Isoform switching in myofibrillar and excitation-contraction coupling proteins contributes to diminished contractile function in regenerating rat soleus muscle

Alessandra Esposito,1* Elena Germinario,1,2* Marika Zanin,1 Philip T. Palade,3 Romeo Betto,2,4 and Daniela Danieli-Betto1,2

1Department of Human Anatomy and Physiology, University of Padova, Padova, Italy; 2Istituto Interuniversitario di Miologia, Padova, Italy; 3Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, Arkansas; and 4Muscle Biology and Physiopathology Unit, Consiglio Nazionale delle Ricerche Neuroscience Institute, Padova, Italy

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Postnatal development of skeletal muscle occurs through the progressive transformation of diverse biochemical, metabolic, morphological, and functional characteristics from the embryonic to the adult phenotype. Since muscle regeneration recapitulates postnatal development of muscle fiber, it offers an appropriate experimental model to investigate the existing relationships between diverse muscle functions and the expression of key protein isoforms, particularly at the single-fiber level. This study was carried out in regenerating soleus muscle 14 days after injury. At this intermediate stage, the regenerating muscle exhibited a recovery of mass greater than its force generation capacity. The lower specific tension of regenerating muscle exhibited a recovery of mass greater than the adult phenotype. The complete recovery of skeletal muscle after injury normally occurs in a few weeks (12, 34), and this aspect identifies regeneration as an excellent experimental model to study the myogenic process, as well as the correlation between functional properties and the expression level of proteins involved in the maturation stages of muscle fibers. A large variability exists between muscle fibers that is associated with the presence of multiple isoforms of diverse important proteins. As a result, myofibrillar and membrane protein isoforms determine the functional diversity in contractile properties among various muscle fibers (4). Muscle tension development and decline depend on sarcoplasmic reticulum (SR) Ca2+ release, the sensitivity of the myofibrillar apparatus to Ca2+, the speed of actin-myosin interaction, and the rate of SR Ca2+ uptake.

The sensitivity of the myofibrillar apparatus to Ca2+ is primarily regulated by troponin, and in particular by troponin C (TnC), the Ca2+ binding subunit of the complex. Mammalian TnC exists in two isoforms, one fast (TnCf) and one slow/ cardiac (TnCs), which differ on the basis of the number of active Ca2+ binding sites (26) and the degree to which Ca2+ binding increases the affinity of TnC for troponin I. These differences greatly affect Ca2+ sensitivity of muscle fiber tension generation, in that TnCs confers a higher sensitivity to Ca2+ than TnCf (27).

Skeletal muscle regeneration is sustained by mononucleated muscle cells, called satellite cells. These cells are quiescent in adult muscle, but are activated, proliferated, and differentiated in response to a muscle injury. New myoblasts fuse to each other, forming multinucleated myotubes, which replace the damaged fibers. Regeneration, in several aspects, recapitulates postnatal development of muscle fibers as, in both processes, mature morphological, biochemical, and functional properties are progressively attained throughout the transformation from immature to adult form. The complete recovery of skeletal muscle after injury normally occurs in a few weeks (12, 34), and this aspect identifies regeneration as an excellent experimental model to study the myogenic process, as well as the correlation between functional properties and the expression level of proteins involved in the maturation stages of muscle fibers.

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Sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) controls the myoplasmic-free Ca\textsuperscript{2+} concentration by transporting the ion into the SR. In this way, SR modulates relaxation. Three SERCA isoforms are encoded by three different genes: SERCA1a, expressed in adult fast-twitch skeletal fibers; SERCA2a, in adult slow-twitch skeletal fibers in cardiac and smooth muscles; and SERCA3, having a wide tissue distribution (48, 49). During regeneration, an alternative splice form of SERCA1, the neonatal SERCA1b, is gradually replaced by the adult SERCA2a (15, 50) and SERCA1a (23), in slow and fast muscles, respectively.

Two Ca\textsuperscript{2+} channels play a major role in skeletal muscle excitation-contraction (EC) coupling: the dihydropyridine-sensitive L-type Ca\textsuperscript{2+} channel receptor (DHPR) and the ryanodine receptor (RyR). In skeletal muscle, membrane depolarization stimulates the DHPR, which acts as a voltage sensor and triggers Ca\textsuperscript{2+} release from the SR by stimulating the RyR. The DHPR is composed of five subunits, of which the α\textsubscript{1}-subunit acts as voltage sensor and also as an L-type calcium channel (5). The α\textsubscript{1}-subunit exists in diverse isoforms: α\textsubscript{1S} and α\textsubscript{1C} isoforms being specific for skeletal and cardiac muscle, respectively. The α\textsubscript{1S} isoform acts in skeletal muscle primarily as a voltage sensor and stimulates Ca\textsuperscript{2+} release from the SR by directly interacting with the RyR. By contrast, depolarization in heart muscle causes the influx of Ca\textsuperscript{2+} through the α\textsubscript{1C} isoform, a Ca\textsuperscript{2+} necessary for Ca\textsuperscript{2+} release and contraction (13, 43). Recent evidence shows that, besides the α\textsubscript{1S} isoform, slow-twitch skeletal muscles also express the α\textsubscript{1C} isoform (31, 32). In contrast, fast-twitch skeletal muscles express the skeletal isoform only. The α\textsubscript{1C} isoform is also expressed in mammalian neonatal skeletal muscle (6) and in regenerating fast-twitch skeletal muscles (33).

