Effects of fatigue on sarcoplasmic reticulum and myofibrillar properties of rat single muscle fibers

D. DANIELI-BETTO, 1 E. GERMINARIO, 1 A. ESPOSITO, 1 D. BIRAL, 2 AND R. BETTO 2

1 Dipartimento di Anatomia e Fisiologia Umana, and 2 Consiglio Nazionale delle Ricerche, Centro di Studio per la Biologia e la Fisiopatologia Muscolare, I-35131 Padova, Italy

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Danieli-Betto, D., E. Germinario, A. Esposito, D. Biral, and R. Betto. Effects of fatigue on sarcoplasmic reticulum and myofibrillar properties of rat single muscle fibers. J Appl Physiol 89: 891–898, 2000.—Force decline during fatigue in skeletal muscle is attributed mainly to progressive alterations of the intracellular milieu. Metabolite changes and the decline in free myoplasmic calcium influence the activation and contractile processes. This study was aimed at evaluating whether fatigue also causes persistent modifications of key myofibrillar and sarcoplasmic reticulum (SR) proteins that contribute to tension reduction. The presence of such modifications was investigated in chemically skinned fibers, a procedure that replaces the fatigued cytoplasm from the muscle fiber with a normal medium. Myofibrillar Ca2+ sensitivity was reduced in slow-twitch muscle (for example, the pCa value corresponding to 50% of maximum tension was 6.23 ± 0.03 vs. 5.99 ± 0.05, P < 0.01, in rested and fatigued fibers) and not modified in fast-twitch muscle. Phosphorylation of the regulatory myosin light chain isoform increased in slow-twitch muscle fibers (14.2 ± 1.0 vs. 19.6 ± 2.5 nmol·min⁻¹·mg fiber protein⁻¹, P < 0.05) and not altered in fast-twitch fibers. No persistent modifications of SR Ca2+ release properties were found. These results indicate that persistent modifications of myofibrillar and SR properties contribute to fatigue-induced muscle force decline only in slow fibers. These alterations may be either enhanced or counteracted, in vivo, by the metabolic changes that normally occur during fatigue development.

Prolonged stimulation of skeletal muscle causes the well-known phenomenon of fatigue. The mechanisms underlying skeletal muscle fatigue are, however, not yet completely understood. Many factors appear to contribute to force decline, the most relevant being 1) reduction of Ca2+ release from sarcoplasmic reticulum (SR), 2) reduction of myofibrillar Ca2+ sensitivity, and 3) reduction of maximum Ca2+ activated tension (11, 12, 29, 36). In addition, mammalian skeletal muscles are composed of variable mixtures of fibers with oxidative and/or glycolytic capabilities, and, consequently, they exhibit different fatigue resistance (19). During sustained exercise, the myoplasmic concentration of several metabolites varies greatly, thus influencing the activity of some of the proteins directly involved in the control of the contractile machinery. Both acidic pH and high P i concentrations, for example, are known to affect force production as well as Ca2+ uptake and release by the SR (14, 15, 21), even though the effective role of acidosis at physiological temperature is less evident (25, 35) than at lower temperature (18).

It has been recently suggested that, in addition to the well-known changes in muscle metabolites, tension fall during fatigue can also be correlated with post-translational modification of myofibrillar and/or SR proteins. Significantly, it has been demonstrated with chemically skinned fibers that, after replacement of the fatigued myoplasm with an environment that simulates the cytoplasm of a rested cell, fatigue alterations are still evident (37, 38). In those studies, frog fast-twitch semitendinosus muscle exposed to repetitive stimulation exhibited increased myofibrillar sensitivity to Ca2+ and a reduction of Ca2+ uptake and caffeine sensitivity of the SR.

