Denervation produces different single fiber phenotypes in fast- and slow-twitch hindlimb muscles of the rat

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Patterson, M. F., G. M. M. Stephenson, and D. G. Stephenson. Denervation produces different single fiber phenotypes in fast- and slow-twitch hindlimb muscles of the rat. Am J Physiol Cell Physiol 291: C518–C528, 2006. —Using a single, mechanically skinned fiber approach, we tested the hypothesis that denervation (0 to 50 days) of skeletal muscles that do not overlap in fiber type composition [extensor digitorum longus (EDL) and soleus (SOL) muscles of Long-Evans hooded rats] leads to development of different fiber phenotypes. Denervation (50 day) was accompanied by 1) a marked increase in the proportion of hybrid IIB/D fibers (EDL) and I/IIA fibers (SOL) from 30% to >75% in both muscles, and a corresponding decrease in the proportion of pure fibers expressing only one myosin heavy chain (MHC) isoform; 2) complex muscle- and fiber-type specific changes in sarcoplasmic reticulum Ca2+-loading level at physiological pCa ~7.1, with EDL fibers displaying more consistent changes than SOL fibers; 3) decrease by ~50% in specific force of all fiber types; 4) decrease in sensitivity to Ca2+, particularly for SOL fibers (by ~40%); 5) decrease in the maximum steepness of the force-pCa curves, particularly for the hybrid I/IIA SOL fibers (by ~35%); and 6) increase occurred of biphasic activation with respect to Ca2+ sensitivity in SOL fibers, indicating the presence of both slow and fast troponin C isoforms. No fiber types common to the two muscles were detected at any time points (day 7, 21, and 50) after denervation. The results provide strong evidence that not only neural factors, but also the intrinsic properties of a muscle fiber, influence the structural and functional properties of a particular muscle cell and explain important functional changes induced by denervation at both whole muscle and single cell levels.

Mechanically skinned fibers; myosin heavy chain isoforms; lineage; sarcoplasmic reticulum; Ca2+ and Sr2+ sensitivity; Long-Evans hooded rat

Mammalian skeletal muscle fibers display a broad spectrum of structural and functional characteristics determined by the complement of homologous, but not identical, molecular structures involved in the excitation-contraction-relaxation cycle (33, 34, 41). Cross-innervation (4, 6), denervation (16, 17, 26, 28, 37), and chronic low-frequency stimulation (7, 20, 33, 34) experiments have produced compelling evidence that the pattern of neural stimulation plays a crucial role in determining the functional and/or structural properties of skeletal muscle (38). However, neural control of fiber phenotype does not extend to the entire complement of cellular structures responsible for muscle fiber function, as demonstrated by cross-innervation studies. For example, when the extensor digitorum longus (EDL) muscle of the rat, a typically fast-twitch muscle, was cross-innervated by the nerve of the soleus (SOL) muscle, a typically slow-twitch muscle, the twitch time course of the cross-innervated muscle remained considerably faster than the twitch of the typical SOL muscle, even 16 mo post cross-innervation (time to peak ~25 ms in 480-day cross-innervated EDL muscle vs. ~35 ms in SOL muscle and ~15 ms in EDL muscle; Refs. 5 and 6). This suggests that factors other than the neural input may contribute to the fiber phenotype.

Although it has been suggested that there are at least as many fiber types as there are motor units in the body of an animal (33, 34), skeletal muscle fibers are primarily classified based on the type of molecular motor protein isoform expressed in that fiber. The molecular motor protein in skeletal muscle is the myosin heavy chain (MHC) and according to the nomenclature used in our laboratories, the MHC isoform detected in a muscle or muscle fiber is identified by a Roman numeral and a lowercase letter (e.g., MHC Ia). A fiber containing only one MHC isoform is referred to as pure fiber and is identified by a Roman numeral and a capital letter (e.g., IIA fiber), whereas a fiber expressing more than one MHC isoform is referred to as a hybrid fiber and is identified by a composite symbol (e.g., I/IIA and IIB/D). Typically, the fastest/glycolytic to the slowest/oxydative rat limb muscle fibers express the MHC isoforms: Iib ↔ Iib/IId ↔ IId ↔ IId/IIda ↔ IIda ↔ IIa ↔ IId/IIda ↔ IId/IIda ↔ IId/IIda ↔ IIa ↔ I, with fibers expressing type II MHC isoforms generally regarded as fast (fast-twitch) fibers and fibers expressing MHC I generally regarded as slow (slow-twitch) fibers.

Recent experiments on regenerated EDL and SOL muscles have revealed that denervated EDL and SOL stimulated with the same slow stimulus pattern expressed slow and fast MHC isoforms in different proportions, which led to the interpretation that the satellite cells in the EDL and SOL muscles from which the regenerated muscles originate, have intrinsically different properties (19). If the precursor cells from which the regenerated muscles develop have different intrinsic properties with respect to factors that affect gene expression and the mechanism regulating transcription (see DISCUSSION), then one could hypothesize that on denervation, when the neural input is removed, the mature muscle cells from EDL and SOL muscles would converge to form different fiber phenotypes. To test this, we used a single fiber approach to examine fiber phenotypes from denervated EDL and SOL muscles not only with respect to the MHC isoform profile but also with respect to Ca2+-handling characteristics of the sarcoplasmic reticulum (SR) and contractile activation properties, which can also play an im-
important role in determining how fast the fiber can be activated and relax (41).

In the adult Long-Evans hooded rat, EDL and SOL muscles do not overlap in their patterns of MHC expression and fiber type composition, with the EDL muscle containing only type IIB and IID fibers and a small fraction of type IIB/D hybrid fibers (14) and SOL containing only type I and IIA fibers and a small fraction of type I/IIA hybrid fibers (14, 35). In this study we used mechanically skinned muscle fiber preparations from Long-Evans hooded rats to investigate MHC isoform expression, SR properties, and contractile activation characteristics at the single fiber level. The results show that at 50 days post-denervation, there was still no overlap in the fiber types present in the EDL and SOL muscles, and that >75% of the fibers in both the EDL and SOL muscles became hybrid with respect to MHC composition, with the hybrid EDL fibers co-expressing MHC isoforms IIB and IID and the hybrid SOL fibers co-expressing MHC isoforms I and IIA. These hybrid fibers also displayed different properties with respect to Ca\(^{2+}\) activation of the contractile apparatus and SR Ca\(^{2+}\) handling, but not with respect to the maximum level of specific force produced. The results are important as they provide strong evidence that not only neural factors but also the intrinsic properties of the muscle fiber determine the functional properties of a particular muscle cell.