Three subtypes of RyR, derived from distinct genes, are expressed in mammalian tissues, with the RyR1 isoform mainly expressed in skeletal muscle, RyR2 in cardiac muscle, while RyR3 has a wider tissue distribution (16, 42). At variance with RyR1 and RyR2, for which the functional role is to release the Ca\textsuperscript{2+} necessary for muscle contraction, there is no clear evidence of a physiological role for RyR3. In adult soleus muscle, RyR1 is the predominant isoform, whereas only traces of the RyR3 can be detected (7, 16). In skeletal muscle, direct contact between RyR and DHPR is important for activation of Ca\textsuperscript{2+} release from the SR. However, morphological analysis revealed that not all RyRs are in direct apposition to DHPRs (2), and it has been suggested that the RyR3 isoform forms uncoupled receptors, which provide a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release effect, thus amplifying Ca\textsuperscript{2+} release (1, 42). RyR3 is highly expressed during the postnatal development of skeletal muscle (1).

In the present study, we decided to focus on 14-day regenerating soleus muscle to investigate, both at the whole muscle and single-fiber levels, diverse functional characteristics and to establish the functional differences between injured and uninjured soleus muscle. Moreover, we investigated, at this mid-recovery point, the existing relationships between diverse muscle functions and the expression of key protein isoforms. The study revealed that all factors responsible for muscle contraction do not recover in parallel and identified factors responsible for the incomplete development of contractile activity.

METHODS

Muscle Regeneration

The study and all procedures were approved by the University of Padova Ethical Committee and by the Italian Health Ministry. Experiments were performed in 2-mo-old male Wistar rats weighing ~200 g. Rats were anesthetized by an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (20 mg/kg) and subjected to acute degeneration of soleus (slow) muscle by injecting 0.5 ml of 0.5% bupivacaine solution (Marcaine, Astra) through a small cutaneous incision. Animals were euthanized by exposure to CO\textsubscript{2}.

Whole Muscle Mechanical Properties

Contractile properties were investigated at 30 ± 1°C in a vertical in vitro muscle apparatus (300B, Aurora Scientific) containing a solution of the following composition: 120 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl\textsubscript{2}, 3.15 mM MgCl\textsubscript{2}, 1.3 mM NaH\textsubscript{2}PO\textsubscript{4}, 25 mM NaHCO\textsubscript{3}, 11 mM glucose, 30 μM d-tubocurarine, pH 7.2–7.4, bubbled with 95% O\textsubscript{2}/5% CO\textsubscript{2}. Stimulation conditions and tension recordings were performed as previously described (24). Twitches were obtained by applying single supramaximal stimuli (0.5-ms duration), and maximal tetanus was obtained by applying 1-s-long trains of stimuli at 60 Hz. The following parameters were measured: contraction time, half relaxation time of the twitch and of the tetanus, and twitch (P\textsubscript{0}) and tetanic tensions (P\textsubscript{0}). The specific force (i.e., force per wet weight) produced by each muscle was calculated. The maximum rate of rise of tetanus and the mean rate of the linear phase of the tetanic relaxation were measured, setting the maximum tension as 1. Muscle responses were recorded via an AT-MIO 16 AD card, and data were analyzed by the LabView computer program (National Instruments). Force-frequency curves were determined by stimulating soleus muscle at 10, 20, 40, and 60 Hz, the last frequency corresponding to the tetanic fusion frequency. Occasionally, P\textsubscript{0} and P\textsubscript{0} were measured in nominally Ca\textsuperscript{2+}-free solution, where Ca\textsuperscript{2+} was substituted with an equivalent concentration of MgCl\textsubscript{2}.

In separate experiments, 30 mM caffeine were added to the medium, and the rate and the amplitude of the tension development were measured. The contracture tension amplitude was normalized to the maximum P\textsubscript{0}. The maximal initial rate of contracture tension development was expressed as normalized caffeine contracture tension per minute.

Single-Fiber Preparation

Muscle fibers were chemically skinned, as previously described (8, 24). Briefly, muscles were tied to a wooden stick and quickly immersed into an ice-cold skinning solution containing 170 mM K-propionate, 2.5 mM Mg-propionate, 2.5 mM Na\textsubscript{2}K\textsubscript{2}ATP, 5 mM K\textsubscript{2}EGTA, 10 mM 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.

pCa-Tension Relationship

pCa-tension curves were obtained by exposing the fiber sequentially to solutions with different free Ca\textsuperscript{2+} concentrations (8). Single-fiber segments (~1.5 mm length) were inserted between two clamps, one fixed and the other connected to a tension transducer (403A force transducer, Aurora Scientific). Fibers were immersed in a relaxing solution (R solution) at room temperature (22–24°C) with the following composition: 170 mM K-propionate, 2.5 mM Mg-propionate, 5 mM Na\textsubscript{2}K\textsubscript{2}ATP, 5 mM K\textsubscript{2}EGTA, 10 mM imidazole buffer, pH 7.0. Fibers were stretched to a sarcomere length of 3 μm, as measured by a video sarcomere length system (Aurora Scientific). 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 R solution. Specific tension for each
single fiber was calculated by normalizing the maximum tension measured at pCa 5 to the fiber cross-sectional area (CSA), as calculated by three different diameter determinations along the fiber length, considering the fiber immersed in solution as a cylinder (9).

