Tension-time index, fatigue, and energetics in isolated rat diaphragm: a new experimental model

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Klawitter, Paul F., and Thomas L. Clanton. Tension-time index, fatigue, and energetics in isolated rat diaphragm: a new experimental model. J Appl Physiol 96: 89–95, 2004. First published September 12, 2003; 10.1152/japplphysiol.00237.2003.—The tension-time index (TTI) has been used to estimate mechanical load, energy utilization, blood flow, and susceptibility to fatigue in contracting muscle. The TTI can be defined, for a rhythmically contracting muscle, as the product of average force development divided by maximum tetanic force times duty cycle [contraction time + (contraction + relaxation time)]. In this study, the TTI concept was applied to isolated diaphragm via a method that allowed TTI to be clamped at a predetermined value. The hypothesis tested was that, at constant TTI, muscle energetics and the extent of fatigue would vary with stimulation frequency. Isolated diaphragm strips were stimulated at 25, 50, 75, or 100 Hz for 4 min, one per second. Duty cycle was continuously adjusted to maintain TTI at 0.07, which was near the highest TTI tolerated for 4 min, at 20-Hz stimulation. At the end of the fatigue run, muscles were either immediately frozen for determination ATP, creatine, and creatine phosphate concentrations (n = 6) or stimulated for evaluation of low- and high-frequency fatigue (n = 5). Results demonstrated no difference in the extent of fatigue or in the final ATP and creatine phosphate concentrations between groups. Large within-run increases in duty cycle were required at low stimulation frequencies, but only small increases were required at the highest frequencies. The results demonstrate that, at a constant TTI, similar fatigue properties predominated at all stimulation frequencies with no clear distinction between high- and low-frequency fatigue. The method of clamping TTI during fatigue may be useful for evaluating energetics and contractile function between treatment groups in isolated muscle when treatment influences baseline contractile characteristics.

THE TENSION-TIME INDEX (TTI) can be defined, for a rhythmically contracting muscle, as the product of average force development divided by maximum tetanic force times duty cycle [contraction time + (contraction + relaxation time)]. It is believed to be a key determinant of the rate of energy consumption (12), muscle blood flow (5), skeletal muscle fatigue (4, 32), and skeletal muscle injury (37). It is most appropriately applied to conditions of isometric or near-isometric contractions (11) and in conditions of intact neuromuscular control (4).

Despite a wide acceptance of the TTI concept and its applications to understanding fatigue and energetics in intact, perfused models of muscle contraction, it has not been extensively applied toward understanding the behavior of isolated muscles. The threshold level of TTI that can be sustained for several minutes is quite low in isolated rat diaphragm preparations because most contraction paradigms result in TTI values falling below 0.08 within 4 min of repeated contractions (13, 14). In contrast, intact diaphragm muscles in humans can generally sustain contractions with TTI values between 0.15 and 0.18 (4).

In isolated preparations, mechanisms that may be responsible for increased susceptibility to fatigue include the influence of tissue core hypoxia and accumulation of metabolites. Additionally, the loss of neuromuscular adaptation makes isolated, artificially stimulated muscle potentially more susceptible to losses in force at a fixed stimulation frequency. In intact neuromuscular systems, for which the TTI concept has proven most valuable, muscle contractile activity is optimized by adjustments in stimulation frequency (rate coding) and by orderly recruitment of motor units (19, 27, 30), both of which are overridden when motor units are synchronously stimulated during in vitro muscle experiments. In an intact neuromuscular control system, slow, oxidative fiber populations that are fatigue resistant (small motoneurons) are preferentially recruited first and can presumably sustain a substantial isometric load for a prolonged period before more fatigue-susceptible fibers are recruited. When all motoneurons are fired synchronously with artificial electrical stimulation, there may be a disproportionate loss of force due to the early activation of fatigue-susceptible motor units. Finally, in vitro, muscle fibers may be more susceptible to mechanisms of “high-frequency fatigue,” thought to be due to compromised transmembrane ion gradients (21). Without an intact circulation and large diffusion gradients, potential accumulation of ions in the extracellular space could further compromise membrane function and make the muscle fatigue more quickly than it would in its intact state.