**MATERIALS AND METHODS**

**Animals**

Two groups of male Long-Evans hooded rats aged 4–6 mo were used: 1) a control group of 6 nonoperated rats and 2) a denervated group of 17 right-side-denervated rats. Denervation of lower leg muscles was performed while the rats were under halothane anesthesia by removal of 3–5 mm of the sciatic nerve below the sciatic notch, as previously described (31). The contralateral (left) hindlimb muscles of denervated animals were not used as control muscles due to altered weight-bearing effects. Similarly, muscles from sham-operated animals were not used because altered muscle use in the sham-operated hindlimb was likely to have affected normal muscle functioning, particularly in the first week after the procedure. All work with animals was approved by La Trobe University’s Animal Ethics Committee.

**Muscle Dissection and Skinned Fiber Preparation**

At the required time [day 0 (control), day 7, day 21, and day 50 post-denervation], the animals were euthanized by halothane overdose and the EDL and SOL muscles were dissected, blotted on filter paper, and placed under paraffin oil. Single fibers were isolated and mechanically skinned under a dissection microscope, as previously described (32). The mechanically skinned fibers were then attached between a forceps fixed to a micromanipulator. The length and the average diameter of the fiber, measured at \(\times 40\) magnification in three locations along the fiber, were then determined at slack length to allow calculation of the cross-sectional area (CSA) and volume of the preparation. The fiber was then placed in pre-release solution (Table 1).

**Solutions**

Solutions for skinned fiber experiments (Table 1) were prepared according to previously published methods (42). Solutions I and II were mixed in varying proportions to give activating solutions in the pCa \((-\log_{10}[Ca^{2+}])\) range of 7.2 to 4.0. Similarly, solutions I and II for the SR load-release experiments were mixed in varying proportions to give solutions in the pSr \((-\log_{10}[Sr^{2+}])\) range of 7.0 to 3.7. All other solutions in Table 1 were used in SR load-release experiments.

**SR Load-Release Experiments**

After being mounted, fibers were exposed for 2 min to pre-release solution and then transferred to the SR-release solution, which contained caffeine and low [Mg\(^{2+}\)]. The resulting force response was indicative of the endogenous SR Ca\(^{2+}\) content and the fiber was kept in this solution for at least 1 min to thoroughly deplete the SR of Ca\(^{2+}\) (12). The SR Ca\(^{2+}\) loading properties of the fiber were then tested using a SR load-release protocol as described in the legend to Fig. 1. Briefly, the fiber underwent a 2 min exposure to a load solution buffered at either pCa 7.1 or 6.2, and thereafter to a full release by exposure to the SR-release solution. The sequence of solution changeovers was repeated such that each fiber underwent a load-release cycle at both pCa 7.1 and pCa 6.2.

**Table 1. Composition of solutions used with mechanically skinned fibers**

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<th>Compound, mM</th>
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<tr>
<td>Mg(^{2+})</td>
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<td>EGTA(_{Total})</td>
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<td>Ca(_{Total})</td>
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<td>Sr(_{Total})</td>
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<td>Sr(_{II})</td>
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**SR Load-Release Experiments**

After being mounted, fibers were exposed for 2 min to pre-release solution and then transferred to the SR-release solution, which contained caffeine and low [Mg\(^{2+}\)]. The resulting force response was indicative of the endogenous SR Ca\(^{2+}\) content and the fiber was kept in this solution for at least 1 min to thoroughly deplete the SR of Ca\(^{2+}\) (12). The SR Ca\(^{2+}\) loading properties of the fiber were then tested using a SR load-release protocol as described in the legend to Fig. 1. Briefly, the fiber underwent a 2 min exposure to a load solution buffered at either pCa 7.1 or 6.2, and thereafter to a full release by exposure to the SR-release solution. The sequence of solution changeovers was repeated such that each fiber underwent a load-release cycle at both pCa 7.1 and pCa 6.2.

**Contractile Activation Experiments**

After the SR function experiments, preparations were transferred to a relaxing solution I (Table 1) and then the Ca\(^{2+}\)- and Sr\(^{2+}\)-activation characteristics were determined by sequential exposure to a series of heavily buffered solutions of increasing [Ca\(^{2+}\)] or [Sr\(^{2+}\)] made from solution I and II and from solution I and Sr\(_{II}\), respectively (1, 32).

For each mechanically skinned fiber segment, the following contractile activation parameters were determined: 1) maximum Ca\(^{2+}\)-activated specific force (force per CSA: Ca\(_{F_{max}}/\text{CSA}\), kN/m\(^2\)) measured from the amplitude of the first force response at pCa 4.0 and from estimated fiber CSA measured in paraffin oil before exposure to aqueous solutions, 2) sensitivities of the contractile apparatus to Ca\(^{2+}\) (pCa\(_{50}\)) and Sr\(^{2+}\) (pSr\(_{50}\)), where [Ca\(_{50}\)] and [Sr\(_{50}\)] are the [Ca\(^{2+}\)] and [Sr\(^{2+}\)] respectively, corresponding to half-maximal activation of the force response and 3) the Hill coefficients associated with the force-pCa and force-pSr curves. The pCa\(_{50}\) and pSr\(_{50}\) values were obtained from fitting the relative force (P\(_F\)) produced by a fiber at each pCa or pSr by the simple Hill equation

\[
P_F = \frac{1}{1 + 10^{(n_H \cdot (pX - pX_{50})} \tag{1}\]