**Caffeine Sensitivity of SR**

In a separate group of fibers, the threshold for SR Ca\(^{2+}\) release in response to caffeine was determined as previously described (9, 24). Fibers were first incubated for 30 s in a Ca\(^{2+}\)-loading solution (pCa 7.0) containing (in mM) 170 K-propionate, 5 Na\(_2\)K\(_2\)ATP, 2.5 Mg-propionate, 5 K\(_2\)EGTA, 0.8 Ca\(^{2+}\), and 10 imidazole buffer, pH 7.0, and then in R solution deprived of EGTA (W solution). The fibers were stepwise challenged with increasing concentrations of caffeine (0.5 mM step, 15 s long) until tension was recorded. Caffeine threshold was defined as the lowest concentration of caffeine that was able to induce a tension that was at least 5% of that generated with 20 mM caffeine, i.e., the concentration that completely depletes Ca\(^{2+}\) from the SR cisterns. The ratio between tension developed at threshold and with 20 mM caffeine was calculated (9, 37).

**SR Ca\(^{2+}\) Uptake and Ca\(^{2+}\) Release Measurements**

In an additional fiber group, spontaneous release of the SR-stored Ca\(^{2+}\) was also analyzed using the method described by Trachez et al. (44). Fibers were maximally loaded with Ca\(^{2+}\) (3 min in pCa 7.0) and then soaked in the R solution for variable periods of time (1, 3, and 5 min). Ca\(^{2+}\) released as a consequence of spontaneous leakage from the SR was buffered by the EGTA present in R solution. Tensions were expressed as percentage of pCa 5 tension. After two washes with the W solution, 20 mM caffeine was added to promote release of Ca\(^{2+}\) remaining in the SR. The peak amplitude of caffeine-induced tension was used to estimate leakage of Ca\(^{2+}\) from SR during the exposure to R solution.

In a different fiber group, Ca\(^{2+}\) uptake by the SR was measured at room temperature (22–24°C) by the light-scattering method (38), as previously described (12, 26). With the light-scattering method, fibers were mounted in a chamber containing R solution and stretched to 180% of slack length to avoid interference in light-scattering measurements caused by actin-myosin interactions (39). Fibers were then incubated in a Ca\(^{2+}\)-loading solution (pCa 6.4) containing (in mM) 170 K-propionate, 5 Na\(_2\)K\(_2\)ATP, 2.5 Mg-propionate, 5 K\(_2\)EGTA, 2.5 Ca\(^{2+}\), and 10 imidazole buffer, pH 7.0. Ca\(^{2+}\) loading activity of the SR was measured by the fiber light-scattering increase after the addition of 5 mM oxalate, which is proportional to the increase in Ca\(^{2+}\) content, with the plateau level of light scattering representing the maximum capacity for Ca\(^{2+}\) uptake of SR (38). The calibration procedures for converting the light-scattering signal to fiber Ca\(^{2+}\) concentration by using 45Ca\(^{2+}\) were described in detail elsewhere (38). The relative increase in light scattering was proportional to the Ca\(^{2+}\)-oxalate precipitate inside the fiber (11, 24). When the light-scattering signal reached a plateau level, Ca\(^{2+}\) release from the SR was initiated by rapidly exchanging the Ca\(^{2+}\)-loading solution with a solution containing (in mM) 170 K-propionate, 5 K\(_2\)EGTA, 10 caffeine, and 10 imidazole buffer, pH 7.0. The releasing solution did not contain Mg\(^{2+}\) and ATP to prevent SR Ca\(^{2+}\) uptake by the Ca\(^{2+}\) pumps. Caffeine-induced Ca\(^{2+}\) release from the SR exhibited an exponential decay from which the initial efflux rate was calculated.

**SDS-PAGE Analysis of MHC and TnC Isoforms**

Muscle homogenate or single fibers were examined by SDS-PAGE for MHC contents. Muscle homogenate was solubilized in the SDS-PAGE sample buffer (62.5 mM Tris, pH 6.8, 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol) containing the Complete protease inhibitor cocktail (Roche). A fragment of each chemically skinned single fiber used for physiological analysis was dissolved in 20 μl of the sample buffer, and an aliquot (5 μl) was analyzed on 7% polyacrylamide gels by the method described by Rossini et al. (36) to determine MHC composition. MHC protein bands were revealed by silver staining, and isoform percentage composition was evaluated by densitometry using a Bio-Rad Imaging Densitometer (GS-670). The remaining 15 μl were utilized for determining the TnC content, according to the method of O’Connell et al. (30). TnC bands were revealed by silver staining.

