Depressed contractile performance and reduced fatigue resistance in single skinned fibers of soleus muscle after long-term disuse in rats

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Udaka J, Terui T, Ohtsuki I, Marumo K, Ishiwata S, Kurilnara S, Fukuda N. Depressed contractile performance and reduced fatigue resistance in single skinned fibers of soleus muscle after long-term disuse in rats. J Appl Physiol 111: 1080–1087, 2011. First published June 30, 2011; doi:10.1152/japplphysiol.00330.2011.—Long-term disuse results in atrophy in skeletal muscle, which is characterized by reduced functional capability, impaired locomotor condition, and reduced resistance to fatigue. Here we show how long-term disuse affects contractility and fatigue resistance in single fibers of soleus muscle taken from the hindlimb immobilization model of the rat. We found that long-term disuse results in depression of caffeine-induced transient contractions in saponin-treated single fibers. However, when normalized to maximal Ca2+-activated force, the magnitude of the transient contractions became similar to that in control fibers. Control experiments indicated that the active force depression in disused muscle is not coupled with isoform switching of myosin heavy chain or troponin, or with disruptions of sarcomere structure or excessive internal sarcomere shortening during contraction. In contrast, our electronmicroscopic observation supported our earlier observation that interfibrillar lattice spacing is expanded after disuse. Then, to investigate the molecular mechanism of the reduced fatigue resistance in disused muscle, we compared the inhibitory effects of inorganic phosphate (Pi) on maximal Ca2+-activated force in control vs. disused fibers. The effect of Pi was more pronounced in disused fibers, and it approached that observed in control fibers after osmotic compression. These results suggest that contractile depression in disuse results from the lowering of myofilirillar force-generating capacity, rather than from defective Ca2+ mobilization, and the reduced resistance to fatigue is from an enhanced inhibitory effect of Pi coupled with a decrease in the number of attached cross bridges, presumably due to lattice spacing expansion.

skeletal muscle; muscle mechanics; troponin

IT IS WELL ESTABLISHED that muscular inactivity, such as space-flight, hindlimb unloading, and bed rest, leads to atrophy, especially in slow skeletal muscle (e.g., 7). Recent studies employing cell and molecular biology technologies (for reviews, see 14, 17, 18, 20) successfully identified hormones, growth factors, and cytokines that cause quantitative changes (i.e., loss of myofilibrillar proteins) in skeletal muscle after disuse. In our previous study (32), we demonstrated that long-term disuse causes preferential loss of the giant elastic protein titin (connectin) during myofibrillogenesis in vivo, leading to marked alteration of the sarcomeric ultrastructure, i.e., 1) myofilament lattice spacing expansion and 2) thick and thin filament shortening. These changes in sarcomeric ultrastructure result in a loss of cross-bridge formation and, hence, a reduction of active force production. However, the changes in sarcomeric ultrastructure may be accompanied by changes in the properties of the sarcoplasmic reticulum (SR), i.e., Ca2+ sequestration and/or Ca2+ release, resulting in an alteration in intracellular Ca2+ mobilization.

In skeletal muscle, contraction is triggered by membrane depolarization, followed by a transient rise in the intracellular Ca2+ concentration. Downstream of the Ca2+ transient, the sensitivity of myofilaments to Ca2+, commonly known as Ca2+ sensitivity, is another determinant in the setting of the magnitude of contractility. With an increase in myoplasmic Ca2+ concentration, the binding of Ca2+ to troponin (Tn) C results in a series of structural changes of the regulatory proteins, i.e., Tn and tropomyosin (Tm) on the thin filament, facilitating the formation of cross bridges with actin, thereby producing contractile force. Reportedly, isoform changes occur in various myofilibrillar proteins upon disuse, viz., from slow to fast type; such as in myosin heavy chain (MHC), TnT, TnI, TnC, and Tm (see 32 and references therein). It is still unknown whether or not these changes contribute to the depressed contractile performance of disused muscle.

