated by three different diameter determinations along the fiber length, considering the fiber immersed in solution as a cylinder.

Sarcoplasmic reticulum caffeine response. The caffeine threshold for sarcoplasmic reticulum Ca\(^{2+}\) release was determined as previously described (10, 33). The minimum tension accepted to identify caffeine threshold was 5% of the maximum tension measured with 20 mM caffeine. Fibers were first incubated for 30 s in a Ca\(^{2+}\) loading solution (pCa 7.0 solution). Then, the fibers in a relaxing solution deprived of EGTA were challenged in a stepwise fashion with increasing concentrations of caffeine until tension was recorded, thus identifying the caffeine concentration threshold (10, 33, 41).

Sarcoplasmic reticulum Ca\(^{2+}\) accumulation properties. Ca\(^{2+}\) uptake by the fiber sarcoplasmic reticulum was measured at room temperature (22–24°C) either by a light-scattering method (39, 41), as previously reported (33), or by a caffeine contracture method (51).

With the first method, fibers were mounted in a chamber containing the relaxing solution and stretched to 180% of slack length to avoid interference in light-scattering measurements caused by actin-myosin interactions. Fibers were then incubated in a Ca\(^{2+}\) loading solution (pCa 6.4). Ca\(^{2+}\) loading activity by the sarcoplasmic reticulum was measured after the increase of the fiber light scattering after the addition of 5 mM oxalate (45). The plateau level of light scattering represents the maximum capacity for Ca\(^{2+}\) uptake of the sarcoplasmic reticulum (40, 45). The calibration procedure for converting the light-scattering signal to fiber Ca\(^{2+}\) concentration was performed by using \(80\text{Ca}^{2+}\) as described in detail (40). The relative increase in light scattering was proportional to the Ca\(^{2+}\) concentration inside the fiber. The proportionality constants for control, 2-day-denervated, and 7-day-denervated fibers were 0.199 ± 0.035 (n = 6), 0.159 ± 0.032 (n = 5), and 0.210 ± 0.031 (n = 6) nmol \(80\text{Ca}^{2+}\)–light-scattering unit \(^{–1}\)μg fiber protein \(^{–1}\), respectively. Accordingly, these values were used in the analysis of data obtained under the relative experimental condition.

The caffeine contracture method is an indirect technique that allows estimating the amount of Ca\(^{2+}\) stored in the sarcoplasmic reticulum after various periods of Ca\(^{2+}\) loading (51). The fibers were first exposed to the relaxing solution containing 20 mM caffeine to deplete the sarcoplasmic reticulum of Ca\(^{2+}\). After being rinsed in the relaxing solution, the fiber was incubated in pCa 7.0 loading solution for 15, 30, 60, 120, and 180 s. The amount of Ca\(^{2+}\) sequestered by the sarcoplasmic reticulum was then determined by exposing the fiber to 20 mM caffeine in a relaxing solution deprived of EGTA. For each fiber, the contracture-force was plotted against loading duration and data were fit, via nonlinear regression \(r^2 = 0.93–0.99\), to an equation of the form \(F = 100(1 − e^{−t/\tau})\), where \(F\) is the normalized contracture force, \(K_{\text{Ca}}\) is the rate constant for Ca\(^{2+}\) uptake, and \(t\) is the loading duration.

**SDS-PAGE of Single Fibers and Whole Muscle**

All of the fibers investigated by the above-described procedures were subsequently analyzed by 7% PAGE and identified by MHC isoforms expressed (11, 13). Briefly, after each analysis, single-fiber segments were placed into a microcapillary and dissolved with 20 μL of SDS-PAGE solubilization buffer (12). Five microliters of each sample (20 samples for each run) were electrophoresed, and proteins were revealed.
by silver staining (11, 13). In the present work, only type 2B fibers were considered, i.e., fibers containing only the 2B MHC isoform and fibers containing the 2B plus minimal traces (<10%) of type 2X MHC (Fig. 1). Our data indicate that the properties of the two groups of fibers did not reveal appreciable differences.