**Western Blot Analysis of SERCA, DHPR, and RyR Isoforms**

To investigate the SERCa isoform expression, muscle homogenate was electrophoresed on a 10% SDS-PAGE slab gel, and then the proteins were transferred to a 0.2-μm nitrocellulose sheet at 350 mA in 25 mM Tris, 192 mM glycerine, and 20% methanol, for 2 h. SERCa isoforms were identified by mouse monoclonal antibodies pan-specific for SERCA1 (D1G8, both adult SERCa1a and neonatal SERCa1b) or SERCa2 (2A7-A1, Biomol), as previously shown (15). Visualization was performed by diaminobenzidine staining. DHPR and RyR content was analyzed by Western blot of total membrane fractions purified from control or 2-wk regenerating soleus muscle, as previously described (10). For DHPR detection, purified membranes were electrophoresed on a 4–10% linear gradient SDS-PAGE slab gel; then the proteins were transferred overnight to a 0.2-μm nitrocellulose sheet at 40 V in 25 mM Tris, 192 mM glycerine, 0.01% SDS, and 10% methanol. The cardiac α1C DHPR subunit was identified by the polyclonal antibody ACC003 (Alomone Laboratories), while the α1S skeletal isoform was recognized by the MAB427 monoclonal antibody (Chemicon International). For RyR isoform identification, purified membranes were electrophoresed overnight on 4–10% linear gradient SDS-PAGE slab gel; proteins were then transferred to a 0.2-μm nitrocellulose sheet at 400 mA for 7 h in 25 mM Tris, 192 mM glycerine, 0.01% SDS, and 10% methanol. RyR1 and RyR3 isoforms were identified by the monoclonal antibody 34C (Developmental Studies Hybridoma Bank). In SDS-PAGE, the two isoforms differ significantly in their apparent molecular mass. Antibodies were visualized by an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). Signal intensities were evaluated by densitometry (GS-700 Imaging Densitometer, Bio-Rad).

**Statistical Analysis**

Means and SE were calculated according to standard procedures. Student’s t-test was used to test for statistical significance of differences between mean values following analyses of variance. Statistical

**Table 1. Isometric contractile properties of 14-day regenerating soleus muscle**

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 7)</th>
<th>Regenerating (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twitch</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt, N</td>
<td>0.17±0.02</td>
<td>0.11±0.02*</td>
</tr>
<tr>
<td>sPtN, N/g</td>
<td>1.39±0.16</td>
<td>1.25±0.17</td>
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<tr>
<td>CT, ms</td>
<td>52.7±3.1</td>
<td>53.0±1.2</td>
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<tr>
<td>HRT, ms</td>
<td>60.2±4.3</td>
<td>57.2±1.7</td>
</tr>
<tr>
<td>Tetanus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt, N</td>
<td>0.89±0.08</td>
<td>0.48±0.06†</td>
</tr>
<tr>
<td>sPtN, N/g</td>
<td>7.41±0.07</td>
<td>5.58±0.56*</td>
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<tr>
<td>MRRT, s⁻¹</td>
<td>6.38±0.60</td>
<td>7.18±0.46</td>
</tr>
<tr>
<td>MRR, s⁻¹</td>
<td>1.58±0.14</td>
<td>1.92±0.11</td>
</tr>
<tr>
<td>HRTT, ms</td>
<td>95.9±2.8</td>
<td>87.1±2.5*</td>
</tr>
<tr>
<td>(P/P_0)</td>
<td>0.187±0.001</td>
<td>0.220±0.001*</td>
</tr>
</tbody>
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Values are means ± SE; n, no. of animals; Pt, twitch tension; sPtN, specific twitch tension; CT, contraction time; HRT, half relaxation time; Pt, maximal tetanic tension; sPtN, specific maximal tetanic tension; MRRT, maximal rate of rise of tetanus; MRR, mean rate of linear relaxation; HRTT, half relaxation tension; Pt/P0, twitch tension-to-tetanic tension ratio. MRRT and MRR were measured, by setting the maximum tension as 1. Significant differences: *P < 0.05; †P < 0.001.
significance was set at P < 0.05. pCa-tension data were fitted by a least squares method using the Table Curve fitting program (Jandel Scientific), according to the following equation: \( y = \max x^2/(x^2 + k^2) \), where \( \max \) is the maximal value of pCa-tension curve, which was normalized to 1, \( x \) is Ca\(^{2+} \) concentration, \( k \) is the Ca\(^{2+} \) concentration at 50% of maximum tension, and \( N \) is the Hill coefficient.

RESULTS

Regenerating Soleus Muscle

Fourteen-day regenerating soleus muscle weighed on average 85 ± 6 mg (n = 7), while the contralateral uninjured soleus was 120 ± 3 mg (n = 7, P < 0.001), i.e., a mean mass difference of 29%. The regenerating areas were distinguishable because of the presence of small central nucleated myofibers. The mean CSA of regenerating muscle fibers (1,350 ± 76 \( \mu \)m\(^2\); 662 fibers from 3 rats), as measured in hematoxylin and eosin-stained cryostat sections, was significantly (P < 0.005) lower than that of the contralateral muscle (2,405 ± 102 \( \mu \)m\(^2\); 756 fibers from 3 rats).

Contrac- tile properties. Twitch contraction and half relaxation times of 14-day regenerating soleus muscle were similar to those of control muscle (Table 1). The absolute \( P_0 \) of regenerating soleus was significantly lower than that of the control, while the specific force was not different. Both the absolute and specific maximal tetanic forces were significantly reduced (−46 and −25%, respectively) with respect to the contralateral uninjured muscle. The twitch-to-tetanus ratio was significantly higher in regenerating muscle than in control (Table 1). The frequency-relationships in regenerating and contralateral muscles (n = 7) were comparable, with the regenerating muscle developing 44.8 ± 1.9% at 10 Hz, 72.5 ± 2.3% at 20 Hz, and 92.1 ± 1.7% at 40 Hz of maximum tension and the control muscle 43.6 ± 3.6, 70.1 ± 2.2, and 89.4 ± 1.9%, respectively. In the regenerating muscle, the maximal rate of rise of tetanic force and the mean rate of the linear phase of relaxation were not significantly different from the control values (Table 1). However, the half relaxation \( P_0 \) time was significantly shorter in regenerating than in control muscles.