During fatigue, inorganic phosphate (Pi) accumulates inside skeletal muscle cells up to 20–30 mM, accompanied by a decrease in pH to ~6.0, resulting in marked reductions in active force production (e.g., 1, 2). It is well known that Pi reverses the force-generating step in the actomyosin cycle and, consequently, depresses active force by decreasing the number of attached cross bridges (e.g., 16). H+ has been reported to directly act on cross bridges (24, 25), as well as on TnC (3, 6, 27), resulting in a reduction in the fraction of attached cross bridges (i.e., reduced active force). In a recent review article, Allen (2) summarized that the fatigue-induced contractile depression is comprised of three phases, i.e., 1) phase 1: acute drop (10–20%) of active force by Pi coupled primarily with the breakdown of creatine phosphate; 2) phase 2: slight decrease in active force associated with a change in Ca2+ transients; and 3) phase 3: persistent and progressive reduction of active force due to the reduction of the Ca2+ release from the SR and the reduced sensitivity of myofilaments to Ca2+ by Pi (and H+). Therefore, in both phases 1 and 3, Pi is the primary cause of the decline in active force during fatigue, resulting presumably from a decrease in the number of attached cross bridges (2). It is therefore likely that the reduced resistance to fatigue in disused muscle is coupled with apparent enhancement of the sensitivity of myofilaments to Pi (and H+).

The present study was undertaken to elucidate whether the active force depression in atrophied skeletal muscle is coupled with the changes in sarcomeric ultrastructure or, alternatively,
with the alteration in intracellular Ca\(^{2+}\) mobilization in a long-term hindlimb immobilization model of the rat. Likewise, we tackled the problem of the reduced fatigue resistance, by investigating whether and how the depressant effect of Pi or H\(^+\) is altered in atrophied muscle. Our analyses revealed that the active force depression is mainly due to the reduced force-producing capability of the sarcomere rather than defective Ca\(^{2+}\) mobilization, and that the reduced resistance to fatigue is associated with a decrease in the number of attached cross bridges, presumably due to expansion of interfilament lattice spacing.

MATERIALS AND METHODS

This study was performed in accordance with the Guideline on Animal Experimentation of the Jikei University School of Medicine, and study protocol was approved by the Animal Care Committee of The Jikei University School of Medicine.

**Disuse atrophy model and muscle mechanics.** Experiments were performed in accordance with our previous work (32) with male Wistar rats (300–330 g). Briefly, hindlimb immobilization was performed with casting tape (type 82002-1, Sumitomo 3M, Tokyo, Japan) changed every week to keep the ankle joint in a fully extended position. No intervention was performed on the right leg (used as control). After 6 wk of immobilization, animals were anesthetized with pentobarbital sodium (50 mg/kg ip), and the whole soleus muscles were dissected from both legs and quickly immersed in oxygenated Ca\(^{2+}\)-free Tyrode’s solution (see 11 for composition) containing 30 mM 2,3-butanedione monoxime (BDM) at 30°C. The wet weight was 167 ± 7 and 113 ± 6 mg (P < 0.05; 18 animals) in control and disused muscles, respectively. No reduction of body weight was observed during immobilization (as in 32). Small bundles (1- to 2-mm-diameter; ~10-mm length) were dissected from the muscles and skinned in relaxing solution [5 mM MgATP, 40 mM BES, 1 mM Mg\(^{2+}\), 10 mM EGTA, 1 mM dithiothreitol, 15 mM phosphocreatine, 15 U/ml creatine phosphokinase, 180 mM ionic strength (adjusted with K-propionate), pH 7.0] containing 1% (wt/vol) Triton X-100 and 10 mM BDM overnight at ~3°C. The preparations were then washed thoroughly with relaxing solution and stored for up to 2 days in relaxing solution at ~3°C. To prevent protein degradation, all solutions contained protease inhibitors: PMSF (0.5 mM), leupeptin (0.04 mM), and E64 (0.01 mM). Active force was measured with the single-fiber preparation, based on our previous studies (10, 11, 12). pCa adjusted by Ca/EGTA. Sarcomere length (SL) was measured by laser diffraction, and adjusted to 2.4 μm during relaxation. The values of maximal Ca\(^{2+}\)-activated force (at pCa 4.5) were 167.7 ± 10.9 and 109.2 ± 4.7 mN/mm\(^2\) (P < 0.05), respectively, with and without disuse in immobilized rats (n = 7, 3 animals), and 159.6 ± 7.6 mN/mm\(^2\) (P > 0.05 compared with the value of control fibers in immobilized rats) in control rats (n = 7, 5 animals) with no casting treatment.