Analysis of MHC isoform of the whole muscle was performed by the method described by Talmadge and Roy (48). Twenty cryostat sections (20 μm in thickness), from control and denervated muscles, were collected in an Eppendorf tube, weighed, and dissolved at a concentration of 2 mg/ml in SDS-PAGE solubilization buffer. Forty micrograms of each sample were electrophoresed on 8% SDS-PAGE slab gels, and protein bands were revealed by silver staining. MHC isoform composition was determined by densitometry of slab gels by using a Bio-Rad imaging densitometer (GS-670).

Myosin light chain and parvalbumin composition was analyzed by two-dimensional gel electrophoresis as previously described (5). Briefly, 20 cryostat sections (16 μm in thickness), from control and denervated muscles, were dissolved in 100 μl of 9.5 M urea, 2% (vol/vol) Nonidet NP-40, 5% (vol/vol) 2-mercaptoethanol, and 1.0% (vol/vol) Pharmalyte (Pharmacia Biotech) of pH range 5–7, 1.0% (vol/vol) Pharmalyte (Pharmacia Biotech) of pH range 3.5–10, and were subjected to isoelectric focusing. SDS-PAGE in the second dimension was performed in 15% (wt/vol) polyacrylamide slab gels. The gels were then stained with Coomassie brilliant blue G250 (Sigma Chemical) and destained, and the relative amount of each protein band (myosin light chain isoforms and parvalbumin) was determined by densitometry of SDS-PAGE slab gels by using a Bio-Rad imaging densitometer (GS-670).

**Whole Muscle Mechanical Characterization**

Contractile properties were investigated in vitro at 22 ± 1°C. Muscle bathing, stimulation conditions, and tension recording were performed as previously described (31, 33). Twitches were obtained by applying single supramaximal stimuli (0.5 ms duration), whereas tetani were obtained by applying trains of stimuli at 80 Hz frequency. The following parameters were measured: contraction time, half-relaxation time of the twitch, twitch tension, and tetanic tension. Twitch tension and tetanic tension were normalized to cross-sectional area of the muscle (specific tension; in mN/mm²). The maximum rate of rise of tetanus was also measured. Muscle responses were recorded via an AT-MIO 16 AD card, and data were analyzed by the LabView computer program (National Instruments).

**Electrophysiology**

Resting membrane potential and action potential were measured in vitro at 22 ± 1°C as previously described (33).

No more than one action potential could be generally recorded from a stimulated single fiber without signs of membrane damage as revealed by a decrease of resting membrane potential. The maximum rate of rise and the maximum rate of fall of the action potential were determined by computing the first derivative of the digitized spike. The conduction velocity of the action potential along the fiber was calculated on the computer-restored cathodic ray oscilloscope tracings by relating the measured distance between the recording and stimulating electrodes, by using a micrometer lens mounted on a stereomicroscope, with the time interval between the stimulus artifact and the action potential onset. The latter was established to begin at the point of intersection of the extrapolated steepest part of the rising phase of action potential with the baseline (7).

**Statistical Analysis**

Means ± SE were calculated from individual values by standard procedures. Results were analyzed by one-way analysis of variance performing multiple comparisons against the control group (SigmaStat, Jandel Scientific). The 0.05 level of probability was established for statistical significance. pCa-tension data were fitted by a least squares method using the Table Curve fitting program (Jandel Scientific), according to the following equation: y = max xH1/(xH1 + kH1), where max is the maximal value of pCa-tension curve, which was normalized to 1; k is the pCa at 50% of maximum tension (i.e., pC50); x and y refer to coordinates shown in Fig. 2; and nH is the Hill coefficient.

**RESULTS**

**Myofibrillar Protein Ca²⁺ Sensitivity of Single Denervated Fibers**

The second day after denervation, type 2B fibers showed a left shift of the pCa-tension curves, which then reversed in direction by displaying a moderate right shift after 7 days (Fig. 2). The pCa threshold for tension development of 2-day-denervated type 2B fibers was almost identical to that of control fibers.