Caffeine contracture. Normally, high caffeine doses determine in rat soleus muscle (n = 5) the development of a typical contracture response that seems to reach a plateau (Fig. 1). By contrast, the exposure to caffeine of 14-day regenerating soleus (n = 5) generated a tension development that does not seem to reach a plateau. The contracture amplitude at the 5th min, expressed as normalized to \( P_0 \), was 0.29 ± 0.07 and 0.14 ± 0.02 in regenerating and in control muscles, respectively (P < 0.06). Moreover, the maximal initial rate of caffeine contracture development was significantly lower (P < 0.05) in regenerating than in control muscles (0.69 ± 0.05 and 1.03 ± 0.11 min\(^{-1}\), respectively).

Expression of key proteins of EC coupling. The unusual contracture response of regenerating soleus muscle suggested the presence of abnormalities in Ca\(^{2+} \) handling, in particular in Ca\(^{2+} \) release properties and/or in DHPR-to-RyR communication. Accordingly, we investigated the expression of DHPR and RyR isoforms in the regenerating muscle.

Western blot analysis in muscle homogenates (n = 3) showed a higher level of the cardiac isoform of DHPR \( \alpha_1 \)-subunit in the 14-day regenerating muscle than in control muscles (Fig. 2), confirming previous observation (31). Since the presence of \( \alpha_1 \)-C-subunit renders the muscle sensitive to extracellular Ca\(^{2+} \) levels (20), \( P_t \) and \( P_0 \) were also measured in muscles bathed by low-Ca\(^2+ \) solution (Ca\(^{2+} \) replaced by Mg\(^{2+} \)), \( P_t \) and \( P_0 \) of control soleus muscle (n = 6) were reduced in the absence of extracellular Ca\(^{2+} \) by 30.6% (P < 0.01) and 18.4% (not significant), respectively. Consistent with the higher content of the \( \alpha_1 \)-C DHPR subunit, \( P_t \) and \( P_0 \) of 14-day regenerating muscle (n = 6) were both significantly decreased (44.8%, P < 0.001, and 30.5%, P < 0.05, respectively).

The analysis of RyR isoform expression (Fig. 2) confirmed previous data (7) showing that normal soleus muscle (n = 6) expressed high levels of RyR1 (84.4 ± 4.3%) and trace amounts of RyR3 protein isoforms (15.6 ± 4.3%). In contrast, RyR1 expression level in neonatal muscle (n = 3) was low (18.3 ± 3.5%) and that of RyR3 was high (81.7 ± 3.5%). In the 14-day regenerating muscle (n = 8), RyR3 expression level (36.3 ± 5.7%) was significantly higher (P < 0.05) than in control muscle (Fig. 2).

We also analyzed the expression of SERCA1 (fast SERCA1a and neonatal SERCA1b) and 2a (slow) protein isoforms in the 14-day regenerating soleus. In agreement with previous data (16), Western blot analysis showed a threefold higher SERCA1 expression level in the regenerating muscle (n = 6) than in control muscle (n = 4). Most probably, the SERCA1 protein detected in the regenerating soleus muscle by the pan-specific antibody corresponds to the SERCA1b neonatal isoform, as recently demonstrated by Zador et al. (50). By contrast, SERCA2a isoform expression was comparable in regenerating and control muscles (data not shown).

MHC composition of regenerating and control soleus muscles (n = 4) was also analyzed. Regenerating muscles showed a slightly lower, not significant, content of type 1 and type 2A MHC isoforms with respect to the contralateral muscles. Type 1 MHC was 85.3 ± 6.5% in control and 82.1 ± 3.8% in regenerating soleus, while type 2A MHC was 14.7 ± 6.5% in control and 8.4 ± 2.1% in regenerating soleus. The main difference between the two muscles was the presence of substantial levels (9.5 ± 1.9%) of the embryonic MHC isoform in the regenerating soleus, not evident in control muscle (Fig. 3).

![Fig. 1. Typical responses to 30 mM caffeine of 14-day regenerating (14d-R) and contralateral uninjured (control) soleus muscles. Tension amplitude of contractures (P) is expressed as percentage of the maximum tetanic tension (P0) generated by the muscles.](http://jap.physiology.org/)
Regenerating Soleus Muscle Fibers

The mean CSA of single fibers isolated from 14-day regenerating soleus muscles (1,960 ± 124 μm², n = 64) was significantly (P < 0.001) smaller than that of fibers isolated from the contralateral uninjured soleus (3,570 ± 227 μm², n = 44). As chemical skimming solutions determine the swelling of isolated fibers, it is not surprising that CSA of chemically skinned fibers is larger than that in cryostat muscle sections. However, it is important to note that the swelling ratio is identical in control and regenerating fibers (1.45 and 1.48, respectively).

pCa-tension relationship. Regenerating fibers showed, on average, a significant right shift of the pCa-tension relationship with respect to control fibers. In Fig. 4A are shown the curves of control fibers expressing only type 1 MHC and the two curves of regenerating fibers expressing either type 1 plus the embryonic MHC isoforms or type 1, type 2A, and embryonic MHC isoforms. Since the values from regenerating fibers, regardless of the MHC profile, were indistinguishable, the data were pooled (Table 2). The pCa threshold for tension development and the pCa₅0, i.e., the amount of Ca²⁺ required to develop 50% of maximum tension, were significantly lower in regenerating fibers with respect to the corresponding values of control fibers. The Hill coefficient (N), an estimate of cooperative interactions between myofibrillar elements, was not significantly different in regenerating fibers with respect to control fibers. The specific tension produced by regenerating single fibers was lower (31%) than that of control fibers.