The primary objective of this study was to reexplore the applicability of the TTI concept to both the metabolic and contractile responses of in vitro muscle fatigue by using isolated rat diaphragm. The hypothesis tested was that, at constant TTI, muscle energetics and the extent of fatigue vary with stimulation frequency. To accomplish this, a new experimental paradigm was developed allowing ongoing TTI to be clamped at a single value as the muscle fatigues at varying stimulation frequencies or duty cycles. This was done in part to establish a suitable method for comparing fatigue characteristics in muscles with differing force-frequency characteristics.
METHODS

Muscle preparation. Adult Sprague-Dawley rats (300–500 g) were anesthetized with intraperitoneal sodium pentobarbital, tracheotomized, and mechanically ventilated. The entire costal diaphragm was dissected out, with associated ribs and central tendon intact, and immediately placed in a physiological salt solution (in mM: 21 NaHCO₃, 0.45 NaSO₄, 0.6 NaHPO₄, 1.0 MgCl₂, 2.0 CaCl₂, 5.9 KCl, 121 NaCl, 11.5 glucose; and 10 μM curare), bubbled with 95% O₂-5% CO₂, as described previously (3, 13). Strips 5–8 mm wide were cut along the plane of the muscle fibers, which included a portion of the central tendon and associated ribs. The strips were then placed in tissue baths containing 25 ml of physiological salt solution, between two platinum stimulation electrodes, continuously bubbled with 95% O₂-5% CO₂, and maintained at 37°C.

After mounting, the maximum required stimulation current and the preload required to reach optimal length (L₀) were determined by use of twitch contractions. All stimulations were done at supramaximal current and at L₀. Force-frequency relationships were determined with 400-ms tetanic stimulations at 20, 30, 40, 50, 60, 80, 100, and 150 Hz, with 20 s of rest between stimulations. Individual contractions before and after fatigue were recorded on a strip chart recorder (Gould), in series with the output to an analog-to-digital converter.

Fatigue model. A system was designed to stimulate the diaphragm strips to contract at a constant TTI throughout the fatigue protocol. The target TTI was predetermined as a set fraction of the baseline maximum tetanic force generated by the muscle at 150 Hz, obtained from preliminary force-frequency measurements.

In this study, the TTI was calculated on a contraction-by-contraction basis as

\[ TTI = \frac{1}{T_{tot}} \int_{t_1}^{t_2} \text{Force} \, dt / P_o \]

where \( t_1 \) and \( t_2 \) are the initiation times of two consecutive contractions or stimulations, \( \text{Force} \) is the ongoing active tetanic force during contraction, \( T_{tot} \) is the total period of contraction and relaxation, and \( P_o \) is the maximum tetanic force of the rested muscle.

For the purposes of this study, TTI was always controlled during fatigue by making continuous adjustments in duty cycle, although the system allowed for maintaining TTI with stimulation frequency as well. Because many studies of isolated rat diaphragm fatigue use stimulation frequencies of 100 Hz, preliminary experiments were performed to determine the TTI that could be sustained for 4 min at this frequency. At 20 Hz, diaphragm muscles are capable of contracting for 4 min at a TTI of 0.07 or below. TTI values set above 0.08 usually required the duty cycle to be increased to 1.0 by the end of the fatigue trial (i.e., continuous contraction). When approaching a duty cycle of 1.0, the muscle quickly loses active tension and cannot sustain the target TTI. Therefore 0.07 was set as the target TTI for all experiments.

Although there was no attempt to limit the duty cycle in these experiments, the experimental paradigm resulted in final duty cycles that ranged from 0.08 to 0.37, depending on stimulation frequency. In preliminary studies, when the duty cycle exceeded ~0.5, the muscles were susceptible to rapid failure and were unable to complete the 4-min protocol. Because the normal duty cycle for contracting diaphragm of an awake rat is 0.34 (8, 31), the results fell within a range that included normal contractile behavior.

Force measurements during fatigue were digitized at 1 kHz (Flash-12, 8-channel analog-to-digital board, Strawberry Tree) and processed by data-acquisition software (Workbench 5 for Windows, Strawberry Tree). Using a summing amplifier, we subtracted a voltage equivalent to the preload on each muscle (passive force) from the total force to determine active force. A moving average of active force, over time, was then determined by calculating a continuous exponential, weighted mean of the signal, with a time constant of 3 s. This system was tested to ensure that it gave an accurate representation of tension-time when contractions were performed at 1 per second. The moving average of tension-time was continuously displayed on a computer screen that was used as an oscilloscope. The target average force (i.e., 7% of baseline max tetanic force at 150 Hz) was marked on the screen, and, during the fatigue run, ongoing adjustments in duty cycle were continuously made to keep the muscle contracting with small oscillations around the target TTI (see Fig. 1). To determine the influence of stimulation frequency on the energetics and fatigability of the muscles, four muscles in each animal were studied simultaneously in four baths, at 25, 50, 75, and 100 Hz, respectively. Other than frequency of stimulation, all tissues were treated identically. In every experiment, contractions during fatigue were performed at 1 per second.