The present study was undertaken to investigate whether fatigue causes similar persistent modifications also in mammalian skeletal muscles. Because of well-known differences in fatigability between muscle fiber types, fatigue was induced in both fast- and slow-twitch rat muscles by a prolonged tetanic stimulation. Whole muscles were chemically skinned before or immediately after fatigue. The SR Ca2+ uptake and Ca2+ release properties, SR caffeine sensitivities, and myofibrillar Ca2+ sensitivities were investigated on single skinned fibers for both muscle fiber types. Our results show that fatigue induces persistent modifications of the myofibrillar and SR properties in mammalian muscle, particularly in slow-twitch fibers.

METHODS

Fatigue protocol. The study was approved by the Ethical Committee of the Medical Faculty of the University of Padova. Soleus (140 ± 6 mg) and extensor digitorum longus (EDL, 130 ± 3 mg) muscles isolated from Wistar male rats (2–3 mo old) were used. The animals were killed under ether anesthesia. The muscles were dissected and immediately placed in a Ringer solution containing (in mM) 120 NaCl, 4.7...
KCl, 2.5 CaCl₂, 3.15 MgCl₂, 1.3 NaHPO₄, 25 NaHCO₃, 11.1 glucose, and 3.75 × 10⁻³ d-tubocurarine. The solution was continuously bubbled with O₂ (95%) and CO₂ (5%); the pH was 7.2–7.4. One muscle from each animal was used as control, and the contralateral was stimulated to fatigue. The muscle was mounted vertically and connected to an isometric force transducer (Harvard 50–7947, South Natick, MA) and stimulated by applying supramaximal stimuli delivered by an electronic stimulator (Grass S44, Quincy, MA) (23). Fatigue was induced at room temperature by a tetanizing stimulation (25 Hz for soleus and 40 Hz for EDL), frequencies known to produce 50% of peak force, of 300-ms duration, repeated every 3 s. Electric stimulation was prolonged for 30 min in soleus and 10 min in EDL until the tetanic force declined to a plateau level that was −30 and 15% of the initial value, respectively.

**Chemical skinning of muscle fibers.** Resting and fatigued muscles were tied to a wooden stick and quickly immersed into an ice-cold skinning solution containing (in mM) 170 potassium propionate, 2.5 magnesium propionate, 2.5 ATP, 5 EGTA, and 10 imidazole, pH 7.0. Chemical skinning was carried out at 0–4°C as previously described (8, 28). At the first, second, fourth, and twenty-third hour, the skinning solution was replaced with fresh solution. After 24 h, skinned muscles were transferred to a skinning solution supplemented with 50% (vol/vol) glycerol and stored at −20°C. Skinned fibers were used within 2–3 wk of preparation. The skinning procedure, by permeabilizing the sarcolemma, allows the complete removal of the myoplasm, but it preserves the SR and myofilaments (27).

**pCa-tension relationship.** Single fiber segments were isolated under a dissecting microscope and transferred to a chamber containing 0.8 ml of a relaxing (Ca²⁺-free) solution containing (in mM) 170 potassium propionate, 2.5 magnesium propionate, 2.5 ATP, 5 EGTA, and 10 imidazole, pH 7.0. The fiber segments were inserted between two clamps (the mean fiber segments' length between the clamps was ~1.5 mm), one of which was connected to a tension transducer (AK Sensonor, Horten, Norway), and stretched up to 30% of their slack length (8). pCa-tension curves (in which pCa indicates −log of Ca²⁺ concentration) were obtained by exposing the fibers sequentially to solutions of different free calcium concentrations at room temperature (22–24°C), as previously described (8). The isometric tension generated in each solution was continuously recorded, and the baseline tension was established as the steady-state voltage output recorded with the fiber in relaxing solution. Specific tension for each single fiber was calculated by normalizing the maximum tension measured at pCa 5 to the fiber cross-sectional area, as calculated by three different diameter determinations along the fiber length, considering the fiber immersed in solution as a cylinder. For rested and fatigued fibers, maximum tension developed in the presence of pCa 5 was determined before and after each experimental protocol. Only fibers showing no significant differences between initial and final values were utilized.