TnC isoforms in regenerating muscle. Since, in mammalian muscle fibers, the pCa-tension relationship is largely affected by the regulatory proteins, troponin and tropomyosin, we investigated the Ca²⁺-modulated TnC isoform content in several of the fibers used for the pCa sensitivity determination.

In control type 1 fibers, two groups can be identified: one group, comprising the majority of fibers (n = 9), containing only the TnCs isoform, and a second group (n = 3) containing only the TnCf. The three TnCf-expressing fibers were not considered in the subsequent analyses.

Regardless of the type of MHC expressed, we recognized in 14-day regenerating fibers two groups containing either TnCs or both TnCs and TnCf isoforms. We did not find fibers expressing only TnCf. Regenerating fibers containing only TnCs (n = 10) showed a pCa curve shifted to the right with respect to the corresponding control fibers, with the thresholds being significantly different (Table 3, Fig. 4B). Regenerating fibers with both TnCs and TnCf (n = 7) showed a further right shift of the pCa-tension curve with respect to the regenerating fibers containing only TnCs, with the pCa threshold being significantly lower (Table 2, Fig. 4B). This curve was significantly shifted to the right with respect to control adult fibers (Fig. 4B). Notably, the contemporary presence of TnCs and TnCf was paralleled by lower force generation capacity of isolated regenerating fibers (Table 3).

MHC composition of regenerating fibers. The MHC content of the single fibers submitted to the tests described below was determined by SDS-PAGE (36). The fibers isolated from control soleus (n = 88) were composed as follows: 73.9% of fibers contained solely type 1 MHC, 4.5% solely type 2A, and 21.6% of fibers contained both type 1 and type 2A MHC. None of the control fibers contained the embryonic MHC isoform.

By contrast, all of the regenerating fibers (n = 152) expressed more than one MHC isoform. About 43% of regenerating fibers contained both type 1 and the embryonic MHC isoforms. The residual regenerating fibers contained, in different proportion, type 1, 2A, and the embryonic MHC isoforms.

Passive Ca²⁺ leakage from the SR. No fibers isolated from control soleus showed significant spontaneous Ca²⁺ release from the SR. By contrast, in 14-day regenerating soleus fibers (n = 19), Ca²⁺ did leak out of the SR after loading, since the caffeine-induced tension 1, 3, and 5 min after Ca²⁺ loading was 67.4 ± 6.5, 56.4 ± 7.2, and 55.4 ± 6.8% of the pCa 5 tension, respectively.

SR caffeine threshold. The mean threshold caffeine concentration able to develop tension, i.e., able to stimulate the release of enough Ca²⁺ from the SR to cause fiber contraction, was significantly (P < 0.05) higher in regenerating (n = 37) than in control (n = 21) fibers (Table 4). The ratio between the tension developed at threshold caffeine concentration and that developed at 20 mM caffeine (a concentration able to completely empty the SR) was not different in control and regenerating fibers. By contrast, the contracture induced by 20 mM caffeine normalized to the pCa 5 tension was significantly higher in regenerating fibers (Table 4).

Fig. 2. α₁-subunit of dihydropyridine receptor (DHPR) and ryanodine receptor (RyR) expression in control and 14-day regenerating (14-d R) adult soleus muscles and in hindlimb muscle homogenate (50 μg of protein in each lane) from 3-day-old rat (neo). The fourth lane shows the expression of DHPR isoforms in adult rat heart. DHPR subunits were identified by the antibody ACC003 (Alomone Laboratories) and MAB 427 (Chemicon) specific for the cardiac (α₁C) and skeletal (α₁S) isoform, respectively. In SDS-PAGE, RyR1 and RyR3 differ in their apparent molecular mass and were both identified by monoclonal antibody 34C (DSHB).
**SR Ca\(^{2+}\) uptake and release.** As previously shown (16), the maximum Ca\(^{2+}\) uptake capacity of regenerating fibers (n = 24), measured by the light-scattering method, was higher (+26.7%) compared with control fibers (n = 40). No significant difference in SR Ca\(^{2+}\) release between regenerating and control fibers was detected (not shown).

**DISCUSSION**

Skeletal muscle regeneration after bupivacaine-induced degeneration was utilized as a model to investigate the correlation between contractility and the expression of specific protein isoforms at an intermediate stage of the functional recovery of soleus muscle. Our analysis, performed both at the whole muscle level and at the single-fiber level, shows that the mass of the regenerating muscle is greater than its force generation capacity, suggesting intrinsic defective EC coupling and/or contractility processes. Part of the lower specific tension is due to the immaturity of EC coupling characteristics of the regenerating muscle. This was confirmed by the persistence of the developmental isoforms of key proteins of EC coupling: the α1C subunit of the DHPR, the RyR3 isoform of the RyR, and neonatal SERCA1b calcium pump. Moreover, data from single fibers demonstrated that the lower specific tension is for the most part associated with the persistence of the embryonic MHC and the simultaneous presence of the TnCs and TnCf.