In Eq. 1, pX stands for pCa or pSr, pX\(_{50}\) is the pCa\(_{50}\) or pSr\(_{50}\), and \(n_H\) is the Hill coefficient. The Hill coefficient is directly related to the maximum steepness of the respective force-pX activation curve and to the minimum number of cooperating Ca\(^{2+}\) or Sr\(^{2+}\) binding sites in the process of force production. It is relevant to point
Fig. 1. The multistep sarcoplasmic reticulum (SR) load-release protocol used for determining SR loading properties of mechanically skinned single fibers. The trace shown was produced by a fiber from an extensor digitorum longus (EDL) control muscle. The fiber was sequentially exposed to the Pre-release solution (2 min), the caffeine-containing release (R) solution (>1 min), the wash solution W1 (30 s), the wash solution W2 (30 s), the pCa 7.1 SR-load solution (2 min), the wash solution W3 (30 s), the R solution (>1 min), the W1 solution (30 s), the W2 solution (30 s), the pCa 6.2 SR-load solution (2 min), the W1 solution (30 s), and the R solution (>1 min). Details of the protocol and composition of individual solutions are given in the text and in Table 1. The vertical bar represents 0.15 mM. The horizontal bar represents 1 min except for the time in the release solution R when it represents 15 s. The wash solutions W1, W2, and W3 were placed in different wells but otherwise were identical in composition.

Data Analysis

Results, expressed as means ± SE, were analyzed using Microsoft Excel 97 and Sigmaplot 4.01 (SPSS) software. Force-pCa (pSr) curves were constructed using Prism software (GraphPad Software). Statistical significance was tested at the P < 0.05 level using ANOVA, followed by the post hoc Newman-Keuls multiple-comparison test, or Student’s t-test for unpaired samples, as appropriate.

RESULTS

Mass of Control and Denervated Muscles

As expected, surgical removal of a section of the sciatic nerve (see MATERIALS AND METHODS) was accompanied by a decrease in the mass of both EDL and SOL muscles from male Long-Evans rats such that 50 days after denervation, the mass of both EDL and SOL muscles decreased by ~80% (Fig. 2). Interestingly, at 7 days post-denervation, the drop in the EDL muscle mass was markedly smaller (~36%) than that recorded for the SOL muscle (~49%). A similar observation at 7 days post-denervation was made by Schulte et al. (39) who used female Wistar rats.

MHC Isoform Composition and Fiber Types in Control and Denervated Rat EDL and SOL Muscles

Electrophoretic analyses of MHC isoform composition in whole muscle homogenates (see Fig. 3A) showed that in all rats used in this study (control and denervated), EDL muscles contained only MHC Iib and IId, whereas the SOL muscles contained only MHC I and Ia. The MHC ratios Iib/IId and
In I/IIa in the respective muscles from control and denervated rats at day 0, 7, 21, and 50 post-denervation are presented in Fig. 3B. The decrease in the respective MHC ratios post-denervation is graded in time and is statistically significant for both EDL and SOL muscles (one-way ANOVA; \(P < 0.002\) for each experimental point. One-way ANOVA: \(P < 0.002\) for both EDL and SOL), followed by Newman-Keuls multiple-comparison tests: \(P < 0.01\) for the IIb/IId ratio between day 0 and day 21, day 21 and day 50 post-denervation in EDL and \(P < 0.05\) for the I/IIa ratio between day 0 and day 7 and day 7 and day 50 post-denervation in SOL.

In agreement with the whole muscle MHC isoform data, all randomly isolated fibers from the EDL and SOL muscles of control and denervated rats were of type IIB, IID, or IIB/D, and of type I, IIA, or I/IIA, respectively. Importantly, denervation was associated with a marked decrease in the proportion of pure fibers and a marked increase in the number of hybrid fibers expressing two MHC isoforms in both EDL and SOL muscles. More specifically, 50-day post-denervation, \(>75\%\) of the fibers sampled from the EDL and SOL muscles were hybrid fibers of type IIB/D (7/8) and I/IIA (10/13), respectively (Fig. 4). This compares with only \(30\%\) hybrid fibers sampled from the control EDL (3/10) and SOL (3/10) muscles. Assuming that the hybrid IIB/D and I/IIA fibers contained the respective MHC isoforms in equal amounts and that all fibers were of similar size, the single fiber results in Fig. 4 predict MHC ratio IIb/IId ratio values of 1.86 and 0.78 in control and 50-day-denervated EDL muscle and MHC I/IIa ratio values of 3.00 and 1.60 for control and 50-day-denervated SOL muscle. These values are remarkably close to the measured MHC isoform ratios in whole muscle homogenates (1.45 and 0.5 for IIb/IId in control and 50-day-denervated EDL muscle and 3.46 and 1.61 for I/IIa for control and 50-day-denervated SOL muscle, respectively). Taken together, these results provide evidence that, following denervation, there is an increase in the proportion of IIB/D and I/IIA hybrid fibers in the EDL and SOL muscles respectively, which accounts for the marked decrease in the MHC isoform ratios IIB/IId (in the denervated EDL) and I/IIa (in the denervated SOL) in muscle homogenates, implying that the denervated EDL muscle becomes functionally slower while the SOL muscle becomes functionally faster.

Fig. 3. Myosin heavy chain (MHC) isoforms detected in EDL and SOL muscle homogenates at different times post-denervation. A: representative electrophoretograms of the MHC isoforms detected in EDL and SOL muscles from control and denervated rats. Lane 1, MHC isoform marker displaying the 4 MHC isoforms expressed in adult rat hindlimb skeletal muscle [in order of decreasing electrophoretic mobility: I, IIb, IId/x (referred to as IId throughout the text) and IIA]. Lane 2, control muscle homogenates. Lane 3, denervated muscle homogenates (EDL 50-day; SOL 50-day). Sample size: 4 \(\mu\)l solubilizing buffer containing 0.4 \(\mu\)g protein. B: ratios of MHC isoforms IIb/IId in EDL muscle and I/IIa in SOL muscle before and after denervation. \(n = 3–5\) for each experimental point. One-way ANOVA: \(P < 0.002\) for both EDL and SOL, followed by Newman-Keuls multiple-comparison tests: \(P < 0.01\) for the IIb/IId ratio between day 0 and day 21, day 21 and day 50 post-denervation in EDL and \(P < 0.05\) for the I/IIa ratio between day 0 and day 7 and day 7 and day 50 post-denervation in SOL.