Experimental protocol. Twenty muscle strips from five animals were studied to evaluate contractile fatigue (n = 5, “fatigue group”). Twenty-three strips from six animals (n = 6, except for 100 Hz stimulation where n = 5) were studied for high-energy phosphate determination after fatigue (“energetics group”). An additional set of eight sham strips from eight animals was incubated in muscle baths over a similar time period and used as nonfatigued controls for high-energy phosphate determination (“control group”). For comparison, “fresh” diaphragm tissue was also obtained by use of freeze clamping, from mechanically ventilated rats. For the fatigue and energetics groups, after a 30-min equilibration at 37°C, approximately 10 min after \( L₀ \) was determined, a force-frequency measurement was performed. After another 15 min of rest, the muscles underwent the 4-min fatigue protocol, one strip at a time. The order in which the muscle strips, with different stimulation frequencies, were fatigued was randomly determined between experiments. In the fatigue group, 20 s after the fatigue protocol ended, each muscle was subjected to stimulations at 20 and at 150 Hz to determine the extent of fatigue at high and low frequency. In the energetics group, the muscle strips were immediately freeze-clamped on completion of the fatigue protocol (timed to be within 1 s of the last fatigue contraction). Frozen tissues were wrapped tightly in foil, weighed, and stored at −80°C for later homogenization and biochemical analysis.

Analysis of high-energy phosphate status. ATP, creatine phosphate (CP), and creatine (Cr) were determined by using a modified method of Bergmeyer (6), as described by Itoya et al. (20). This method was previously used by our laboratory for evaluation of cardiac energy metabolites (24). Frozen tissues (~50–100 mg) were placed in a mortar and pestle that were equilibrated to liquid N₂ temperature and ground under liquid N₂ with 0.5 ml of 0.6 N perchloric acid. The N₂ was allowed to evaporate, and the frozen powder was placed in a vial containing an additional 0.75 ml of ice-cold perchloric acid, vortexed, and then centrifuged at 16,000 g for 1 min. One milliliter of super-
natant was pipetted to a new tube, and the pellet was kept for protein analysis. A 1 M solution of K$_2$CO$_3$ was titrated (~350 µM) into the supernatant to adjust the pH ~7.0. The sample was allowed to sit (on ice) for 30 min to precipitate KCIO$_4$ and then was centrifuged again. The final supernatants were pipetted into aliquots and stored at ~80°C for later enzymatic analysis and were thawed shortly before measurements were performed. The CrP and ATP in the supernatants were stable up to 6 h (6), and repeated measurements from frozen supernatants were stable over several weeks. Careful volume corrections were made for each step in the dilution process.

Cr, ATP, and CrP were assayed by using the enzymatic methods that are described and validated in detail by Bergmeyer (6). ATP and CrP measurements utilized the hexokinase and glucose 6-phosphate dehydrogenase reactions, and measurements were made by following changes in NADPH at an absorbance of 339 nm. Cr was measured by using the action of creatine kinase on Cr to make CrP and ADP. The ADP was then measured by using the pyruvate kinase and lactate dehydrogenase reactions, and the amount of NADH consumed by lactate dehydrogenase (absorbance 339 nm) was monitored. All assays were performed in duplicate and repeated until values agreed within 10%.

Protein assay. All values were normalized to protein content of the sample and to wet weight. The values expressed per protein showed the least variance within groups and were, therefore, used for final analysis. To determine the protein concentration of the sample, stored protein pellets were thawed and washed with acetone. Dried pellets were resuspended in 500 µl of 1 N NaOH and heated for 10 min at 95–100°C and then placed on ice or kept frozen at ~20°C until assayed (standards were treated similarly). Total dissolved protein was determined by use of the Lowry method (28). Samples were read by using a spectrophotometer at 750-nm absorbance and compared with a standard curve.