**Ca²⁺ uptake and Ca²⁺ release measurements.** Ca²⁺ uptake by the SR was measured at room temperature (22–24°C) either by the light-scattering method (27), as previously described (7, 23), or by a caffeine contracture method (37). With the light-scattering method, fibers were mounted in a chamber containing relaxing solution and stretched to 180% of slack length to avoid interference in light-scattering measurements caused by actin-myosin interactions (27). Fibers were then incubated in a Ca²⁺ loading solution (pCa 6.4) containing (in mM) 170 potassium propionate, 5 NaK₂ATP, 2.5 magnesium propionate, 5 K₂EGTA, 2.15 Ca²⁺, and 10 imidazole buffer, pH 7.0. Ca²⁺-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 Ca²⁺ content, with the plateau level of light-scattering representing the maximum capacity for Ca²⁺ uptake of SR (27). The calibration procedures for converting the light-scattering signal to fiber Ca²⁺ concentration by using ⁴⁵Ca²⁺ were described in detail elsewhere (27). The relative increase in light scattering was proportional to the Ca²⁺ concentration inside the fiber. The proportionality constants for type 1 and type 2 fibers were 0.260 ± 0.035 (n = 6 fibers) and 0.200 ± 0.031 (n = 6 fibers) nmol ⁴⁵Ca²⁺/light-scattering unit⁻¹·μg protein⁻¹, respectively.

When the light-scattering signal reached a plateau level, Ca²⁺ release from the SR was initiated by rapidly exchanging the Ca²⁺-loading solution with a relaxing solution containing (in mM) 170 potassium propionate, 5 K₂EGTA, 10 caffeine, and 10 imidazole buffer, pH 7.0. To prevent SR Ca²⁺ uptake by the Ca²⁺-pumps, the releasing solution did not contain Mg²⁺ and ATP. Caffeine-induced Ca²⁺ release from the SR was monitored by the maximal decrease in light scattering from which the initial Ca²⁺ efflux rates were calculated. To evaluate the possible contribution of SR leakage to the caffeine-induced Ca²⁺ release, spontaneous release of the SR-stored Ca²⁺ was analyzed before and after fatigue by use of the method described by Trachez et al. (33). Fibers were maximally loaded with Ca²⁺ (3 min in pCa 7.0) and then soaked in the relaxing solution for variable periods of time (1, 3, and 5 min). Ca²⁺ release as a consequence of spontaneous leakage from the SR was buffered by the EGTA present in relaxing solution. After two washings with the washing solution, 20 mM caffeine was added to promote release of Ca²⁺ remaining in the SR. The peak amplitude of caffeine-induced tension was used to estimate leakage of Ca²⁺ from SR during the exposure to relaxing solution. However, no SR leakage was found in rested and fatigued muscle fibers (not shown).

The caffeine contracture method is an indirect technique that allows estimation of the amount of Ca²⁺ stored in the SR after various periods of Ca²⁺ loading (37). Fibers were first exposed to the relaxing solution containing 20 mM caffeine to deplete the SR of Ca²⁺. After being rinsed in the relaxing solution, the fiber was incubated in pCa 7.0 loading solution for 15, 30, 60, 120, and 180 s. After each loading period, the fiber was immersed in a relaxing solution deprived of EGTA (washing solution) and exposed to 20 mM caffeine, and the tension developed was measured. For each fiber, the contracture force was plotted against loading duration, and data were fitted, via nonlinear regression (r² = 0.93–0.99), to the equation F = 100(1−e⁻kt), where F is the measured tension normalized to the maximum tension developed, k is the rate constant for Ca²⁺ uptake, and t is the loading duration (37).

**Caffeine sensitivity of the SR.** Caffeine sensitivity of SR Ca²⁺ release was also analyzed indirectly by following the minimal caffeine-induced tension development (7, 28). Fibers were allowed to accumulate Ca²⁺ into the SR by incubation in a pCa 7.0 loading solution (same composition of relaxing solution with 0.8 mM Ca) for 30 s at room temperature. After Ca²⁺ loading, fibers were immersed in the washing solution (see above) and then challenged in a stepwise manner with increasing concentrations of caffeine until tension was recorded. Caffeine threshold was defined as the lowest concentration of caffeine that was able to induce an appreciable tension (28).