For the measurement of Ca\(^{2+}\) sensitivity of active force, [Ca\(^{2+}\)]\(_{s}\) was varied from pCa 7.0 to 4.5. The curves were fitted to the Hill equation and the values of pCa\(_{50}\) (midpoint of the force-pCa curve) and nH (Hill coefficient) were obtained, as in our previous study (10). All mechanical studies were conducted at 15°C.

**Saponin treatment.** Saponin-treated single fibers were obtained based on the method of a previous paper (5). Briefly, small muscle bundles (see above) were treated in relaxing solution containing 30 μg/ml of saponin for 30 min at 3°C. After dissection of a single fiber, SL was set at 2.40 μm in relaxing solution. The fiber was immersed in a low-[Ca\(^{2+}\)]-containing solution (pCa 6.5) for 2 min, allowing the sarcoplasmic reticulum (SR) to sequestrate Ca\(^{2+}\). Then, 50 mM caffeine was applied to the fiber to induce the release of Ca\(^{2+}\) from the SR and the subsequent transient contraction. The magnitude of the SR-based contraction was constant in both control and disused fibers when the sequestration time was varied (i.e., longer than 2 min), indicating thereby that Ca\(^{2+}\) was nearly fully sequestrated into the SR. Also, a caffeine concentration of 50 mM was considered sufficient to release all of the Ca\(^{2+}\) in the SR, since a second application of the same concentration of caffeine did not elicit contraction (data not shown). The composition of the solutions was the same as that used for Triton X-100-treated fibers, except that the EGTA concentration was set at 0.5 mM to minimize its buffering capacity. Thereafter, the force-pCa protocol was performed as described above in the presence of 100 μM ryanodine to suppress Ca\(^{2+}\) release from the SR.

**Troponin exchange.** The Tn exchange was performed in Triton X-100-treated single fibers based on our previously published procedure (21, 30, 31). The whole Tn complex [fast skeletal Tn (fsTn): fsTnT-fsTnI-fsTnC] was purified from rabbit fast skeletal muscle and stored at a concentration of ~19 mg/ml at ~80°C for less than approximately 1 year. Briefly, after measuring maximal Ca\(^{2+}\)-activated force (pCa 4.5) at SL 2.4 μm, a single fiber, from either control or disused muscle, was bathed in rigor solution (10 mM BES, 150 mM K-proionate, 2.5 mM EGTA, 5 mM MgCl\(_2\), pH 7.0) containing 2 mg/ml fsTn and 80 mM BDM for 1 h at 25°C. Then, the fiber was washed with normal relaxing solution at 15°C for 10 min with gentle agitation to remove excess fsTn, resulting in a <10% increase in the band intensity of each Tn subunit upon fsTn reconstitution (21, 30, 31).

**Electron microscopy.** Intact or skinned fibers were used based on our previous studies (8, 32). After measuring SL by laser diffraction (in either skinned or intact preparations) during relaxation (oxygenated Ca\(^{2+}\)-free Tyrode’s solution used for intact preparations), the solution was replaced with a fixation solution containing 2% (vol/vol) glutaraldehyde. For observation of sarcomere structure during contraction, skinned fibers were contracted by an activating solution (pCa 4.5) under isometric conditions, and 30 s after the solution switch (at which time active force becomes maximal in control and disused fibers; see 32), fixation was performed. Longitudinal sections as well as cross sections (~50 nm thickness) were then observed with an electron microscope (H-7500, Hitachi, Japan). NIH image software was used to quantify SL in the electron micrographs (32).