Statistical analysis. All values are expressed as means ± SE. Significant differences between means were determined by ANOVA with stimulation frequency being the factor of interest and individual rat handled as a random variable. All analyses were performed by use of SAS JMP (SAS Institute, version 3.2.2 software package). Post hoc analyses were done with the use of Tukey’s test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

The average baseline tensions for all tissues were 9.1 ± 0.5 N/cm$^2$ at 20 Hz and 26.4 ± 1.1 N/cm$^2$ at 150 Hz, with no significant differences between groups. Figure 2 illustrates the loss of force, measured by contractions at both 20 and 150 Hz after the fatigue protocol. Muscles fatigued at the same TTI but, with widely varying frequencies of stimulation, showed no difference in the extent of contractile fatigue at either low or high frequencies. The loss of force was ~50% of initial force at both 20 and 150 Hz (Fig. 2), suggesting a relatively parallel shift downward in the force-frequency relationship due to the fatigue protocol.

The total ATP present in the tissue immediately after the fatigue protocol was not significantly changed from baseline levels at any stimulation frequency or between groups (Fig. 3). In contrast, [CrP] and the ratio of [CrP]/([CrP]+[Cr]), where brackets denote concentration, fell precipitously with levels seen in nonstimulated muscle, but these measurements were not different between muscles fatigued at different stimulation frequencies and widely varying duty cycles. Because measurements of [CrP] and [ATP] are rarely expressed per milligram protein, for comparison with other studies, samples were also normalized to wet weight and compared with the literature in Table 1 (1, 16, 18, 25). Note the wide variation in reported values for [CrP], [ATP], and [Cr] in rat diaphragm.

The duty cycle required to maintain a constant TTI at each frequency gradually increased throughout the fatigue run as tension generation fell (see Fig. 1). The duty cycle that was needed at the end of the fatigue protocol to maintain a constant TTI was inversely related to stimulation frequency (Fig. 4). This was expected, because the tension generated increases with the frequency of stimulation. Interestingly, at the highest stimulation frequency, the adjustment needed in duty cycle to maintain a TTI of 0.07 was minimal over the 4-min stimulation period.

DISCUSSION

These results demonstrate that the extent of fatigue and the energetic state at the end of the fatigue trial appear to be...
in which this variable was recorded. This graph came from 4 animals in each of the 2 groups, the only experiments by the inset equation. Values are means required over the course of the 4-min fatigue run. Furthermore, a target TTI of 0.07, and this changes very little over the 4-min stimulation protocol (see Fig. 4). It is evident that the muscles may have been able to sustain values of TTI much higher than 0.07 at this stimulation frequency. In contrast, at 25-Hz stimulation, an ever-increasing duty cycle from 0.20 to 0.35 was required over the course of the 4-min fatigue run. Furthermore, as described in METHODS, decreasing frequency to 20 Hz or increasing the target TTI to 0.08 at low frequencies takes the muscle to the very edge of maximum contractile performance over 4 min. What mechanism could account for this apparent difference in response between low and high stimulation frequencies?

One explanation rests on the possibility that, in response to artificial stimulation at low frequencies, force development in a muscle with mixed fiber type is likely to reflect a greater contribution from slow-twitch fibers. These fibers (type I) make up only ~26% of the cross-sectional area of rat costal diaphragm (26) and probably contribute a proportionately smaller force per area ratio than larger fibers (17). The remaining square area of the muscle is made up of 23% type Iia fibers (considered equivalent to fast-twitch, fatigue-resistant fibers) and 52% type IIX (considered equivalent to fast-twitch, fatigue-resistant fibers) (26). There is a very low population of fast-fatigable Iib fibers in rat diaphragm (34). The predominant effect of the slow-fiber population on force at low stimulation frequencies is predictable on the basis of the differing shapes of the force-frequency relationships of different categories of motor units. For example, when the soleus is used as a representative of the predominant behavior of slow fibers [i.e., 65% slow oxidative and 35% fast oxidative in the soleus (7)], at 25 Hz a tetanic force of ~75% maximum is attained (33). In contrast, when the extensor digitorum longus (EDL) is used as a rough approximation of the behavior of fast categories of fiber types in the diaphragm [i.e., ~45% fast oxidative and intermediate oxidative and 55% fast glycolytic in the EDL (7)], only ~18% of maximum tetanic force is achieved at 25 Hz, about the same magnitude as the twitch-to-tetany ratio in this type of muscle fiber (33). Thus, at 25 Hz, a predictably larger proportion of net force development would be contributed by tetanic contractions of slow fibers. The faster fiber populations would not be fully tetanized and instead would be more likely to undergo repetitive twitchlike contractions at 25 Hz. In addition, they must be contracting in a partially shortened state, because surrounding slow fibers have already shortened the entire muscle against its series elastic elements. Thus it is likely that the contribution of fast fibers to force development at low frequencies would not be particularly effective. In contrast, at 100-Hz stimulations, all fibers would contribute synchronously to isometric contraction, reaching almost full tetany and sharing in the net isometric force development. This would most likely make contractions at high frequency more economical, necessitating only brief durations of contraction to perform a given TTI and effectively sharing the isometric load across a greater population of fibers for shorter periods of time.