![Fig. 1. SDS-PAGE analysis of myosin heavy chain (MHC) isoform composition of extensor digitorum longus single skeletal muscle fibers. Lane a, MHC isoforms from mixed soleus and extensor digitorum longus muscle cryostat sections; lanes b–e, MHC isoforms of single extensor digitorum longus muscle fibers; lane e, MHC isoform of a soleus muscle fiber.](image-url)
[6.31 ± 0.03 (n = 35) and 6.34 ± 0.04 (n = 18), respectively], whereas it was significantly increased at 7 days [6.15 ± 0.03 (n = 21); *P < 0.05, vs. both control and 2-day-denervated type 2B fibers]. The *pCa*₅₀ was not significantly changed during the first week of denervation [5.93 ± 0.04 (n = 35), 5.99 ± 0.07 (n = 18), and 5.89 ± 0.03 (n = 21) in control, 2-day-denervated, and 7-day-denervated fibers, respectively]. The Hill coefficient, an estimate of cooperative interactions between myofibrillar elements (11), showed a significant reduction (*P < 0.05) after 2 days [2.28 ± 0.17 (n = 18)] compared with control [2.93 ± 0.14, (n = 35)] and an increase after 7 days [3.32 ± 0.20, (n = 21); *P < 0.05 vs. the 2-day-denervated fibers].

The specific tension of single skinned fibers, calculated by normalizing the maximum tension to the fiber cross-sectional area, was not modified at 2 days [13.8 ± 1.4 N/cm² (n = 19)] and was slightly reduced at 7 days [10.9 ± 0.7 N/cm² (n = 16)], compared with control [12.6 ± 1.4 N/cm² (n = 17)].

The rate of tension development of denervated type 2B fibers was transitory and significantly reduced 2 days after denervation with respect to the control, showing significant differences between 2-day- and 7-day-denervated fibers (*P < 0.01) [0.28 ± 0.04 (n = 17), 0.17 ± 0.02 (n = 11), and 0.29 ± 0.03 mN/s (n = 9) in control, 2-day denervated, and 7-day denervated, respectively].

**Two-Dimensional Analysis of Myosin Light Chains**

To evaluate whether phosphorylation of the regulatory (or phosphorylatable) myosin light chains may have affected the myofibrillar Ca²⁺ sensitivity of denervated EDL fibers (46), we performed two-dimensional analyses of myosin light chains. Figure 3 shows that phosphorylation of the regulatory light chain (2F-P) was not changed after 2 days, whereas it was greatly reduced after 7 days of denervation. Densitometric analyses confirm indeed that the percentage of phosphorylated 2F myosin light chain was 42.7 ± 3.2 (n = 9), 42.8 ± 4.6 (n = 6), and 27.6 ± 5.4 (n = 8) in control, 2 days after denervation, and 7 days after denervation, respectively, with the difference between control and 7-day-denervated muscle being significant (*P < 0.01). In addition, surprisingly, the light chain 3F-to-light chain 2F protein band ratio was significantly reduced (*P < 0.01) 2 days after denervation and returned to control values at 7 days [control, 0.25 ± 0.01 (n = 9), 2-day denervated, 0.17 ± 0.02 (n = 6); 7-day denervated 0.22 ± 0.02 (n = 8)].

Last, the expression level of parvalbumin progressively increased during the first week of EDL muscle denervation. In fact, the parvalbumin-to-myosin light chain 2 ratio was 0.65 ± 0.06 (n = 9), 0.78 ± 0.08 (n = 6), and 0.83 ± 0.05 (n = 8) in control, 2-day-denervated, and 7-day-denervated muscles, respectively, with the increase at 7 days being significant (*P < 0.05) compared with control.

**Sarcoplasmic Reticulum Ca²⁺ Uptake and Release**

Light-scattering analysis of sarcoplasmic reticulum properties of type 2B fibers shows that denervation caused a significant decrease in the sarcoplasmic reticulum Ca²⁺ accumulation capacity at 2 days after denervation. Importantly, this trend reversed at 7 days after denervation to produce a significant increase in the Ca²⁺ accumulation capacity (Table 1). Significant changes, i.e., a first decrease and then an increase, were also observed in the rate of Ca²⁺ uptake. Accordingly, *K*ₐ₅₀, calculated by the caffeine method (51), was 1.66 ± 0.11 (n = 12), 1.39 ± 0.13 (n = 9) and 2.18 ± 0.18 (n = 12) in control, 2-day-denervated and 7-day-denervated fibers, respectively, with a significant difference (*P < 0.05) between the denervated fibers (Fig. 4).