Fig. 4. The proportions of fiber types in the fiber populations dissected from control and 50-day-denervated EDL and SOL muscles. EDL fibers examined: \(n = 10\) (control), \(n = 8\) (50-day). SOL fibers examined: \(n = 10\) (control), \(n = 13\) (50-day). Fibers were typed based on their MHC isoform composition, as described in MATERIALS AND METHODS. The proportions of hybrid fibers in both EDL and SOL muscles 50-day post-denervation are statistically different from those in the respective control muscles (Fisher’s exact probability test, two-sided \(P\) value mid-\(P = 0.01284\) for EDL and 0.02468 for SOL).
SR Ca\(^{2+}\) Loading Properties of Control and Denervated EDL and SOL Fibers

In a mechanically skinned fiber preparation, the relative levels of Ca\(^{2+}\) loading in the SR are indicated by the relative areas under the force responses elicited upon exposure of the preparation to the caffeine-and-low-Mg\(^{2+}\) SR Release solution (25). In a previous study from one of our laboratories (12), we showed that typically fast-twitch EDL muscle fibers and slow-twitch SOL fibers display quite different SR Ca\(^{2+}\)-loading properties. When exposed to an intracellular Ca\(^{2+}\) concentration that is close to the endogenous level (pCa = ~7.1), the Ca\(^{2+}\) loading of the SR is near capacity in slow-twitch SOL fibers, but is only at ~40% of capacity in fast-twitch EDL fibers. Therefore, upon exposure to a higher [Ca\(^{2+}\)] (pCa ~6.2) the SR of typical fast-twitch EDL fibers, unlike that of typical slow-twitch SOL fibers, loads markedly more Ca\(^{2+}\) than it does at the endogenous pCa.

It should be noted that the relatively low level of SR Ca\(^{2+}\) loading in fast-twitch fibers facilitates the uptake of Ca\(^{2+}\) into the SR when myoplasmic Ca\(^{2+}\) rises, thus promoting fast relaxation. In contrast, if the SR is close to capacity, as is the case with the slow-twitch fibers, the rate of Ca\(^{2+}\) uptake will be markedly reduced, contributing to the slower time course of the twitch response. Thus the level of SR Ca\(^{2+}\) loading at physiological pCa (~7.1) is an important factor in determining the time course of the force response (12).

In this study, the ratio between the SR Ca\(^{2+}\) content after 2-min loading at pCa 6.2 and at 7.10, estimated from the relative areas under the force responses (see Fig. 5 and MATERIALS AND METHODS), has been used here as an indicator of the SR Ca\(^{2+}\)-loading properties of individual fibers from control and denervated muscles.

The results obtained with all fibers from the EDL and SOL muscles (regardless of fiber type) are summarized in Fig. 5A. As expected, the ratios for the SR Ca\(^{2+}\) content at pCa 6.2 and 7.1 for EDL and SOL fibers from control rats (“zero” denervation days) were significantly different from each other, with average values >2 for EDL fibers, indicating a relatively empty SR and close to 1 for SOL fibers, indicating that in these fibers the SR was effectively maximally loaded. Denervation does not statistically significantly modify, as a whole, the ratio for the SR Ca\(^{2+}\) content at pCa 6.2 and 7.1 for SOL fibers, but it causes a decline of the ratio in EDL fibers at 21-d post-denervation, such that the SR properties in the EDL fibers converge toward those of the SOL fibers.

When only pure type II fibers from the EDL and type I fibers from SOL muscles are considered, the marked fiber type-related difference with respect to the ratio for SR Ca\(^{2+}\) loading at pCa 6.2 and pCa 7.1 observed in control muscles was maintained, with some exceptions as described below, throughout the 50-day denervation period (Fig. 5B). The pure type IIA fibers from the SOL muscles displayed a complex pattern with respect to the ratio of SR Ca\(^{2+}\) loading at pCa 6.2 and pCa 7.1, with one fiber from control muscle displaying a ratio of 1.07, one fiber from day 7 denervated muscle displaying a ratio of 0.74, and another fiber from day 27 denervated muscle displaying a ratio of 1.66. This finding is not surprising given that pure type IIA control SOL fibers have been previously reported (1) to also display hybrid characteristics with respect to the myosin light chain composition, containing both fast and slow myosin light chain isoforms. One pure SOL type I fiber from a 50-day denervated rat displayed a ratio of 2.46, which was typical of type II EDL fibers, indicating that the SR properties in that SOL fiber changed toward those characteristic of the type II EDL fibers before the fast MHC IIa isoform was expressed. In hybrid fibers of type I/IIA from denervated SOL muscles, there was also a large degree of variation with respect to the ratio of SR Ca\(^{2+}\) loading at pCa 6.2 and pCa 7.1, with values ranging between 1.01 and 1.75. Thus, it appears that in denervated SOL fibers, the SR characteristics of individual fibers are not tightly correlated with the MHC isoform(s) expressed, thus conferring unusual functional properties to the respective fibers.

Considering now the hybrid fibers from EDL (IIB/D) muscles, the ratio between the SR Ca\(^{2+}\) content at pCa 6.2 and 7.1 significantly (P < 0.05, double-sided t-test) decreased from 2.32 ± 0.03 (n = 3) in control muscles to 1.6 ± 0.2 (n = 6) in denervated muscles (7- to 50-day post-denervation). Be-
cause the percentage of the IIB/D hybrid fibers markedly increased in the denervated EDL muscle (Fig. 3A), these data indicate that changes in the SR loading characteristics observed in the total fiber population of denervated EDL muscle result predominantly from changes in the SR characteristics of the hybrid (IIB/D) and not of the pure IIB and IID fibers.