**Gel electrophoresis.** Based on our previous studies (21, 30, 31, 32), acrylamide gels (15%) were run and stained with Coomassie brilliant blue stain to quantify Tn subunits, and the optical density (OD) of the band of interest was determined using One-D-Scan (v. 2.03, Scana, Fairfax, VA). Proteins were identified by comparison with known standards. We quantified the Tn exchange ratio by comparing the ratio of TnI to actin with that obtained in rabbit psoas muscle (as in 30). For the separation of MHC I and MHC II (see 32), single fibers were solubilized, and run on 8% acrylamide gels. Silver staining was performed (Wako Pure Chemical Industries, Osaka, Japan), and band intensities were quantified.

**Statistics.** Significant differences were assigned using the paired or unpaired Student’s t-test (as appropriate). “N.S.” denotes insignificant. Linear regression analysis was used to test correlations between parameters. In all cases, statistical significance was verified with a P value of <0.05. All data were expressed as means ± SE, with n representing the number of experiments.

RESULTS

Effect of disuse on contractile force in saponin-treated single fibers. First, we investigated the effects of disuse on active force development by using saponin-treated single fibers. Figure 1A shows chart recordings for caffeine (50 mM)-induced contractions in control and disused single fibers after loading of Ca\(^{2+}\) (at pCa 6.5 for 2 min) compared with maximal Ca\(^{2+}\)-activated force subsequently obtained at pCa 4.5 in the same fibers. Upon application of caffeine, a transient contraction was observed in fibers from control and disused muscles.
found that the MHC shift varied from fiber to fiber within the same muscle (Fig. 2A). Figure 2B illustrates a disuse-induced change in the fiber type in our model. We found that the percent of type I/II fibers (expressing both MHC I and MHC II) increased, with a concomitant decrease in type I fibers (expressing solely MHC I) and minimal change in type II fibers (expressing solely MHC II).

We then investigated by using Triton X-100-treated disused fibers whether the isoform switching correlates with Ca²⁺ sensitivity of force or maximal Ca²⁺-activated force. Figure 2C plots the MHC expression vs. active force (Ca²⁺ sensitivity or maximal Ca²⁺-activated force) in individual single skinned fibers taken from disused muscle. No significant correlation was found to exist between the parameters in either graph.

Effect of fsTn reconstitution on active force in control vs. disused fibers. To directly investigate whether Tn isoform changes underlie the disuse-induced reduction in Ca²⁺ sensitivity of force in our model, we reconstituted thin filaments of Triton X-100-treated single skinned fibers of control and disused muscles with the identical Tn (fsTn; see MATERIALS AND METHODS), and tested Ca²⁺ sensitivity of force.

As shown in Fig. 3A, endogenous Tn subunits (i.e., TnT, TnI, and TnC) were clearly exchanged (>90% as indexed by

with a similar time course, and the absolute force was smaller in disused fibers (Fig. 1, A and B). In both control and disused fibers, the caffeine-induced contractions were almost completely abolished in the presence of 100 μM ryanodine (Fig. 1A). When normalized to maximal Ca²⁺-activated force (bottom); n = 8 (4 animals). *P < 0.05. The Hill coefficient n_H of the force-pCa curve was 2.10 and 1.81 in control and disused fibers, respectively. Type I fibers were used. Cont, control without disuse; Dis, disuse.

Relation between MHC expression and active force in disused fibers. We previously reported that the MHC isoform shift occurs in soleus muscle upon disuse, viz., from MHC I to MHC II (more precisely MHC IIB), while no detectable MHC II was observed in control muscle (32). In the present study, we