Similarly, sarcoplasmic reticulum Ca²⁺ release properties of type 2B fibers were also modified during the first week of denervation. After 2 days of denervation,}

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**Table 1. Ca²⁺ transport activities of the sarcoplasmic reticulum of type 2B fibers from denervated rat EDL muscle**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>2-day Denervated</th>
<th>7-day Denervated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca²⁺ uptake capacity, µmol/mg fiber protein</td>
<td>0.54 ± 0.06 (15, 3)</td>
<td>0.28 ± 0.03* (9, 3)</td>
<td>0.70 ± 0.06‡ (12, 3)</td>
</tr>
<tr>
<td>Rate of Ca²⁺ uptake, nmol min⁻¹·mg fiber protein⁻¹</td>
<td>43.6 ± 4.8 (15, 3)</td>
<td>24.3 ± 2.4 (9, 3)</td>
<td>62.6 ± 8.4‡ (12, 3)</td>
</tr>
</tbody>
</table>

Values are means ± SE; nos. in parentheses are no. of fibers and of animals, respectively. EDL, extensor digitorum longus. *P < 0.05; †P < 0.01 vs. controls. ‡P < 0.01 vs. 2-day-denervated fibers.
DENERVATION OF TYPE 2B FIBERS

Table 2. Contractile properties of denervated rat EDL muscle

<table>
<thead>
<tr>
<th></th>
<th>Control (10)</th>
<th>2-day Denervated (4)</th>
<th>7-day Denervated (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contraction time, ms</td>
<td>40.0 ± 1.1</td>
<td>41.3 ± 2.7</td>
<td>60.3 ± 3.7*‡</td>
</tr>
<tr>
<td>Half-relaxation time, ms</td>
<td>30.3 ± 1.9</td>
<td>26.6 ± 5.6</td>
<td>56.2 ± 5.7†‡</td>
</tr>
<tr>
<td>Twitch specific tension, mN/mm²</td>
<td>73.1 ± 5.5</td>
<td>60.9 ± 4.2</td>
<td>82.6 ± 6.1‡</td>
</tr>
<tr>
<td>Tetanic specific tension, mN/mm²</td>
<td>220.9 ± 14.7</td>
<td>176.2 ± 15.4</td>
<td>128.2 ± 6.1†‡</td>
</tr>
<tr>
<td>Maximum rate of rise of tetanus, mN/ms</td>
<td>8.90 ± 0.47</td>
<td>8.06 ± 0.22</td>
<td>4.75 ± 0.53†</td>
</tr>
</tbody>
</table>

Values are means ± SE; nos. in parentheses are no. of experiments. *P < 0.05 vs. control. †P < 0.001 vs. control. ‡P < 0.05 vs. 2-day-denervated muscles.

CONTRACTION and half-relaxation times were unmodified 2 days after denervation, whereas they were both significantly increased after 7 days (Table 2). Twitch tension, normalized to the cross-sectional area, showed a significant difference between 2 and 7 days of denervation. After 2 days, there was a small reduction (−16.7%) in twitch tension, whereas after 7 days, there was a small increase (+12.9%) compared with control. In contrast, tetanic tension progressively decreased throughout the entire denervation period studied. The maximum rate of rise of tetanus was significantly reduced in 7-day-denervated muscles (Table 2).

The mass of EDL muscle was slightly increased 2 days after denervation [168.7 ± 9.8 mg (n = 4)] compared with control [148.0 ± 6.4 mg (n = 10)] and slightly reduced after 7 days [131.2 ± 9.4 mg (n = 6)], in agreement with previous reports (43). The mean fibers radius was unmodified after 2 days (19.3 ± 0.3 µm, 162 fibers) and significantly decreased (P < 0.05) after 7 days (17.0 ± 0.2 µm, 180 fibers) compared with control (19.3 ± 0.2 µm, 225 fibers).