Importantly, at day 50 post-denervation, there was a significant difference ($P < 0.002$, double-sided $t$-test) with respect to the ratio of SR Ca$^{2+}$ loading at Ca$^{2+}$ 6.2 and Ca$^{2+}$ 7.1 between EDL type IIB/D hybrid fibers (1.71 ± 0.08; $n = 4$) and SOL type I/IIA hybrid fibers (1.21 ± 0.06; $n = 5$), indicating that the two predominant hybrid fiber populations of the day 50 denervated EDL and SOL muscles display dissimilar properties with respect to Ca$^{2+}$ handling by the SR.

**Contractile Properties**

The Ca$^{2+}$- and Sr$^{2+}$-activation characteristics (maximum Ca$^{2+}$-activated specific force, force-pCa parameters, force-pSr parameters) of individual fibers were determined by exposure of the skinned fiber preparation to a series of heavily buffered [Ca$^{2+}$] or [Sr$^{2+}$] solutions (see **MATERIALS AND METHODS**).

**Maximum Ca$^{2+}$-activated specific forces.** The maximum Ca$^{2+}$-activated specific forces (Ca$_{Fmax}$/CSA) results for EDL and SOL fibers from normal and denervated muscles are shown in Fig. 6A. There was a marked decline in the maximal level of specific tension that could be developed in both EDL and SOL fibers post-denervation irrespective of fiber type (Fig. 6B). This is an important result because it shows that the marked reduction of whole muscle force following denervation is dependent not only on the reduction in muscle size post-denervation, but also on the reduced ability of individual muscle fibers to produce maximum specific force, probably because of the reduced fraction of the CSA occupied by the contractile apparatus in denervated fibers (46).

**Ca$^{2+}$ activation curves.** Examples of Ca$^{2+}$ activation curves for representative fiber types are presented in Fig. 7, whereas those in Fig. 8A show the mean pCa$_{50}$ values for all fibers from control and denervated EDL and SOL muscles, regardless of the fiber type. The control SOL fibers were significantly more sensitive to Ca$^{2+}$ than the control EDL fibers ($P < 0.001$, double-sided $t$-test). For SOL fibers there was a highly significant (one-way ANOVA; $P < 0.0001$) decrease in Ca$^{2+}$ sensitivity post-denervation. The decrease in the average value of pCa$_{50}$ by 0.24 pCa units between fibers from control and day 50 denervated SOL muscles represents a decrease in the sensitivity to Ca$^{2+}$ by a factor of 1.7.

For the EDL fibers, there was a much smaller, albeit statistically significant (one-way ANOVA; $P < 0.03$), decrease in Ca$^{2+}$ sensitivity in denervated muscles by about a factor of 1.25 (0.1 pCa unit), such that at day 50 post-denervation, the pCa$_{50}$ values for fibers from denervated EDL and SOL muscles were essentially the same (5.95 ± 0.03, $n = 14$ vs. 5.95 ± 0.02, $n = 14$, respectively).

There was also a statistically significant ($t$-test, $P < 0.05$) difference between pCa$_{50}$ values of pure type II fibers from control EDL muscle and type I fibers from control SOL muscle, and a highly statistically significant (one-way ANOVA; $P < 0.01$) decrease in pCa$_{50}$ for type I fibers from denervated muscles (6.23 ± 0.02, $n = 6$ for controls; 6.12 ± 0.04, $n = 4$ for 7-day denervated muscles; 5.95 ± 0.05, $n = 2$ for 21-day denervated muscle and 5.90 ± 0.07, $n = 3$ for day 50 denervated muscle). However, there was no statistically significant ($P > 0.05$) decrease in pCa$_{50}$ values for pure type II EDL fibers in the denervated muscles.

At day 50 post-denervation, the hybrid fibers of type I/IIA from the SOL muscle showed a statistically significant ($P < 0.015$) decrease in pCa$_{50}$ values compared with the hybrid fibers from SOL controls (5.96 ± 0.03, $n = 9$ vs. 6.14 ± 0.05, $n = 3$). No statistically significant difference associated with denervation was observed for IIB/D hybrid fibers from EDL muscle, with the IIB/D hybrid fibers from the day 50 denervated EDL muscle displaying a similar sensitivity to Ca$^{2+}$ as I/IIA hybrid fibers from the day 50 denervated SOL muscle. Taken together, these results indicate that, while all fiber types from the SOL muscle become markedly less sensitive to Ca$^{2+}$ soon after denervation, there is a smaller change in the sensitivity to Ca$^{2+}$ in EDL fibers. Furthermore, the hybrid fibers from the day 50 denervated EDL and SOL muscles, which are predominant in these muscles, display similar sensitivities to Ca$^{2+}$ even though they contain different MHC isoforms.
nCa significantly (determined via one-way ANOVA) decreased for fibers from the EDL and SOL muscles post-denervation, indicating a reduction in the steepness of the force-pCa curves (Fig. 8B). Notably, the nCa values stabilized after 21 days of denervation for EDL and SOL fibers and were significantly smaller than those for controls (Newman-Keuls post hoc multiple test, P < 0.05 for EDL; P < 0.01 for SOL). There was no significant difference between pure type II and hybrid IIB/D fibers from control EDL muscles (4.40 ± 0.42, n = 7 vs. 4.62 ± 0.98, n = 3), but there was a significant difference between type I and I/IIA fibers from control SOL muscles, with the nCa for type I fibers being significantly lower than the nCa for hybrid I/IIA fibers (2.81 ± 0.15, n = 6, vs. 4.34 ± 0.7, n = 3; P < 0.03, double-sided t-test). Denervation did not cause a significant decrease in nCa values for pure type I SOL fibers (2.81 ± 0.15, n = 6 for control rats vs. 2.70 ± 0.29, n = 4 for day 50 denervated rats), but did for the hybrid type I/IIA fibers (4.34 ± 0.70, n = 3 for control rats vs. 2.82 ± 0.23, n = 9, for day 50 denervated rats) such that the Hill coefficients for the two fiber types reached similar values in day 50 denervated SOL muscles. The nCa values for the IIB/D hybrid fibers from the day 50 denervated EDL muscle (3.43 ± 0.45; n = 7) also significantly decreased compared with those from control EDL muscle but were not statistically significantly different from those for the I/IIA hybrid fibers from the 50-day denervated SOL muscle. Thus, the main contributors to the overall decrease in the steepness of the Ca^{2+}-activation curves post-denervation were the hybrid fibers, which displayed significantly smaller Hill coefficients in the denervated muscle.