Caffeine contracture experiments were performed by exposing the whole muscle to a 30 mM caffeine solution and measuring the subsequent contracture, both in resting con-
ditions and 30 s after the fatiguing protocol. The contracture
tension was expressed as percentage of twitch tension.

Single-fiber SDS-PAGE. Single chemically skinned fibers
were identified by their myosin heavy chain (MHC) compo-
sition (9). At the end of each experiment, the fiber segment
was dissolved with 20 μl of SDS-PAGE solubilization buffer
(62.5 mM Tris, pH 6.8, 2.3% SDS, 5% 2-mercaptoethanol,
10% glycerol) and analyzed by 7% PAGE (26) to identify the
MHC isoform composition. The evaluation of the experimen-
tal data was limited to type 1 (slow-twitch, fatigue-resistant)
fibers from soleus muscle containing only the MHC1 isoform
(Fig., lane c) and from type 2 fast-twitch, fatigue-sensitive
EDL muscle fibers containing only the MHC2B isoforms (Fig.
1, lane d), or those fibers of EDL that besides the MHC2B
contained traces of MHC2X only (Fig. 1, lane e).

Myosin light chain isoform analysis. Myosin light chain
composition was analyzed by two-dimensional gel electro-
phoresis as previously described (3). About 20 cryostat muscle
sections (20 μm thin) were dissolved in 100 μl of 9.5 M
urea, 2% (vol/vol) Nonidet NP-40, 5% (vol/vol) 2-mercapto-
ethanol, 1.0% (vol/vol) Ampholine (LKB) of pH range 5–7,
and 1.0% (vol/vol) Ampholine (LKB) of pH range 3.5–10 and
subjected to isoelectric focusing. SDS-PAGE in the second
dimension was performed in 15% (wt/vol) polyacrylamide
slab gels. The relative amounts of phosphorylated and non-
phosphorylated myosin light chain bands were determined by
densitometry of SDS-PAGE slab gels by using a Bio-Rad
imaging densitometer (GS-670).

Statistical analysis. Means and SE were calculated from
individual values by standard procedures. Results were an-
alyzed by one-way ANOVA performing multiple comparisons
against the control group (SigmaStat, Jandel Scientific). The
0.05 level of probability was established for statistical signif-
icanve. pCa-tension data from each muscle fiber were fitted
by a least squares method using the Table Curve fitting
program (Jandel Scientific) according to the equation
\[ y = \max x^k/(x^n + k^n) \] 
where max is the maximal value of pCa-
tension curve, which was normalized to 1, \( k \) is the pCa at 50%
of maximum tension (pCa50), and \( N \) is the Hill coefficient.

RESULTS

The occurrence and relevance of posttranslational
modifications during fatigue were investigated by using
single muscle fibers chemically skinned immedi-
ately after the fatiguing protocol and by comparing their contractile properties with those of resting fibers.
The chemical skinning procedure allows the complete
removal of fatigue milieu and, by replacement with a
physiological medium, should reintroduce the original
capacity of the fiber to produce 100% of tension (36).

pCa-tension relationships. The pCa-tension relation-
ship of chemically skinned type 1 fibers from soleus
muscle was significantly shifted to the right after fa-
tigue compared with rested fibers (Fig. 2A). In fact, the
pCa threshold, i.e., the lowest concentration of calcium
inducing a detectable tension, and the pCa50 were
significantly lower in fatigued than in rested fibers
(Table 1). The Hill coefficient, an estimate of the cooper-
activity among the elements participating to the activ-
ation of the contractile apparatus (8), was unmodified
by fatigue (Table 1), suggesting that only calcium sen-
sitivity of myofilaments was altered.

In comparison with the behavior of soleus fibers, the
cpCa-tension relationship of fast-twitch fibers isolated
from EDL muscle was only modestly affected by fa-
tigue. In fact, minor, not significant, increases in the
pCa threshold for tension development were observed
in the EDL fatigued fibers (Fig. 2B); no modifications of
the pCa50 and of the Hill coefficient were evident (Ta-
ble 1).