Fig. 1. Sarcoplasmic reticulum (SR) function and Ca²⁺-activated force in saponin-treated single fibers. A: chart recording showing caffeine-induced contraction in control and disused fibers compared with maximal Ca²⁺-activated force (pCa 4.5). Same fiber was used throughout experiment. Sarcotubules (SL), 2.40 μm. Caff, 50 mM caffeine; Rya, 100 μM ryanodine. Double arrowheads indicate the points at which solution was switched from caffeine to Ca²⁺(Mg²⁺) or on a percent scale compared with maximal Ca²⁺-activated force (bottom); n = 8 (4 animals). *P < 0.05. Significance was not detected in the bottom graph (NS). C: active force at various pCa’s in saponin-treated single fibers in the presence of 100 μM ryanodine (normalized at pCa 4.5). Inset, pC₅₀, n = 8 (4 animals). *P < 0.05. The Hill coefficient n_H of the force-pCa curve was 2.10 and 1.81 in control and disused fibers, respectively. Type I fibers were used. Cont, control without disuse; Dis, disuse.

Fig. 2. Effect of myosin heavy chain (MHC) isoform switching in disuse. A: example of differential expression of MHC. Three fibers (1, 2, and 3) were taken from the same soleus muscle with disuse and run on a gel. Despite being from the same muscle, the MHC I/II ratio varied. MHC I/II (in %): 1) ~100/0; 2) ~0/100; 3) ~18/~82. B: graphs showing disuse-induced changes in the fiber type. n = 50 for control and disused fibers (8 animals). C: effect of MHC isoform switching on active force production in disuse. Force-pCa protocols were conducted on single fibers, taken from disused muscles (n = 24; 4 animals). pC₅₀ (left) and maximal Ca²⁺-activated force (right) were plotted against the MHC I/II (MHC I + MHC II) ratio for each fiber. No significant correlation was observed for either pC₅₀ or maximal force.

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TnI) for their rabbit fast skeletal counterparts in both control and disused fibers. Ca\(^{2+}\) sensitivity of force was not significantly changed upon fsTn reconstitution, in either control or disused fibers, as manifested by the force-pCa curves almost overlapping before and after the exchange procedure (Fig. 3B). Accordingly, the difference in the value of maximal force (bottom) was similar before and after fsTn reconstitution (Fig. 3C). Maximal Ca\(^{2+}\)-activated force was little affected by Tn exchange in both control and disused fibers (Fig. 3D), indicating that our Tn reconstitution protocol does not aggravate contractile properties (cf. 21, 30). The difference in the value of maximal force between control and disused fibers was similar before and after fsTn reconstitution (Fig. 3D).

Comparison of sarcomere structure during contraction in control and disused fibers. We reported in a previous work that the diameter of myofibrils becomes smaller upon disuse (32), as in other atrophy models (e.g., 7). Figure 4A compares the sarcomere structure in Triton X-100-treated control and disused fibers during maximal contraction (i.e., 30 s after the onset of contraction). We found that in both control and disused fibers, the Z-line structure was well preserved, and the A-band structure was similarly disarrayed. Also, when SL was set at \(\sim 2.4 \mu m\) during relaxation, it was shortened to \(\sim 2.0 \mu m\) in the center of the fiber at the peak of contraction (i.e., internal sarcomere shortening, \(\sim 0.4 \mu m\)) in both control and disused fibers (Fig. 4B). Therefore, the magnitude of internal sarcomere shortening was similar in both control and disused fibers (i.e., \(\sim 16\%\)).

Figure 4C, top, shows the chart tracings of Triton X-100-treated control and disused single fibers when activated at pCa 4.5 at SL 2.4 \(\mu m\). We observed sustained contractions in fibers with and without disuse. As summarized in Fig. 4C, bottom, the magnitude of the reduction of maximal force was less than 5% at 30 s (at which time fixation with glutaraldehyde was performed; see above) and \(\sim 10\%\) at 2 min, with and without disuse. Differential inhibitory effect of Pi or H\(^+\) on maximal Ca\(^{2+}\)-activated force in control and disused fibers. Finally, to investigate the molecular mechanism of the reduced fatigue resistance in disused muscle, we tested the inhibitory effects of Pi (or H\(^+\)) on maximal Ca\(^{2+}\)-activated force in control vs. disused fibers. Figure 5A shows the effect of Pi on maximal Ca\(^{2+}\)-activated force (pCa 4.5) in control vs. disused fibers treated with Triton X-100. We found that the inhibitory effect of Pi (see Fig. 5A, bottom) was more marked in disused fibers, over the range up to 20 mM. Even after reconstitution with fsTn the inhibitory effect of Pi was still more marked in disused fibers (Fig. 5B).