**Table 2. pCa-tension relationship in regenerating single fibers**

<table>
<thead>
<tr>
<th>pCaTH</th>
<th>Control (n = 29)</th>
<th>Regenerating (n = 36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCa(_{50})</td>
<td>6.55 ± 0.04†</td>
<td>6.55 ± 0.04†</td>
</tr>
<tr>
<td>N</td>
<td>2.66 ± 0.15</td>
<td>2.66 ± 0.15</td>
</tr>
<tr>
<td>Maximum tension, mN/mm(^2)</td>
<td>125.9 ± 5.2</td>
<td>87.2 ± 9.2†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of fibers. All control fibers contained the sole type 1 myosin heavy chain (MHC); 43.3% of the 14-day regenerating expressed type 1 and embryonic MHC, while 56.7% of fibers had type 1, 2A, and embryonic MHC: pCa\(_{TH}\), lowest pCa giving a detectable tension; pCa\(_{50}\), pCa value corresponding to 50% of maximum tension; N, Hill coefficient; maximum tension corresponds to the tension produced at pCa 5. Significant differences: *P < 0.05; †P < 0.001.

**Regenerating Soleus Muscle**

The analyses performed at the whole muscle level confirmed that the 14-day regenerating soleus muscle developed a lower maximal absolute force than uninjured control. Since the decrease in maximal P\(_0\) (-46%) was greater than the reduced mass (-26%), the specific P\(_0\) was significantly lower than in control (-25%), suggesting that, after 14 days, the muscle did not completely recover its force generation capacity. The intrinsic inability of the regenerating muscle to develop tension could be attributed to immature characteristics of contractile properties, myofibrillar organization, and/or EC coupling. Observations in developing muscles have indicated that transitions from neonatal to adult myosin phenotypes could influence the contractile properties of developing muscle. In fact, it has been suggested that the expression of neonatal MHC isoforms may contribute to the weaker force observed in developing muscles (19). Accordingly, specific tension of regenerating single fibers was significantly lower than that of control fibers.

A further observation made at the muscle level is that the 14-day regenerating soleus generated a more ample contracture after exposure to high caffeine. This result suggests the presence of some alteration in the EC coupling properties of regenerating muscle compared with control. In skeletal muscle, the Ca\(^{2+}\) necessary for contraction is released through RyR1. However, our data show that RyR1 is decreased, whereas RyR3 is present in 14-day regenerating muscles at elevated levels. It is thus possible that, in regenerating muscles, as

**Table 3. pCa-tension relationships of regenerating fibers grouped on the basis of TnC isoforms content**

<table>
<thead>
<tr>
<th>pCa(_{TH})</th>
<th>Control (TnCs (n = 9))</th>
<th>Regenerating (TnCs (n = 10))</th>
<th>Regenerating (TnCs + TnCf (n = 7))</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCa(_{50})</td>
<td>6.56 ± 0.06†</td>
<td>5.92 ± 0.12†</td>
<td>5.76 ± 0.07†</td>
</tr>
<tr>
<td>N</td>
<td>2.28 ± 0.39</td>
<td>2.09 ± 0.24</td>
<td>0.18 ± 0.50</td>
</tr>
<tr>
<td>Maximum tension, mN/mm(^2)</td>
<td>116.7 ± 27.1</td>
<td>81.4 ± 13.6</td>
<td>45.3 ± 10.0*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of fibers. TnCs and TnCf; slow and fast troponin C isoform, respectively. Significant differences: *P < 0.05 and †P < 0.002 with respect to the control; ‡P < 0.002 with respect to 14-day regenerating fibers.
demonstrated in neonatal muscles (1), full activation of SR Ca\(^{2+}\) release to achieve an optimal force may require the involvement of the RyR3 isoform. The caffeine contracture response is a good test to investigate the contribution of RyR3 to Ca\(^{2+}\) release. It has been demonstrated, in fact, that the amplitude of the response to caffeine in intact preparation of diaphragm is amplified by RyR3 (1, 35) and that in RyR3\(^{-/-}\) mice the lack of RyR3 determines a lower response to caffeine with respect to the wild type (1, 35). Accordingly, we found in regenerating soleus muscle a sustained development of caffeine contracture in soleus muscle that could be attributed to the higher RyR3 content. Consistent with this interpretation is the relatively stronger contracture induced by 20 mM caffeine (ratio between tension developed at 20 mM caffeine and at pCa 5) in single regenerating fibers, which could be explained by amplification of Ca\(^{2+}\)-induced Ca\(^{2+}\) release in the presence of RyR3 (28). Apparently in contrast, SR caffeine threshold of individual fibers reveals that the mean caffeine concentration able to stimulate Ca\(^{2+}\) release from the SR was significantly higher in the regenerating fibers compared with control fibers, independent of the overall higher RyR3 expression level. However, this result could be, at least in part, attributed to lower Ca\(^{2+}\) sensitivity of the myofibrillar proteins in regenerating fibers.

Caffeine contracture is a diagnostic test utilized to evaluate malignant susceptibility to hyperthermia (MH), which is a skeletal muscle disorder caused by genetic defects of RyR1, whose excessive stimulation may cause the hemorrhagic release of Ca\(^{2+}\) (29). However, also mutations of the \(\alpha_S\)-subunit of DHPR appear to cause MH (25), suggesting that, in MH, the whole EC-coupling mechanism is disturbed. Based on this knowledge, we speculate that also the expression of the \(\alpha_C\)-isoform of DHPR might affect the large caffeine response of regenerating soleus, probably by contributing to the higher SR Ca\(^{2+}\) uptake capacity of the 14-day regenerating fibers (15). It has been demonstrated that the \(\alpha_C\) cardiac isoform is expressed early during skeletal muscle regeneration and is gradually replaced by the \(\alpha_S\) skeletal muscle isoform (33). As an additional confirmation, the presence of \(\alpha_C\) was functionally established by the higher dependence on external Ca\(^{2+}\) of tension developed in regenerating the control muscle, as also previously shown (20).