As expected by the very short denervation time, densitometric analysis of MHC isoform composition from SDS-PAGE gels demonstrated that MHC expression level was not significantly modified in the first week of denervation. The MHC of the EDL control contained 4.9 ± 0.7% 2A isoform, 25.8 ± 3.0% type 2X, 61.0 ± 2.9% type 2B, and 8.1 ± 0.9% type 1 (n = 6); the 2-day-denervated MHC contained 6.8 ± 2.2% 2A isoform, 19.9 ± 2.9% type 2X, 60.9 ± 1.0% type 2B, and 12.3 ± 1.8% type 1 (n = 3); and 7-day-denervated MHC contained 5.6 ± 0.9% 2A isoform, 30.8 ± 3.2% type 2X, 51.7 ± 6.6% type 2B and 11.9 ± 2.9% type 1 (n = 3).

Also in agreement with previous reports (2, 27), we observed that denervation induced substantial alterations of electrophysiological properties of EDL (Table 3). The resting membrane potentials were significantly reduced after 2 days, which persisted by 7 days. Major changes of action potentials were observed 7 days after

Fig. 4. Caffeine contracture force recorded after Ca\(^{2+}\) loading for various time periods. Values are means ± SE of data obtained from control (12 fibers, 3 muscles), 2-day-denervated (9 fibers, 3 muscles), and 7-day-denervated (12 fibers, 3 muscles) type 2B fibers. \(*P < 0.02, \text{7-day-denervated fibers vs. control.}\)

Fig. 5. Percentage distribution of caffeine threshold for sarcoplasmic reticulum Ca\(^{2+}\) release of control, 2-day-denervated, and 7-day-denervated extensor digitorum longus single type 2B muscle fibers. Total number of type 2B fibers examined was 43, 22, and 27, respectively, from 4 animals in each group. \(^*P < 0.05\) vs. control.

Type 2B fibers showed marked increase of the sensitivity to caffeine of sarcoplasmic reticulum Ca\(^{2+}\) release (Fig. 5). Specifically, the mean caffeine concentration able to induce Ca\(^{2+}\) release was significantly reduced in 2-day-denervated fibers from 8.95 ± 0.50 to 2.36 ± 0.32 mM (Fig. 5). The higher sensitivity to caffeine exhibited by denervated fibers was confirmed by the observation that the ratio between the tension developed by 20 mM caffeine and that developed at pCa 5, an estimate of the amount of Ca\(^{2+}\) released from the sarcoplasmic reticulum by caffeine (41), was significantly\((P < 0.05)\) increased in 2-day-denervated [1.07 ± 0.08 (n = 22)] and 7-day-denervated type 2B fibers \([0.98 ± 0.06 (n = 27)]\) compared with control \([0.80 ± 0.06 (n = 43)]\).

**Functional Changes of EDL Muscle During the First Week of Denervation**

Contraction and half-relaxation times were unmodified 2 days after denervation, whereas they were both significantly increased after 7 days (Table 2). Twitch tension, normalized to the cross-sectional area, showed a significant difference between 2 and 7 days of denervation. After 2 days, there was a small reduction (−16.7%) in twitch tension, whereas after 7 days, there was a small increase (+12.9%) compared with control. In contrast, tetanic tension progressively decreased throughout the entire denervation period studied. The maximum rate of rise of tetanus was significantly reduced in 7-day-denervated muscles (Table 2).

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Also in agreement with previous reports (2, 27), we observed that denervation induced substantial alterations of electrophysiological properties of EDL (Table 3). The resting membrane potentials were significantly reduced after 2 days, which persisted by 7 days. Major changes of action potentials were observed 7 days after...
Table 3. Electrophysiological properties of denervated rat EDL muscle