Another potential explanation of this phenomenon is that, at the two extremes of frequency, muscle fibers presumably operate at different locations on their individual force-pCa\(^{2+}\) relationships. At low-frequency stimulation, the average muscle fiber is likely to be operating on some portion of the ascending limb of its force-pCa\(^{2+}\) curve, a region where force can be greatly affected by shifts in Ca\(^{2+}\) sensitivity and Ca\(^{2+}\) release, both of which can occur with some forms of fatigue (15, 36). Different fiber types have different force-pCa\(^{2+}\) curves (17), but viewing the composite muscle still makes this scenario likely. At high stimulation frequencies, the fibers are more likely to operate on the plateau of the force-pCa\(^{2+}\) relationship, where Ca\(^{2+}\) binding sites on troponin are satu-

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### Table 1. Comparison of resting energy metabolites in diaphragm with literature values

<table>
<thead>
<tr>
<th>Study</th>
<th>[ATP], (\mu\text{mol/g})</th>
<th>[CrP], (\mu\text{mol/g})</th>
<th>[Cr], (\mu\text{mol/g})</th>
<th>Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present results (control fresh)</td>
<td>3.5±0.3</td>
<td>11.6±0.7</td>
<td>3.8±0.2</td>
<td>perchloric extraction</td>
</tr>
<tr>
<td>Present results (control in tissue bath)</td>
<td>3.0±0.4</td>
<td>9.5±0.8</td>
<td>4.2±0.4</td>
<td>perchloric extraction</td>
</tr>
<tr>
<td>Fitzgerald et al. (16)</td>
<td>2.8±0.48</td>
<td>6.6±1.09</td>
<td>16.8±0.6</td>
<td>perchloric extraction</td>
</tr>
<tr>
<td>Gupta and Goad (18)</td>
<td>4.1±0.05</td>
<td>6.1±0.15</td>
<td>15±0.5</td>
<td>perchloric extraction</td>
</tr>
<tr>
<td>Koerts-de Lang et al. (25)</td>
<td>5.0±0.1</td>
<td>18±0.6</td>
<td>15±0.5</td>
<td>perchloric extraction</td>
</tr>
<tr>
<td>Adler et al. (1)</td>
<td>3.8±0.4</td>
<td>10.3±1.5</td>
<td>15±0.5</td>
<td>perchloric extraction</td>
</tr>
</tbody>
</table>

Values are means ± SE, expressed per gram wet weight for diaphragm. CrP, creatine phosphate; Cr, creatine; brackets denote concentration.

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Fig. 4. Duty cycle needed to maintain TTI of 0.07, measured at the end of the fatigue protocol. Curve represents data fit to an exponential decay, described by the inset equation. Values are means ± SE (n = 8 for all groups). Data for this graph came from 4 animals in each of the 2 groups, the only experiments in which this variable was recorded.
rated. At this operating point, individual fibers would be less responsive to shifts in Ca\(^{2+}\) sensitivity or decrements in Ca\(^{2+}\) release. In this way, stimulation at higher frequencies might be able to sustain higher TTI values by overcoming limitations of excitation-contraction coupling associated with fatigue. This idea, although consistent with current thinking, is, of course, complete speculation.