Two-dimensional analysis of myosin light chains. The myofibrillar calcium sensitivity of skeletal and cardiac muscles may be influenced by the state of
phosphorylation of the regulatory myosin light chains, which are phosphorylated by a specific Ca2+/calmodu-
lin-dependent kinase and dephosphorylated by a type 1
myofibrillar phophatase (30). We investigated whether the changes in the pCa-tension relationship of
fatigued muscle fibers were attributable to changes in
the phosphorylation states of the regulatory light
chains. Accordingly, two-dimensional analysis of myo-
sin light chains was performed to identify changes in
protein phosphorylation resulting from fatigue. As
shown in Fig. 3, the regulatory light chains (labeled 2F)
of EDL fibers were present as two distinct protein
bands with the same molecular weight but different
isoelectric point, both in rested and fatigued muscles.
After fatigue, the EDL muscle exhibited a significant
increase in the amount of the phosphory-
lated regulatory light chains (2F-P), which changed
from 44.3 ± 3.3% (n = 4) on rested muscles to 61.3 ±
3.8% (n = 4) on fatigued muscles (Fig. 3, left). Con-
versely, the regulatory myosin light chains (2S) of
soleus fibers were not phosphorylated in the rested
muscle and were not phosphorylated after fatigue (Fig.
3, right).

SR Ca2+ uptake and release. It has been previously
demonstrated, using purified SR vesicles (4) and skinned
fiber preparations (27), that nonfatigued fast-twitch
muscle fibers possess mean SR Ca2+ uptake capacities
that are at least double those of slow-twitch muscle
fibers. We have confirmed those initial observations
and extended them to include other measures of SR
function and, most importantly, changes in SR func-
tion resulting after fatigue (Table 2). The initial Ca2+
release rate induced by 10 mM caffeine was 30% higher in EDL type 2B fibers than in soleus type 1 fibers, whereas in isolated SR vesicles it is reported that the Ca\(^{2+}\) release rate of fast SR is at least four times that of slow SR (29). However, this apparent difference is attributed to the well-known higher sensitivity to caffeine of slow-twitch muscle fibers compared with fast-twitch fibers (28, 29; see also the caffeine threshold data shown below).

Fatigue did not modify the mean total SR Ca\(^{2+}\) uptake capacities of either EDL type 2B or soleus type 1 muscle fibers (Table 2). On the other hand, the rate of Ca\(^{2+}\) uptake by the SR measured by the light-scattering method was significantly increased in fatigued soleus fibers, whereas it was unmodified in fast EDL fibers (Table 2). Similar results were obtained by using the caffeine contracture procedure (37), in which the SR of fatigued soleus type 1 fibers accumulated calcium at a higher rate than did rested fibers (Fig. 4A). No differences in Ca\(^{2+}\) uptake rate were evident in EDL type 2B fibers (Fig. 4B). The values of K\(_{Ca}\), i.e., the rate constant for Ca\(^{2+}\) uptake, were 2.08 ± 0.29 in rested type 1 fibers and 3.06 ± 0.28 in fatigued fibers (P < 0.05), whereas the values in type 2B fibers were 1.66 ± 0.11 and 1.78 ± 0.38, respectively. It is worth noting that, because of the higher sensitivity to caffeine of slow than of fast muscles (see above), the method used in Fig. 4 (37) does not allow appreciation of the known higher Ca\(^{2+}\) uptake rate of fast muscle than of slow muscles (28, 29).

With the light-scattering method, it is also possible to evaluate the SR Ca\(^{2+}\) release properties of single muscle fibers by stimulating Ca\(^{2+}\) release with 10 mM caffeine after Ca\(^{2+}\) filling of the SR (23, 27). The initial SR Ca\(^{2+}\) release rates of chemically skinned type 1 soleus and of type 2 EDL muscle fibers were not modified after fatigue (Table 2).