<table>
<thead>
<tr>
<th></th>
<th>Control (22, 3)</th>
<th>Denervated (17, 3)</th>
<th>7-day Denervated (15, 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RMP, mV</strong></td>
<td>-72.6 ± 1.2</td>
<td>-65.4 ± 1.3*</td>
<td>-61.5 ± 1.0*</td>
</tr>
<tr>
<td><strong>MRR, V/s</strong></td>
<td>146.9 ± 10.3</td>
<td>120.8 ± 13.2*</td>
<td>46.0 ± 5.0†</td>
</tr>
<tr>
<td><strong>MRF, V/s</strong></td>
<td>63.6 ± 2.9</td>
<td>46.4 ± 4.4*</td>
<td>20.5 ± 2.6†</td>
</tr>
<tr>
<td><strong>AP amplitude, mV</strong></td>
<td>84.7 ± 1.9</td>
<td>64.7 ± 2.4*</td>
<td>53.3 ± 2.7*</td>
</tr>
<tr>
<td><strong>Conduction velocity, m/s</strong></td>
<td>2.3 ± 0.2</td>
<td>1.8 ± 0.2*</td>
<td>1.0 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE; nos. in parentheses are no. of fibers and of animals, respectively. RMP, resting membrane potential; MRR, maximum rate of rise; MRF, maximum rate of fall; AP, amplitude of action potential. *P < 0.05 vs. control. †P < 0.05 vs. 2-day-denervated muscles.

denervation. The maximum rates of rise and fall of action potential were both reduced at 7 days by 69 and 68%, respectively, whereas the reductions at 2 days were smaller (18 and 27%). The amplitudes of action potentials and conduction velocities were also progressively reduced in a significant manner during the first week of denervation (Table 3).

**DISCUSSION**

The nerve-dependence of type 2B muscle fibers is a debated question, at least as far as the expression of MHC isoform is concerned. In fact, because type 2B fibers develop in the absence of both mechanical load and neural stimulation (8, 37, 42, 47), according to some authors, type 2B fibers should be considered a default phenotype. However, some recent reports show a definite role of innervation for the expression of type 2B MHC (15, 29) and for the postnatal differentiation of type 2B fibers (1).

The present work shows that, during early phases of muscle denervation, when changes in MHC expression are not evident yet, there are substantial modifications of myofibrillar and sarcoplasmic reticulum properties in type 2B fibers from fast-twitch muscle. These effects appear to be even more marked than those observed in denervated type 1 fibers from soleus muscle (33), and they contradict the hypothesis that type 2B fibers are insensitive to the action of the nerve. Because these changes of type 2B fiber properties occurred before any modifications in the MHC isoform expression, our results corroborate the opinion that early changes of muscle contractile properties are primarily due to alterations of the muscle activation process (24, 27, 31, 33, 43, 44, 53). Consistently, changes of the excitation-contraction coupling process were noted in the denervated rat EDL muscle (16).

Denervated type 2B fibers showed a higher myofibrillar Ca\(^{2+}\) sensitivity at 2 days and a lower sensitivity at 7 days. Ca\(^{2+}\) sensitivity of myofibrillar proteins of the different fiber types is distinctive according to the expression of specific regulatory proteins, in particular of troponin and tropomyosin isoforms (10, 35, 42). For example, threshold for tension development is mainly attributed to the specific fast or slow isoform of troponin C (35). Even though 1-wk denervation of hindlimb muscles is not enough to change the expression level of myofibrillar proteins of fast muscles (23, 26), Ca\(^{2+}\) sensitivity could be influenced by several other factors and mechanisms that may operate in denervated muscle, like posttranscriptional modifications of involved proteins that include oxidation of sulfhydryl groups (3, 52), glycation (22), deamination (4), and phosphorylation of myofibrillar proteins. It is indeed known that phosphorylation of myosin light chain 2, by a specific Ca\(^{2+}\)/calmodulin-dependent protein kinase, causes a leftward shift of the pCa-tension relationship in fast-twitch fibers (28, 46). Our data show that the level of myosin light chain 2F phosphorylation significantly decreased 7 days after denervation, providing a possible explanation for the rightward shift of pCa-tension curves. The lower phosphorylation level of myosin light chain 2F we noted is consistent with the reported broad decrease of phosphorylation of fast-phenotype markers during denervation (50). It is interesting to note that 2 days of denervation seem enough to alter myosin light chain 3F-to-myosin light chain 2F stoichiometric ratio. Because a relationship between the maximal shortening velocity and the amount of myosin light chain 3F (6, 18) has been demonstrated, the lower expression level of myosin light chain 3F could explain, at least in part, the reduced rate of tension developed by the type 2B fibers 2 days after denervation.