Figure 5C shows the inhibitory effect of lowering pH (down to pH 6.2) on the maximal force in control vs. disused fibers (treated with Triton X-100). Similar to the finding with Pi, the inhibitory effect of lowering pH was more marked in disused fibers, with and without fsTn reconstitution (Fig. 5, C and D).

We then tested the inhibitory effect of Pi on maximal Ca\(^{2+}\)-activated force in control vs. disused fibers treated with saponin (i.e., in the presence of the inner-membrane system; see Fig. 1A). Similar to our earlier observation with Triton X-100-treated fibers (32), thick filament density was found to be less (\(\sim 15\%\)) after disuse in the presence of the inner-membrane system (Fig. 6, A and B). Accordingly, we performed an experiment using dextran T-500 (molecular weight, \(\sim 500,000\); 2% wt/vol; as in 32) to reduce the lattice spacing of disused fibers, and tested the effect of Pi. Pi exhibited a greater inhibitory effect in disused fibers (Fig. 6C); however, osmotic compression of disused fibers increased active force and reduced the inhibitory effect of Pi to a similar level as that observed in control fibers.

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DISCUSSION

In the present study, we demonstrated by using saponin-treated single soleus fibers from rats that long-term disuse depresses caffeine-induced transient contractions and reduces resistance to fatigue. Analysis with Triton X-100-treated fibers provided evidence that MHC or Tn isoform switching cannot account for the disuse-induced contractile depression. The myofilament density was less in disused muscle, and the reduced resistance to fatigue (as indexed by the inhibitory effect of Pi) was abolished by osmotic compression. We discuss these findings in relation to altered cross-bridge formation in disused muscle.

First, we found that the magnitude of caffeine-induced transient contractions was decreased in disused fibers (Fig. 1). As revealed in our previous work (32), marked structural changes occur in soleus muscle upon disuse, such as thinning of myofilaments, disarray as well as shortening of thick and thin filaments, expansion of interfilament lattice spacing, and mitochondrial irregularity. These structural changes may affect the SR properties, resulting presumably in decreases in Ca\(^{2+}\) sequestration and subsequent Ca\(^{2+}\) release and, hence, attenuation of caffeine-induced contractile depression. The myofilament density was less in disused muscle, and the reduced resistance to fatigue (as indexed by the inhibitory effect of Pi) was abolished by osmotic compression. We discuss these findings in relation to altered cross-bridge formation in disused muscle.

The magnitude of caffeine-induced contractions was similar in control and disused fibers when normalized to maximal Ca\(^{2+}\)-activated force, the reduced transient contractions are likely coupled with the depressed sarcomeric function, as manifested by the decreases in maximal Ca\(^{2+}\)-activated force and Ca\(^{2+}\) sensitivity of force (Fig. 1). Consistent with our previous study using Triton X-100-treated fibers, thick filament density was found to be less in disused fibers than in control fibers in the presence of the inner membrane system (Fig. 6). Although this experiment was conducted under the relaxing condition in intact muscle, it is likely that an expansion of the lattice spacing underlies, at least in part, the depressed contractile performance observed in saponin-treated preparations (cf. 32).