It is still curious that the maximum force at 150 Hz, measured after the fatigue protocol, dropped by ~50%, yet there was almost no need to increase the duty cycle to sustain a TTI of 0.07 at the 100-Hz stimulation. One possible explanation is that more delayed relaxation times contributing to net tension-time after tetanic stimulation occurred as fatigue developed. We did not quantify this, but slowing of relaxation time is characteristic of fatigued muscle (35). Prolongations of half-relaxation time of 10 ms or more would be expected (35), and these could substantially contribute to the total integrated force development when duty cycle lasts as little as 70 ms (i.e., the duty cycle required at 100-Hz stimulation and TTI 0.07).

Before performing the study, we hypothesized that at very high frequencies of stimulation there would be a greater degree of contractile fatigue and an inability to sustain the target TTI because of recruitment of high-frequency fatigue mechanisms that are believed to involve local increases in extracellular K\(^+\), particularly in the T tubules. (21). It has been shown that high-frequency stimulation results in greater degrees of fatigue than low-frequency stimulation (10). However, this hypothesis was disproved for this experimental design because none of the expected distinctions between low- and high-frequency fatigue was observed. If anything, as discussed in the previous paragraphs, we discovered that at low-frequency stimulation the muscle “appears” more susceptible to fatigue. No doubt, a unique high-frequency fatigue phenomenon is evident under other experimental conditions; for example, it may be more likely in conditions of prolonged contraction times (21).

Critique of the fatigue model. One limitation of this model of fatigue is that the pattern of contraction was not normal for in vivo diaphragm over the majority of fatigue runs. During the largest portion of the fatigue trials, except at 25-Hz stimulation, the duty cycle was below what is considered to be normal in the awake rat, i.e., 0.34 at rest (8, 31). Furthermore, contraction frequency was 60 times per minute, where the resting rat breathes at a rate of 90–100 per minute (31). As discussed, our study was limited somewhat by the design, because we made online adjustments in duty cycle to compensate for losses of peak force development. Had we begun the experiments with a normal duty cycle, it would not have been possible to compensate with duty cycle for losses in peak force and still keep TTI constant. We possibly could have overcome some of these problems by prolonging Ttot, because it would give a longer time for relaxation, but that would have made the contraction rate even less physiological. It is unlikely that changing Ttot would have substantially altered the conclusions, because Kelsen and Nochomovitz (22) have demonstrated that Ttot has little effect on the fatigue process in the isolated rat diaphragm, over and above its effect on TTI.

The results provide a good illustration of the difficulties and differences of measuring in vitro fatigue of a muscle bundle with mixed fiber population compared with an in vivo muscle with normal abilities to recruit selective motor units. In vivo, maximum performance can be attained by selective and orderly recruitment during contraction. Presumably, fatigue-resistant motor units are recruited first during contraction and are able to sustain submaximal contractions in an energy-efficient manner (19, 27, 30). Later in the contraction, faster motor units are recruited that can substantially support force development and further contribute to shortening. These faster units generally operate at a shortened duty cycle compared with the muscle as a whole and therefore are able to sustain intermittent activity for prolonged periods. It is very likely that the ability to sustain motor activity at the so-called “threshold” TTI, which has often been described for respiratory muscles (2, 4), is highly dependent on an intact motor unit recruitment organization and is unlikely ever to be entirely duplicated in an in vitro environment.

TTI and muscle energetic status. With all of the considerations discussed previously with regard to potential differences in recruitment patterns, it is difficult to conceive of how the in vitro muscle could possibly have the same net energy state after being stimulated at different frequencies. For example, if a greater contribution to tetanic force development came from slow fibers during low-frequency stimulation, and those fibers have greater capacity to sustain energy availability through oxidative phosphorylation, we might expect to see a preservation of high-energy phosphates with low-frequency stimulation, but no such preservation was evident.

The relationship between the tension-time product during tetany and energy consumption or energy liberation is highly linear for oxidative fibers, e.g., soleus (12). It is also linear for fast fibers, e.g., EDL (12), when tetanic contractions are ~6-s duration, although the EDL consumes nearly 2.9 times the energy of the soleus for a given tension-time integral (12), an indication of a muscle designed for lower economy but more rapid shortening velocity. Although the measurement of high-energy phosphate concentrations, immediately after the last fatiguing contraction, is not a complete picture of the energetic requirements of muscles, it is one window in time of what is happening to the composite muscle. The measurement reflects the net accumulated difference between the high-energy phosphate costs from contraction and the gains by various metabolic pathways over the course of the 4-min stimulation period. The results suggest that, if different fiber types contribute differently to force development over this range of stimulation patterns, the differences in the individual energetic status of the different fibers must sum to zero.