After 2 days of denervation, we observed a significant decrease of sarcoplasmic reticulum Ca\(^{2+}\) uptake capacity and rate of type 2B fibers, followed by a marked increase at 7 days. Apparently, the alterations on Ca\(^{2+}\) uptake rate cannot be attributed to modifications in Ca\(^{2+}\)-ATPase content, because no changes in the expression of both sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase Ca\(^{2+}\)-pump isoforms (SERCA-1 and SERCA-2) occur during the first week of denervation (43). On the other hand, the rise of Ca\(^{2+}\) uptake capacity in the 7-day-denervated type 2B fibers is consistent with the increase of calcequestrin-to-Ca\(^{2+}\)-pump protein ratio (38) and the proliferation of junctional sarcoplasmic reticulum membranes reported in 15-day-denervated rabbit fast muscle (53).

Changes of sarcoplasmic reticulum Ca\(^{2+}\) release properties in 2-day-denervated fibers is likely associated to the substantial elevation of sensitivity to opening of the Ca\(^{2+}\) release channel, as indicated by the higher caffeine sensitivity of Ca\(^{2+}\) release. Because, in leakage tests performed according to Trachez et al. (49), the spontaneous Ca\(^{2+}\) release was smaller in fibers from 2-day-denervated muscles than in controls, it is unlikely that the changes are related to membrane leakiness (unpublished observations).

Therefore, our results indicate that the SR Ca\(^{2+}\) uptake and release, key properties in the excitation-coupling process, are under nervous control in type 2B fibers. Fiber Ca\(^{2+}\) sensitivity also appears to be affected by innervation. The observed changes in type 2B fibers after denervation were more marked than in type 1 fibers from soleus muscle (33). Taking into
account the very low level of neuromotor activity discharged on type 2B with respect to type 1 fibers (21), it is possible that 2B fibers are particularly sensitive to the lack of nerve trophic factors, as hypothesized by others (14, 19, 20).

Moreover, because in type 2B fibers, as early as 2 days after denervation, alterations take place that partly disappear (sensitivity to caffeine of sarcoplasmic reticulum Ca$^{2+}$ release, myosin light chain 3F expression level) and partly reverse (Ca$^{2+}$ sensitivity of myofibrillar proteins and sarcoplasmic reticulum Ca$^{2+}$ accumulation properties) 7 days after denervation, one can hypothesize that the initial dramatic effects of inactivity caused by nerve deprivation are attenuated or even reverted by muscle fibration, a cell membrane electric activity that mimics a chronic low-level stimulation and that is known to occur at 2–3 days after denervation (30).

Last, our results confirm that denervation causes substantial early changes of contractile and membrane properties of the whole rat fast-twitch EDL muscle (17). Differently from soleus muscle (33), and despite of the observed modifications in sarcoplasmic reticulum and myofibrillar properties, changes of contractile properties were not evident at 2 days of denervation. It is possible that the alterations of the membrane and of myofibrillar and sarcoplasmic reticulum properties, detected in single type 2B fibers abolish each other’s effects, resulting in the lack of significant alterations in the contractile properties of the whole muscle. On the other hand, in the 7-day-denervated EDL muscle, we observed a general slowing of contractile properties with a significant increase of contraction and relaxation times, as well as of the maximum rate of rise of tension. The observed increase of whole muscle parvalbumin level in the 7-day-denervated EDL muscle, expected to contribute to shorten the relaxation time (25), probably only attenuates the slowing of contractile properties. Moreover, the resting membrane potential, the amplitude of the action potential and the conduction velocity were progressively reduced during the first week of denervation. Because MHC isoform composition does not change in the first week of denervation (our results; Ref. 23), the reduction of maximal rate of rise of tension, a property normally ascribed to actin-myosin cycling rate (35, 42), could be attributed to posttranscriptional modifications, such as, for example, the reduction of myosin light chain 2F phosphorylation. It is also possible that the reduction of impulse conduction velocity contributed to the reduction of the rate of rise of tension, as has been proposed for the denervated fast muscle of the cat (27).

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