Given the simple relationship between the threshold for contraction (pCa_{10}, pCa_{50}, and nH; see Eq. 2), it follows that...
the decrease in the value of the Hill coefficient contributes to an increase in pCa_{10} value at constant pCa_{50}. Thus the decrease in pCa_{50} observed with different fiber types is compensated by the decrease in the value of n_H such that the threshold values change less than the pCa_{50} values in the denervated fibers. For example, the decrease in pCa_{50} values by 0.11 and 0.24 pCa units for the EDL and SOL fibers from day 50 denervated muscles (Fig. 8A) would correspond to decreases in the pCa_{10} values by only 0.06 and 0.17 pCa units, respectively.

**Sr^{2+} Activation Curves**

Recent results from our laboratories (29) showed unequivocally that TnC isoform expression determines fiber type differences with respect to Sr^{2+} sensitivity (pSr_{50}) and to the relative sensitivity of the contractile activation process to Ca^{2+} and Sr^{2+} (pCa_{50}-pSr_{50}). Examples of Ca^{2+}- and Sr^{2+}-activation curves for representative fiber types are shown in Fig. 7. If only the TnC slow isoform (TnC-s) is present, then the fiber is relatively sensitive to Sr^{2+} and pCa_{50}-pSr_{50} < 0.7. In contrast, if only TnC fast isoform (TnC-f) is expressed, then the fiber is ~10-fold less sensitive to Sr^{2+} and pCa_{50}-pSr_{50} > 1.2. When both fast and slow TnC isoforms are expressed in the same fiber, the isometric force-pSr activation curve appears biphasic (see Fig. 7C), displaying a plateau region at submaximal levels of activation whose position on the ordinate is closely related to the proportion of the functional TnC isoforms present and is well fitted by the composite Hill equation described by Eq. 3.

After denervation, the sensitivity to Sr^{2+} remained essentially the same (pSr_{50} 4.62–4.66) in EDL fibers, whereas the trend was for it to decrease in SOL fibers (Fig. 9). There was little change in sensitivity to Sr^{2+} after denervation of pure EDL fibers, but there was a marked and significant decrease in the sensitivity to Sr^{2+} of pure type I SOL fibers up to 21-day post-denervation, when it stabilized. Thus, 50 days post-denervation, the sensitivity to Sr^{2+} of type I SOL fibers decreased by a factor of ~3 (pSr_{50} : 5.20 ± 0.12, n = 3 compared with 5.73 ± 0.05; n = 6 for controls).

Biphasic behavior with respect to Sr^{2+} activation was never encountered in pure or hybrid type II fibers from control or denervated EDL muscles, but was detected in a large proportion of hybrid fibers of type I/IIA from SOL, particularly from 50-day denervated muscles, where 7 of 9 hybrid I/IIA fibers displayed biphasic behavior. An example of such a Sr^{2+} activation curve is shown in Fig. 7C. Importantly, the pSr_{50} values corresponding to the two components, pSr_{50/1} and pSr_{50/2} were close to the pSr_{50} values characteristic of pure type I (5.69 vs. 5.73 ± 0.05; n = 6) and pure type II fibers (4.70 vs. 4.61 ± 0.04; n = 7) from control muscles, further supporting the view that this differential sensitivity to Sr^{2+} is associated with the two isoforms of TnC functional in the respective fibers. Interestingly, there were two pure type I SOL fibers expressing only the slow MHC I isoform, one from a day 21 denervated muscle and the other from a day 50 denervated muscle, that also displayed biphasic behavior with respect to Sr^{2+} activation, suggesting that in these fibers both the TnC-s and TnC-f isoforms were functional. In fibers from normal muscles TnC-f was present only when a fast MHC isoform was also expressed (29). The fact that no fast MHC isoforms could be detected in these two type I fibers indicates that the functional expression of TnC-f in denervated type I fibers precedes expression of a fast MHC (MHC IIA). Thus it appears that the majority of the hybrid I/IIA fibers from the 50-day denervated SOL muscle contain both the slow and the fast TnC isoforms, whereas the IIB/D hybrid fibers from the 50-day denervated EDL muscle contain only the TnC-f isoform. Note that the sizes of the fiber segments from the denervated muscles were too small for detection of TnC isoforms by SDS-PAGE.

**DISCUSSION**

This study offered the opportunity to characterize, at the single-fiber level, changes that occur in the MHC isoform composition and functional properties of denervated muscle, shedding light on the time course of cellular events underlying denervation-induced changes to skeletal muscle function.

The study provides strong evidence that the intrinsic properties of a particular muscle play a major role in determining the fiber phenotype after the removal of neural input.