On the whole, the analysis of regenerating muscle shows that the EC coupling mechanism could have an important role in the reduced specific tension of regenerating muscle.

### Regenerating Single Muscle Fibers

Specific force-generating capacity of permeabilized fibers isolated from 14-day regenerating soleus muscle, measured per CSA, was lower than that of control fibers. This result is similar to that reported recently (18) and is consistent with the reduced specific tension generated by the whole muscle. This result suggests that the presence of the embryonic MHC causes the reduction of maximal tension development in regenerating fibers. As a confirmation, we have data showing that the large variability of recorded tension in regenerating muscle fibers is related to the diverse proportion of embryonic MHC content. In fact, four fibers expressing type 1 MHC and trace amounts of embryonic MHC display a higher mean specific tension (89.4 ± 13.8 N/mm\(^2\)) than six fibers that contained a higher level of embryonic MHC (56.9 ± 17.4 N/mm\(^2\)). Moreover, independently of MHC content, regenerating fibers expressing both TnCs and TnCf developed lower specific tension than fibers containing solely TnCs. This result indicates that immature fibers (containing embryonic MHC and/or mixed TnCf and TnCis isoforms) are also the weakest.

An additional result refers to Ca\(^{2+}\) activation of contraction of regenerating fibers, which shows substantial changes with respect to control and could be correlated with the expression of TnCis isoforms (and of MHC). Analysis in individual skinned fibers offers an excellent condition to study the interactions of Ca\(^{2+}\) with the myofibrillar protein. We examined the contractility of single fibers, identified by their MHC composition, to understand the functional contribution of fiber-specific MHC and TnCis isoforms to Ca\(^{2+}\) sensitivity. The force-pCa relationship of all 14-day regenerating fibers was significantly shifted to the right, indicating a lower Ca\(^{2+}\) sensitivity. This result diverges from a recent report showing that the right shift was evident in soleus fibers after 7 days of regeneration, but was no longer present after 14 days (18). However, the different rat strain and the older age of animals utilized could explain the different recovery time course. While the force-pCa relationship of control, uninjured, soleus fibers was generated by fibers containing solely type 1 MHC, regenerating fibers showed a large heterogeneity with respect to the MHC composition as well as to TnCis isoforms. The majority of 14-day regenerating fibers (57%) contained type 1, 2A, and embryonic MHC isoform. The residual regenerating fibers contained solely type 1 and the embryonic MHC isoform. Regardless of the MHC composition, the force-pCa relationship of regenerating fibers was invariably shifted to the right with respect to control fibers, even the fibers containing solely type 1 MHC. Since MHC does not influence the pCa-tension relationship of myofibrillar protein, it appears improbable that the right shift could be attributed to the presence of the embryonic MHC isoform. Therefore, we analyzed the TnCis isoforms of regenerating fibers. About 70% of regenerating fibers contained the TnCs isoform, while the others contained both the TnCs and TnCf isoforms. In the regenerating fibers expressing solely TnCs, regardless of the MHC isoform, pCa-tension relationship was significantly shifted to the right with respect to the control fibers containing TnCs. This indicates that the lower Ca\(^{2+}\) sensitivity of the regenerating fibers cannot be referred to the TnCis isoforms only. In fact, evidence shows that troponin T, troponin I, and tropomyosin isoforms are also important determinants of myofibrillar Ca\(^{2+}\) sensitivity (8, 14, 17, 40, 45). However, regenerating fibers expressing both TnCs and TnCf showed further shifting of the pCa-tension curve to the right. The higher Ca\(^{2+}\) thresh-

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### Table 4. Sarcoplasmic reticulum caffeine sensitivity of regenerating single fibers

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 21)</th>
<th>Regenerating (n = 37)</th>
</tr>
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<tbody>
<tr>
<td>(\text{Th, mM})</td>
<td>0.76±0.10</td>
<td>1.32±0.18*</td>
</tr>
<tr>
<td>(P_{\text{a}}/P_{\text{b}})</td>
<td>0.21±0.03</td>
<td>0.21±0.02</td>
</tr>
<tr>
<td>(P_{\text{s}}/P_{\text{a}})</td>
<td>0.74±0.05</td>
<td>1.08±0.11††</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n\), no. of fibers. \(P_{\text{a}}/P_{\text{b}}\), ratio between the tension developed at caffeine threshold level (Th) and 20 mM caffeine; \(P_{\text{s}}/P_{\text{a}}\), ratio between the tension developed at 20 mM caffeine and at pCa 5. *P < 0.05; †P < 0.02.
old value of fibers expressing both TnC isoforms is consistent with the lower Ca\(^{2+}\)-binding affinity of the TnC1 isoform compared with TnCs (14, 47).

In conclusion, the present work illustrates the stage of development mirrored in 14 days of regeneration by soleus muscle. The maturation of regenerating fibers is largely incomplete. This apparent immaturity is probably correlated to the expression of α1C and RyR3 isoforms, as well as of embryonic TnC and MHC isoforms.

ACKNOWLEDGMENTS

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REFERENCES