In this preparation, we also analyzed the caffeine sensitivity of SR Ca\(^{2+}\) release. The mean caffeine threshold concentrations capable of inducing significant tension were 1.30 ± 0.22 and 7.89 ± 0.51 mM, respectively, for type 1 and type 2 nonfatigued fibers. The corresponding values for fatigued fibers were 2.03 ± 0.06 and 7.93 ± 0.54 mM, demonstrating no significant alterations in caffeine threshold in mammalian muscle fibers as a function of fatigue status.

Whereas fatigue did not cause persistent modifications in caffeine sensitivity of SR Ca\(^{2+}\) release, the caffeine sensitivity of the whole muscle contractility was dramatically affected by fatigue (Fig. 5). This fig-

Table 1. Effect of fatigue on the pCa sensitivity of tension development of type 1 and type 2 rat fibers from soleus and EDL skeletal muscles

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<th>pCa_{TH}</th>
<th>pCa_{50}</th>
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<td>Soleus</td>
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<tr>
<td>Rosted (14)</td>
<td>6.80 ± 0.04</td>
<td>6.23 ± 0.03</td>
<td>2.49 ± 0.16</td>
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<tr>
<td>Fatigued (17)</td>
<td>6.66 ± 0.04*</td>
<td>5.99 ± 0.05*</td>
<td>2.81 ± 0.12</td>
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<tr>
<td>EDL</td>
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<tr>
<td>Rosted (31)</td>
<td>6.26 ± 0.03</td>
<td>5.91 ± 0.02</td>
<td>4.04 ± 0.25</td>
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<tr>
<td>Fatigued (31)</td>
<td>6.37 ± 0.03</td>
<td>5.88 ± 0.04</td>
<td>4.07 ± 0.35</td>
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Values are means ± SE for the numbers of the analyzed single fibers given in parentheses. EDL, extensor digitorum longus; pCa_{TH}, lowest pCa giving a detectable tension; pCa_{50}, pCa value corresponding to 50% of maximum tension; N, Hill coefficient. Student’s t-test was applied for comparison between groups. *Statistical differences between the rested and the fatigued muscles for values of P < 0.01.
ure demonstrates that the caffeine contractures produced by both EDL and soleus muscles were significantly reduced by fatigue.

**DISCUSSION**

The decline in force induced by fatigue in skeletal muscle is ascribed mainly to the accumulation of metabolites and to decreases in the free calcium concentration of the myoplasm (11, 12, 29, 36). Recovery of a fatigued muscle takes variable time, depending on the ability of the muscle to restore the normal ionic and metabolite levels. One would predict that chemically skinned fibers, which have carefully controlled metabolic and ionic environments, should demonstrate no evidence of fatigue if alterations in soluble metabolites were the only factors responsible for fatigue. However, previous results on frog muscle (37, 38) and the present results on mammalian muscle fibers demonstrate that fatigue-related changes of myofibrillar protein properties and of SR activities are still evident in the chemically skinned muscle fiber preparation devoid of metabolite perturbations. Moreover, our results show that, in mammalian muscles, the effects of fatigue that persisted after chemical skinnng were different in fast- and slow-twitch fibers.

The persistent changes that we demonstrated in chemically skinned fibers could be attributed to posttranslational modifications of proteins, which include enzymatic and nonenzymatic modifications. Enzymatic posttranslational modification of proteins comprises, for example, phosphorylation and dephosphorylation, cleavage, methylation, glycosylation, and ADP-ribosylation. Nonenzymatic modifications, instead, involve chemical-physical perturbations of proteins such as, for example, oxidation, glycation, and deamination.