**Distinct Fiber Phenotypes in Denervated EDL and SOL Muscles**

The fibers in the denervated EDL and SOL muscles of Long-Evans hooded rats converged to form two distinct hybrid fiber phenotypes specific to the EDL and SOL muscles, respectively. Thus, 50 days post-denervation, >75% of the fibers in EDL and SOL muscles were hybrid with respect to MHC content (IIB/D in EDL and I/IIA in SOL). Most of the fibers present in the 50-day denervated muscles must have been preexisting fibers rather than new fibers originating from proliferating satellite cells since it is established that, in the absence of innervation, regenerating fibers express only type IIb and IIld MHCs (9, 18, 19), and there was no trace of MHC isoforms IIb and/or IIId in any of the denervated...
SOL muscles. It is also important to point out that the rapid and marked atrophy exhibited by the rat muscles over 2 mo post-denervation is due to a massive reduction in the cytoplasmic fiber volume of individual fibers and not to a significant decrease in the number of muscle fibers (46). Consequently, the most likely scenario is that upon denervation, the muscle fibers of the SOL and EDL muscles atrophy and gradually undergo molecular transformation, converging to two different hybrid phenotypes: IIB/D in the EDL muscle and IIA in the SOL muscle. The complete lack of overlap between fiber types or MHC present in the denervated muscle 50 days post-denervation, together with the emergence of the two predominant and distinct hybrid fiber phenotypes in the EDL and SOL muscles support the idea that these two fiber phenotypes represent stable fiber populations in the denervated muscles with potential to transform back, upon reinnervation, into one of the two major pure fiber phenotypes found in the normally innervated muscles: types IIB and IID in the EDL and types I and IIA in the SOL. The two hybrid fiber types display distinct characteristics not only with respect to MHC isoform composition but also with respect to SR Ca\(^{2+}\) handling and contractile activation. One likely explanation for the different fiber phenotypes in long-term denervated SOL and EDL muscles may lie in the different myoblast lineages of the two muscles, conferring on the fibers different intrinsic properties with respect to factors that affect gene expression and the mechanism regulating transcription. For example in rodents, the SOL muscle fibers develop predominantly from primary myotubes, whereas the EDL fibers develop mainly from secondary myotubes (30) and there is evidence that myotubes derived from primary and secondary myoblasts have distinct myogenic programs of MHC expression (40). The development of SOL and EDL fibers from two different populations of myoblasts with distinct myogenic programs can directly explain the observation made here that upon removing the external neural input, the fibers in the two muscles converge to form two distinct hybrid fiber phenotypes specific to the myonuclei lineages in the respective muscles. Conversely, the results from this study provide support to the hypothesis that myonuclei from primary and secondary myotubes have distinct myogenic programs. This interpretation also helps explain the restricted adaptive range of individual muscle fibers, as initially suggested by Stockdale (43) for chicken muscle, that the extent of muscle fiber transformation in response to changes in the pattern of neural stimulation will also depend on the distinct myogenic programs of the nuclei present in that particular fiber.

This work also highlights that the fiber type composition of the normally innervated EDL and SOL muscles in the Long-Evans hooded male rats is different from that in Wistar-Kyoto rats. In Wistar-Kyoto rats, the EDL muscles contain in addition to the predominant IIB and IId MHC isoforms/type IIB and type IID fibers, 8–20% IIA MHC/IIA fibers, and small amounts of MHCl/ type I fibers (13, 17, 47). In a previous study (2), we also found that type IIA and IID fibers were present in sizeable proportions in both EDL (9.6 and 17.3%) and SOL (7.5 and 8.2%) muscles of Wistar-Kyoto rats. This was not the case with the Long-Evans rats, where the fiber types present in the EDL muscle do not overlap with the fiber types in the SOL muscle.

In Wistar rats, denervation of the EDL muscle results in fiber transformation such that ~75% of fibers are of type IIA at day 60 post denervation, rising to ~90% by day 130 post-denervation (47). Thus, the pattern of fiber transformation upon denervation differs not only between EDL and SOL muscles from the same rat, but also between EDL muscles of different rat strains that have a different fiber type composition before denervation. The pattern of denervation-induced changes in MHC isoform composition in the EDL muscles nevertheless follows the general pattern of denervation-induced changes in MHC composition for fast-twitch muscles: from faster to slower MHC isoforms (34), although it appears from this study and others (13, 17, 47) that denervated EDL muscle does not express new MHC isoforms that were not expressed in the muscle before denervation.

The most direct explanation as to why the fibers from denervated EDL and SOL muscles of Long-Evans and Wistar rats do not converge toward the same phenotypes is that the SOL and EDL muscles of the Long-Evans rats originate from close to pure lineages of primary and secondary myoblasts, respectively, whereas the lineage of the SOL and EDL fibers of Wistar rats from primary and secondary myoblasts, respectively, is less well defined. This interpretation would also readily explain the incomplete fiber transformation revealed, but not further explored, by cross-innervation SOL and EDL experiments (5, 6).

**Common Trends Induced by Denervation**

It is important to point out that although the emphasis in this study was on showing that the intrinsic properties of a particular muscle play a major role in determining the fiber phenotype after the removal of neural input, this study also shows that there are several common trends induced by denervation irrespective of the muscle or the fiber types present. Thus, upon denervation (day 50), both EDL and SOL muscles atrophy markedly and contain mainly hybrid fibers. Furthermore, the maximum Ca\(^{2+}\)-activated specific force declines by a similar factor irrespective of the muscle or fiber type. These results can be directly explained by the massive reduction in the cytoplasmic (and myofibrillar) fiber volume of individual fibers (46). Another common trend from this study is that the sensitivity to Ca\(^{2+}\) described by the parameter pCa\(_{50}\) converges to a similar value (~5.95) for fibers in both muscles, irrespective of the MHC or TnC isoforms expressed. The Hill coefficient n\(_{Ca}\) describing the steepness of the force-pCa curves also significantly decreases in hybrid fibers from both EDL (type IIB/D) and SOL (type I/IIA) muscles such that at 50-day post-denervation, when the two types of fibers are predominant in the two muscles, the n\(_{Ca}\) values are not significantly different between the two fiber types. These results indicate that these Ca\(^{2+}\)-activation parameters are not simply determined by the MHC and TnC isoforms present.

**Denervation and EDL Muscle**

**Changes in SR properties.** In relation to SR Ca\(^{2+}\)-loading properties, pure type IIB and type IID EDL fibers from denervated muscles maintained control-like SR loading properties. However, type IIB/D EDL hybrids displayed SR Ca\(^{2+}\)-loading properties that were shifted toward the slow phenotype, suggesting that the SR Ca\(^{2+}\) loading at pCa 7.1 was closer to capacity. Evidence of increased SR Ca\(^{2+}\) loading in fibers from denervated EDL muscles was also reported for day 7 denervated Wistar rat (13) and day 14 denervated rabbit (44) EDL muscles. Because the time course of the twitch response is slower and the height of the twitch response is greater when the
SR of EDL fibers is loaded with Ca\(^{2+}\) above endogenous levels (36), the presence in denervated EDL muscles of a large population of hybrid IIB/D fibers that are more loaded with Ca\(^{2+}\) explains why the EDL twitch becomes slower and the twitch to tetanus ratio rises post-denervation (13, 22). In relation to SR loading properties, Schulte et al. (39) showed that denervation caused a marked decrease in the density of the fast isoform of the sarco(endo)plasmic reticulum Ca\(^{2+}\)-adenosine triphosphatase (SERCA1) in EDL muscles of Wistar rats 14 days after denervation. The slow SERCA isoform (SERCA2a) was not detected in denervated EDL muscles for up to 28 days post-denervation. Reduced pump density and activity in denervated EDL fibers (24) could also contribute to prolongation of the twitch time course and increased peak response. Results obtained in this study also explain the previously reported increased sensitivity to caffeine of denervated EDL muscle (15) because a higher level of SR Ca\(^{2+}\) loading is known to increase the SR sensitivity to caffeine (2, 21). Although an increased level of SR Ca\(^{2+}\) loading may increase the level of Ca\(^{2+}\) released from the SR for a certain stimulus intensity, the marked shift in the voltage dependence of K\(^{+}\) contractures to more negative membrane potentials is more likely due to the shift of voltage sensor charge movements to more negative potentials (8). In summary, it appears that changes in the properties of surface and SR membranes in the EDL muscle post denervation markedly affect its function.