It is well established that MHC isoform switching occurs upon disuse in soleus muscle in various models, i.e., from slow type (MHC I) to fast (MHC II) type (e.g., 4, 7). Indeed, the MHC expression switches from MHC I to MHC IIB in our model (32). The augmentation of MHC II (that has a higher ATPase rate than MHC I) in slow skeletal muscle reportedly results in acceleration of the rate of rise of active force (22, 23). To quantify the contribution of the MHC isoform shift to the disuse-induced depression in steady-state active force, we investigated the relation between maximal Ca\(^{2+}\)-activated force and Ca\(^{2+}\) sensitivity of force in individual fibers of disused muscle. As shown in Fig. 2, however, we found no significant correlation between the parameters. Therefore, the slow-to-fast MHC isoform shift is not likely to underlie the depressed contractile performance in disuse.
In addition to the thick-filament-based proteins, i.e., MHC and myosin-binding protein C (e.g., 28), and titin (32), changes in the isoform or protein content reportedly occur in thin-filament regulatory proteins. Indeed, previous studies demonstrated that TnT, TnI, and TnC switch from slow to fast type (19, 29, 36) and that the \(\mathrm{H}^{2+}\)-Tm expression is decreased accompanied by a concomitant increase in the \(\mathrm{H}^{2+}\)-Tm expression (36). Among these proteins, TnT is well known to have distinct features in the slow and fast isoforms; viz., slow and fast muscles express, respectively, acidic and basic isoforms of TnT, the former of which confers higher sensitivity to \(\mathrm{Ca}^{2+}\) (26, 36). Therefore, albeit by a small magnitude, a heterogeneous Tn complex of slow and fast subunits may negatively affect the \(\mathrm{Ca}^{2+}\) regulation on the thin filament. However, we found that both maximal \(\mathrm{Ca}^{2+}\)-activated force and \(\mathrm{Ca}^{2+}\) sensitivity of force were less in disused fibers than in control fibers.

![Diagram A](image1)

![Diagram B](image2)

![Diagram C](image3)

![Diagram D](image4)

Fig. 5. Inhibitory effects of Pi or lowering pH on maximal \(\mathrm{Ca}^{2+}\)-activated force in Triton X-100-treated single fibers of soleus muscle with and without disuse. A: effect of Pi on maximal force. A: absolute data (top); bottom is same as in top, but values are normalized at 0 mM Pi (indicated by an arrow). *\(P < 0.05\). B: effect of 20 mM Pi on maximal \(\mathrm{Ca}^{2+}\)-activated force in Triton X-100-treated control and disused single fibers reconstituted with fast skeletal troponin (fsTn) (force normalized at 0 mM Pi). *\(P < 0.05\). C: effect of lowering pH on maximal force. C: absolute data (top); bottom is same as in top, but values are normalized at pH 7.0 (indicated by an arrow). *\(P < 0.05\). D: effect of lowering pH (from 7.0 to 6.2) on maximal force in Triton X-100-treated control and disused fibers reconstituted with fsTn (force normalized at 0 mM Pi). *\(P < 0.05\). n = 5–7 (3 animals). Type I fibers were used. Cont, control without disuse; Dis, disuse.

![Diagram E](image5)

![Diagram F](image6)

Fig. 6. Thick filament density and inhibitory effect of Pi in control and disused muscles with the inner-membrane system. A: cross-sectional micrographs from control and disused intact muscles, at (near) the M-line (SL \(\sim 3.0 \mu\text{m}\) measured by laser diffraction during relaxation before fixation). Bar, 100 nm. B: summary of thick filament density in control (Cont) and disused (Dis) muscles. n = 11 (3 animals). *\(P < 0.05\). Type I and I/II fibers were pooled for this analysis (as in 32). C: inhibitory effects of Pi on maximal \(\mathrm{Ca}^{2+}\)-activated force (pCa 4.5) in saponin-treated single fibers of soleus muscle. Left: absolute data. Right is same as in left, but values are normalized at 0 mM Pi (indicated by an arrow). Black-filled circles, control (no disuse); open circles, disuse; gray-filled circles, disuse in the presence of 2% (wt/vol) dextran T-500 (Dx). *\(P < 0.05\) compared with control; #\(P < 0.05\) compared with disuse. n = 5–7 (3 animals). Type I fibers were used.
after reconstitution with the whole fsTn (Fig. 3), ruling out the possibility that contractile depression results from isoform changes (and/or degradation) of Tn subunits. Given, however, a previous observation that β-Tm shows a higher Ca^{2+} sensitivity of force than its α counterpart (e.g., 34), presumably via enhancement of myosin attachment to actin, a possibility still remains that the depressed contractile performance in disuse is coupled with the decrease in the β-Tm expression.