**Myofibrillar properties of fatigued fibers.** For skinned slow-twitch rat muscle fibers, fatigue causes a significant reduction in myofibrillar protein sensitivity to

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<th>Table 2. Ca^{2+} transport activities of the sarcoplasmic reticulum of soleus and EDL rested and fatigued fibers</th>
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<td>Ca^{2+} uptake capacity, μmol/mg fiber protein</td>
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<td>Rate of Ca^{2+} uptake, nmol·min^{-1}·mg fiber protein^{-1}</td>
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<td>Initial Ca^{2+} release rate, nmol·min^{-1}·mg fiber protein^{-1}</td>
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Values are means ± SE; nos. of fibers are in parentheses. Ca^{2+} uptake capacity, rate of Ca^{2+} uptake and initial caffeine-induced Ca^{2+} release rate of rested and fatigued type 1 soleus fibers (i.e., fibers expressing type 1 MHC isoform only) and of rested and fatigued type 2B fibers expressing type 2B MHC isoforms plus fibers that, besides type 2B, contained traces of type 2X MHC isoforms) EDL fibers. *P < 0.05 vs. resting fibers values.

![Fig. 4. Caffeine contracture tension recording after sarcoplasmic reticulum Ca^{2+} loading for various time periods of soleus and EDL single muscle fibers (A and B, respectively). Values are means ± SE for number of examined fibers indicated in parentheses. *P < 0.05 vs. resting fibers values.](image)

![Fig. 5. Caffeine contracture of rested (open symbols) and fatigued (solid symbols) soleus (circles) and EDL (triangles) whole rat muscles. Values are means ± SE for number of examined muscles indicated in parentheses; caffeine contractures in fatigued muscles were always significantly lower than in control muscles.](image)
calcium, indicating that, to produce the same tension as in rested fibers, a higher free calcium concentration is needed. In contrast to slow-twitch fibers, fast-twitch fibers did not show a fatigue-dependent right shift of pCa-tension curves.

Even though calcium sensitivity may be influenced by fatigue, we observed that the maximal Ca$^{2+}$-activated tension of fatigued skinned fibers was identical to that of rested fibers. This result indicates that changes in myofibrillar calcium sensitivity caused by fatigue in soleus skinned fibers reside in modifications of regulatory proteins, which reduce the number of cross bridges at a given pCa, but not when myoplasmic calcium concentration is above that for saturation of troponin C. However, in intact fibers, a reduced maximal calcium activated force, as well as a reduced myofibrillar calcium sensitivity, has been observed (36). Thus, besides the changes in the regulatory proteins, other factors may influence the number of or the tension developed by cross bridges, such as, for example, reduced intracellular Ca$^{2+}$ and the accumulation of myoplasmic P$_i$, known to influence maximal Ca$^{2+}$-activated tension (14, 15, 21).

On the basis of results with skinned fibers, repetitive stimulation of fast-twitch muscle fibers is known to cause a leftward shift in the pCa-tension relationship as a consequence of myosin light chain-2 phosphorylation, and it is also known that this is mediated by a specific Ca$^{2+}$/calmodulin-dependent endogenous protein kinase (20, 30). Phosphorylation of the regulatory light chain affects Ca$^{2+}$ sensitivity of fast-twitch fibers prevalently at high and moderate pCa values (30). Phosphorylation of the regulatory light chain may represent a mechanism activated by mammalian skeletal muscle to counteract the effects of fatigue. However, this adaptation is true only for fast-twitch myosin. In fact, the regulatory light chain of slow-twitch muscles is not phosphorylated in the resting state, and stimulation does not modify this condition (see Fig. 3). On the other hand, it is possible that fatigue causes a right shift also in fast-twitch fibers, but this occurrence may be counteracted by light chain phosphorylation.

A possible mechanism operating in slow-twitch muscle to account for changes in myofibrillar calcium sensitivity during fatigue is oxidation of SH groups, which has been shown to modify Ca$^{2+}$ sensitivity (1, 39). However, this mechanism has been shown to also reduce maximal Ca$^{2+}$-activated tension. Because we did not observe significant modification of maximal tension, either this mechanism is not working or its effect is not relevant. An additional possible mechanism operating during fatigue is glycation (17) and/or deamination (2) of myofibrillar proteins. In particular, the glycation mechanism appears to be plausible during fatigue, because it has been observed that both pH and phosphate affect glycation of proteins (31). Finally, Williams (37) hypothesized also that extensive stimulation might produce some transient disarrangement of myofilaments that could involve regulatory proteins. In fact, removal, even partial, of regulatory proteins strongly affects myofilaments calcium sensitivity (22).