Changes in properties of contractile apparatus. The pCa50 of EDL fibers decreased by \(-0.1\) pCa units after \(day\ 50\) denervation and the steepness of the force-pCa curve also decreased (\(n_{\text{Ca}}\) decreased), requiring a larger pCa range to fully activate the contractile machinery. Evidence was also provided that only the TnC-f isoform was functional in all EDL fibers examined from control and denervated muscles, indicating that the decrease in sensitivity to Ca\(^{2+}\) and \(n_{\text{Ca}}\) must be due to denervation-induced changes in myofilament components other than TnC. Germinario et al. (13) also reported a small decrease in Ca\(^{2+}\) sensitivity (pCa50) at \(7\)-day post-denervation in Wistar EDL fibers. These results rule out the possibility that the marked shift to more negative membrane potentials of the contraction threshold (31) and of the curve relating the strength of the K\(^{+}\) contractures to the membrane potential in intact denervated EDL muscle is due to an increased sensitivity of the contractile apparatus to Ca\(^{2+}\) (8). The marked decrease of maximally Ca\(^{2+}\)-activated specific force in fibers from denervated EDL muscles after 21 days of denervation explains to a large extent the decrease in specific maximum tetanic force in long-term denervated rat EDL muscle previously reported by Finol et al. (11).

Denervation and SOL Muscle

Changes in SR properties. In relation to the SR Ca\(^{2+}\)-loading properties, the hybrid I/IIA fibers that become prevalent in SOL muscle after \(day\ 50\) of denervation displayed properties that are intermediate between typical type I and type II fibers with some type I fibers exhibiting properties similar to those of typical type II EDL fibers. This trend is consistent with the results of Schulte et al. (39) showing that after \(day\ 28\) of denervation the ratio between SERCA1 and SERCA2a in SOL muscle increases due to a decrease in SERCA2a expression. The SR Ca\(^{2+}\) loading capacity also increases in fibers from denervated SOL muscle (27). This is also consistent with a shift toward the SR characteristics of fast-twitch fibers, which have a severalfold greater SR Ca\(^{2+}\) loading capacity compared with slow-twitch fibers (12). After 14 and 28 days of denervation, there is also a net decrease in the total SERCA protein per total muscle protein (39), implying a decrease in the density of SR Ca\(^{2+}\) pumps in denervated SOL muscles. These changes in SR properties would promote increased Ca\(^{2+}\) release due to increased SR Ca\(^{2+}\) capacity and slower reuptake due to reduced SR Ca\(^{2+}\) pump capacity leading to a prolongation of the twitch time course and increased twitch peak in the denervated rat SOL muscle as reported in several studies (11, 23, 27). Unlike the denervated EDL fibers, the denervated SOL fibers do not display marked changes in the asymmetrical charge movements associated with the voltage sensors (8).

Changes in the properties of contractile apparatus. MHC isoform changes observed in this study for SOL are consistent with the general pattern of denervation-induced changes in MHC composition in slow-twitch muscles reported in other studies showing a transition from slower to faster MHC isoforms (33). However, the 50-day denervated muscles did not contain a larger proportion of pure type IIA fibers, but a marked increase in the proportion of hybrid I/IIA fibers coexpressing both TnC-s and TnC-f isoforms. Denervation of the SOL muscle gave rise to a decrease in sensitivity to Ca\(^{2+}\) that was more marked and occurred earlier in SOL than in EDL fibers, such that the significant difference in pCa50 values seen for fibers from control SOL and EDL muscles completely disappeared for fibers from the 50-day denervated preparations. The increase in the proportion of fibers expressing both TnC isoforms in denervated SOL fibers explains the more accentuated decrease in pCa50 in SOL fibers than in EDL fibers and the marked decrease of \(n_{\text{Ca}}\) values for hybrid I/IIA fibers from denervated SOL muscles. There was also a more pronounced decrease in specific maximum Ca\(^{2+}\)-activated force after denervation in SOL than in EDL fibers (Fig. 6), in agreement with previous results reported by Midrio et al. (27) for denervated SOL muscle from Wistar rats. The results from this study can fully explain the drop in specific tetanic tension reported by Finol et al. (11) for 42-day denervated rat SOL muscle. Because the contractile apparatus is the major contributor to the muscle mass, the larger decrease in total muscle mass in SOL than in EDL muscle 7-day post-denervation (Fig. 2), is consistent with the more marked changes in the properties of the contractile apparatus and Ca\(^{2+}\) regulatory system in the SOL than in the EDL muscle at this point in time after denervation. Thus it appears that denervation of the SOL muscle causes more marked changes in the contractile apparatus and the regulatory system than in the membrane components of the denervated fibers.

In conclusion, the different MHC isoform profiles, SR loading properties, and contractile activation characteristics of single fibers from 7- to 50-day-denervated EDL and SOL muscles provide strong evidence that the intrinsic properties of a particular muscle play a major role in determining the fiber phenotype after the removal of neural input. The single fiber results reported here can also be used to explain a number of important functional changes induced by denervation at the whole muscle level.
REFERENCES