Consistent with the results of our previous studies (30, 31), the n_H value of the force-pCa curve was not significantly changed upon fsTn reconstitution. We consider that this is because n_H does not purely reflect thin-filament cooperative activation but is a complex parameter influenced not only by thin-filament cooperative activation but also by other processes such as cross-bridge formation. Clearly, future studies are needed to systematically investigate to what extent n_H reflects thin-filament cooperative activation.

Internal sarcomere shortening below the optimal SL may lower active force development in striated muscle (12, 13, 15, 32). Therefore, one may argue that internal sarcomere shortening (and/or disarray of thick and thin filaments) may be more pronounced in disused muscle than in control muscle, resulting in depressed active force production (below SL ~ 2.4 μm; see SL-force curves in 32). However, as shown in Fig. 4, we found that the magnitude of internal sarcomere shortening was similar in control and disused fibers. Also, disruptions of the sarcomere, e.g., at Z-lines, were not observed in either fiber during contraction. It is therefore safe to consider that despite marked structural changes in disused fibers via preferential titin loss (32), the magnitude of sarcomeric disarray is similar in control and disused fibers during contraction.

We found that the inhibitory effect of Pi (or H^+) was more pronounced in disused fibers than in control fibers (Figs. 5 and 6). This may be the basis for the previous observation by Yu et al. (36) who demonstrated that the tetanic force of the unloaded soleus declined very rapidly during continuous high-frequency stimulation. They argued that the slow-to-fast TnT isoform switching may confer the reduced fatigue resistance, with the result of TnT isoform change from slow to fast type after hindlimb suspension. Also, the Solaro group reported that slow skeletal TnI (ssTnI) provides short-term protection from acidic pH in cardiac muscle during development (33) as well as in cardiac muscle expressing ssTnI in transgenic mice (35). However, in the present study, the magnitude of the inhibitory effect of Pi (or H^+) was more pronounced in disused muscle than in control muscle after fsTn reconstitution (Fig. 5), making it unlikely that the Tn isoform switching underlies the reduced fatigue resistance. It was found that the inhibitory effect of Pi was attenuated in the presence of dextran T-500 in disused fibers and became similar to what was observed in control fibers. This suggests that the greater inhibitory effect of Pi observed in disused fibers is due to the reduced number of attached cross bridges, resulting from disuse-associated expansion of interfilament lattice spacing (Fig. 6). Indeed, our previous studies have indicated that the inhibitory effect of Pi or H^+ is strongly dependent on the fraction of attached cross bridges; viz., the inhibitory effect becomes less with an increase in the number of attached cross bridges, such as at longer SL (11) or in the presence of MgADP (that increases attached cross bridges; see 9). Therefore, we consider that the reduced fatigue resistance (i.e., enhanced sensitivity of myofilaments to Pi or H^+) in disused muscle is associated predominantly with lattice spacing expansion, rather than with isoform switching of sarcomeric proteins.

In conclusion, we demonstrated by using single fibers that the reduced force-producing capability of myofilaments, rather than defective Ca^{2+} mobilization, underlies the disuse-associated skeletal muscle weakness. The isoform switching of MHC or Tn subunits is unlikely to account for the depressed contractile performance. The inhibitory effect of Pi was more pronounced in disused muscle, in the absence and presence of the inner-membrane system, presumably underlying the reduced resistance to fatigue. The filament density was less in disused muscle than in control muscle in the presence of the inner-membrane system, and indeed, the enhanced force depression by Pi disappeared when myofilament lattice was compressed. These findings support our view that the decrease in the number of attached cross bridges due to, at least in part, lattice expansion results in contractile depression and reduced fatigue resistance. This line of investigation may be useful to provide a better understanding for the prevention and treatment of muscle weakness in various disuse states.

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DISCLOSURES

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