**SR Ca$^{2+}$ flux properties of fatigued fibers.** Tension decline during fatigue is associated with substantial changes in the intracellular milieu, which, in turn, are mainly responsible for the progressive ineffective delivery of calcium to the myofilaments, likely attributable to an altered excitation-contraction coupling mechanism and changes in SR calcium content and Ca$^{2+}$ release (11, 12, 29, 36).

The present results showed that the SR of slow-twitch fibers chemically skinned immediately after fatigue accumulates Ca$^{2+}$ at a higher rate than that of fibers skinned before fatigue, whereas no appreciable modifications were evident in fast-twitch fibers. Moreover, the SR Ca$^{2+}$ release properties of both fast- and slow-twitch chemically skinned fibers were not modified by fatigue. In a fast-twitch frog skeletal muscle, a significant reduction both in the rate constant of SR Ca$^{2+}$ uptake and in the caffeine sensitivity of SR Ca$^{2+}$ release was reported (37, 38). The discrepancy between these data and ours may be ascribed to species-specific mechanisms.

Studies on isolated SR demonstrate that strenuous exercise causes either reduction of SR Ca$^{2+}$ uptake (5, 6, 13) or no modifications (10). It is possible that these conflicting results may be due to different SR isolation techniques and/or to differences in the type of exercise and fiber population of the muscles studied. In addition, calcium phosphate precipitation within the SR occurring in late fatigue (14) may alter the properties of SR membranes isolated from fatigued muscles that, in turn, could be responsible for the lower Ca$^{2+}$ uptake capacity observed.

SR Ca$^{2+}$ uptake activity is attributed to a specific Ca$^{2+}$ pump, which is located in the SR. The activity of cardiac and slow-twitch skeletal muscles Ca$^{2+}$ pumps can be modulated by phosphorylation. Direct phosphorylation by a Ca$^{2+}$/calmodulin-dependent protein kinase activates the Ca$^{2+}$ pump (16), and phosphorylation by cAMP-dependent and Ca$^{2+}$/calmodulin-dependent protein kinases of phospholamban, a regulatory protein associated with the pump protein, further stimulates the Ca$^{2+}$ pump (24). Thus the higher SR Ca$^{2+}$ uptake rate observed in fatigued slow-twitch fibers is consistent with the possible activation of the Ca$^{2+}$ pump by phosphorylation either directly or indirectly through phospholamban.

In intact single muscle fiber fatigue, however, a marked reduction of the rate of Ca$^{2+}$ removal from cytoplasm has been observed (36). It is worth noting that the incubation of skinned fibers in conditions that mimic those produced by fatigue have been demonstrated to influence the Ca$^{2+}$ uptake rate. For example, high P$_i$ concentration stimulates (14, 32), whereas acidosis reduces, the SR Ca$^{2+}$ uptake (34). Thus the slowing in the Ca$^{2+}$ uptake rate observed in intact fibers is likely the result of the effects of fatigue metabolites combined with those of posttranslational modifications of the Ca$^{2+}$ pump and/or phospholamban. Additionally, it appears that fatigue causes a number of modifications, some metabolic and others
not, that lead to the slowing of the net Ca\textsuperscript{2+} uptake rate.

Even though we have demonstrated fatigue-dependent alterations in calcium uptake properties, our results show that SR Ca\textsuperscript{2+} release properties of skinned fibers were not significantly modified by fatigue. Thus any calcium release defects seen in intact muscles should be ascribed to changes either in metabolite levels (36), in action potential characteristics, or in the activation process of the T-tubular charge sensor (12, 29, 36).

In conclusion, these results demonstrate that posttranslational enzymatic and/or nonenzymatic modifications of proteins responsible for myofibrillar and SR properties contribute to force decline caused by fatigue in mammalian slow-twitch muscle fibers. Conversely, posttranslational changes of proteins appear not to play a role in fatigue of fast-twitch muscle.

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