Another variable that can be calculated is the total number of twitch simulations that each muscle received, summed over Ttot. The values at the end of the fatigue trials can be approximated from the data in Fig. 4 by multiplying final duty cycle by the stimulation frequency. Interestingly, to maintain a TTI of 0.07, the average number of twitch stimulations per second varied only ~1 twitch per second across all stimulation frequencies (i.e., 9–10 twitches per second at all frequencies). Therefore, a given TTI during fatigue simply depends on the number of twitches per second seen by the muscle, independent of the interval it is provided. Because all fibers are stimulated maximally with each twitch, one hypothesis that might explain the results is that the TTI, energetics, and mechanical cost of contractions are a simple function of the number of twitches received by the muscle per time.

Measurements of high-energy phosphates are prone to experimental difficulties. As described in Table 1, varying tech-
niques of sample collection, status of the animal or tissue, and specific biochemical protocols can greatly affect the results from different laboratories. Degradation of the sample is believed to be in part due to residual activity of creatine kinase and interconversion between CrP and ATP during extraction (9). This effect, which theoretically should be blocked by the extreme acidification of the sample during extraction, has been described as being responsible for differences between in vivo measurements with $^{31}$P-NMR and ex vivo measurements using chemical analysis (9). In the method described here, the homogenization and acidification steps are done at liquid $N_2$ temperatures, which may eliminate potential degradation seen in other studies (9). One indicator of the fidelity of the measurements was the ratio [CrP]/[ATP] (∼3.3 in both groups of samples in this study) and the CrP-to-total [Cr] ratio. The former falls within the range of what is generally measured in vivo when using $^{31}$P-NMR and not generally seen when using chemical analysis (9); the latter suggests a general lack of conversion of CrP to Cr. Nevertheless, measurements of high-energy phosphates using any extraction method should always be viewed with some caution, if for no other reason than there are very inconsistent results between laboratories.

**Practical considerations for fatigue testing.** Isolated diaphragm preparations are frequently utilized to study the influence of drugs or other treatments on skeletal muscle fatigueability. A practical problem that our laboratory and many others have dealt with is the fact that drugs and other treatments often affect the relationship between baseline force and frequency of stimulation (3, 13, 23). Therefore, comparison of fatigueability between groups is compromised because muscles under different treatments may contract with greater or lesser force or greater or lesser energy requirements throughout the fatigue protocol at a given stimulation frequency. This problem is rarely addressed, and results are often misinterpreted. One method, used by Khawli and Reid (23), was to match the relative force of the two muscles being compared by adjustments in stimulation frequency. The results of the present study suggest that such a method is a perfectly appropriate approach to the problem, because frequency has little effect on the fatigue process within these ranges of TTI. However, an even better approach would be to match TTI throughout the fatigue protocol, as we have done in this model. This approach would compensate for any transient changes in force-frequency (e.g., via potentiation mechanisms) that are known to evolve during the fatigue process and could vary with differing treatments. There is always the possibility that some interventions may alter the relationship between TTI and fatigue seen in this study, which could also reveal new dimensions to the biology of fatigue.

It has been proposed that a link between TTI and fatigue may be sarcolemmal injury (37). Zhu et al. (37) have shown, in their model of fatigue, that TTI (up to a value of 0.4–0.6) is proportional to sarcolemmal injury. The degree of injury is inversely proportional to the ability of the muscle to generate tension after their stimulation protocol. All of our groups were stimulated with identical TTI. Although we did not measure sarcolemmal injury, one could postulate that the degree of injury was the same in all groups, and this could account for the similar decreases in tension-generating ability. However, on the basis of the work of Zhu et al., we would expect to find <10% of the fibers to be injured at this duty cycle, which seems out of proportion to the large (∼50%) decreases in force generation that resulted after our stimulation protocol. Other mechanisms involved in fatigue development, such as changes in pH or lactate (29) and changes in calcium handling (15, 36), may link the degree of fatigue to TTI. However, at this time the data provided in this study do not directly support or reject any particular fatigue mechanism.

In conclusion, we have shown that the amount of fatigue developed by repetitive, isometric contractions is very carefully matched to TTI in this range of contractile activity and is independent of the frequency of stimulation. This approach allows for comparison of in vitro fatigue between differently treated samples in which energy state and contractile activity are matched between treatment groups.

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