Fatigue and recovery of dynamic and steady-state performance in frog skeletal muscle

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Sy"me, Douglas A., and Dillon M. Tonks. Fatigue and recovery of dynamic and steady-state performance in frog skeletal muscle. Am J Physiol Regul Integr Comp Physiol 286: R916–R926, 2004.—Muscle fatigue reflects alterations of both activation and cross-bridge function, which will have markedly different affects on steady-state vs. dynamic performance. Such differences offer insight into the specific origins of fatigue, its mechanical manifestation, and its consequences for animal movement. These were inferred using dynamic contractions (twitches and cyclic work as might occur during locomotion) and steady-state performance with maximal, sustained activation (tetani, stiffness, and isokinetic force) during fatigue and then recovery of frog (Rana pipiens) anterior tibialis muscle. Stiffness remained unaltered during early fatigue of force and then declined only 25% as force dropped 50%, suggesting a decline with fatigue in first the force-generating ability and then the number of cross bridges. The relationship between stiffness and force was different during fatigue and recovery; thus, the number of cross bridges and force per cross bridge are not intimately linked. Twitch duration increased with fatigue and then recovered, with trajectories that were remarkably similar to and linear with changes in tetanic force, perhaps belying a common mechanism. Twitch force increased and then returned to resting levels during fatigue, reflecting a slowing of activation kinetics and a decline in cross-bridge number and force. Net cyclic work fatigued to the degree of becoming negative when tetanic force had declined only 15%. Steady-state isokinetic force (i.e., shortening work) declined by 75%, while cyclic shortening work declined only 30%. Slowed activation kinetics were again responsible, augmenting cyclic shortening work but greatly augmenting lengthening work (reducing net work). Steady-state measures can thus seriously mislead regarding muscle performance in an animal during fatigue.

Twitch; work; work loop; twitch; stiffness

FITTS (19) contrasts two definitions of fatigue, i.e., failure to maintain force during repeated stimulation vs. failure to maintain the required or expected power output, and suggests that power may be a more suitable measure as it reflects both the kinetics and kinematics of muscle contraction. Due to the multifaceted nature of fatigue (e.g., 1, 19, 54, 55), such distinctions are important, both when quantifying fatigue and in interpreting how it is manifest and the nature of its consequences. Fatigue must be defined in the context of a particular aspect of performance and the cellular processes in question (24). The basis for these arguments suggests a strategy to study fatigue and its impact on animal movement. Fatigue per se is the consequence of changes in different cellular processes, which in turn affect various aspects of muscle contraction. Therefore, by contrasting changes in muscle performance/contraction that rely on different cellular processes, it is possible 1) to further clarify the causes of fatigue, 2) to determine their relative importance at different stages of fatigue and recovery, and 3) to understand how they impact various facets of the mechanical performance of muscle and hence its ability to power animal movement. Thompson et al. (50) used a similar approach to study the etiology of fatigue in frog muscle.

Here we consider fatigue in the muscle cell, as distinct from central fatigue and failure at the neuromuscular junction. In this light, deficits in mechanical performance with fatigue are due to changes in cross-bridge activity (myofibrillar fatigue) and changes in the magnitude and kinetics of activation (failure of activation) (17). The former encompasses force per cross bridge and the cross-bridge cycle as it impacts both force and shortening. The latter includes the calcium handling machinery and its impact on the magnitude and kinetics of the intracellular free calcium transient, troponin-calcium kinetics, the cross-bridge cycle, and ultimately the number of working cross bridges. MacIntosh and Rassier (31) warn that the coexistence of fatigue and potentiation will compromise the ability to quantify fatigue, and while we do not attempt to separate these effects we echo their caution in interpreting results, particularly during the early stages of fatigue.

In addition to the dichotomy of activation vs. cross bridges, differences between steady-state performance (during maximal, sustained activation) and dynamic performance (during cyclic activation and relaxation) can be particularly useful in determining what mechanisms are altered and how this impacts animal movement (4, 28, 46, 47). With these distinctions in mind, we measured steady-state and dynamic performance of frog anterior tibialis muscle during fatigue and recovery to further understand how fatigue is caused and how it impacts muscle performance in relation to animal movement. Steady-state measures included 1) maximal isometric tetanic force (dependent on the number of strongly attached cross bridges and the force produced by each cross bridge), 2) muscle stiffness during tetanic contractions (reflecting the proportion of attached cross bridges; see MATERIALS AND METHODS for cautions in interpretation), and 3) the force produced during isokinetic shortening after maximal isometric activation (reliant on the number of attached cross bridges, the force they produce, and their ability to cause muscle shortening; this force is also a direct index of work done while shortening). These measures reflect cross-bridge activity but not the time course of activation; they are useful in providing insight into the mechanisms of fatigue, but they tell us less about the impact of

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fatigue on animal movement. Dynamic measures included 1) twitch force and kinetics (which depend on the time course and magnitude of activation and cross-bridge kinetics; they will also reflect characteristics of the series compliance, which we have assumed to be passive and not impacted by fatigue, although this is not certain), and 2) work performed during cyclic sinusoidal length cycles and phasic activation using the work-loop technique (26). These latter measures are dynamic actions that reflect both cross-bridge function and the time course of activation and thus can provide insight into the effects of fatigue on work that is available for locomotion. Furthermore, unlike work measured using isokinetic shortening contractions, work measured using cyclic contractions can be used to describe both the work done by the muscle during shortening and also the work required to relengthen the muscle (as would be required of its antagonist). The energy required to lengthen muscles can be of great consequence when considering the function of a muscle in a moving animal. Cyclic work is thus likely a superior indicator of a muscle’s ability to contribute to animal movement and the impact of fatigue on movement.

MATERIALS AND METHODS

Muscle preparation. All procedures and animal husbandry followed approved University of Calgary animal care guidelines. Leopard frogs (Rana pipiens) were killed by decapitation and pithing. Small bundles (n = 7, length 7.5–11.0 mm, wet mass 2.0–9.5 mg) were dissected from the anterior tibialis muscle in physiological saline (in mM: 115 NaCl, 3 KCl, 2 CaCl2, 20 NaHCO3, 2 NaH2PO4, 5 glucose, pH 7.8) on a chilled stage (5°C). To minimize potential differences between preparations in fiber type distribution, bundles were always taken from the central region of the muscle. The bundle was then placed in a chamber filled with circulating physiological saline at room temperature (22.3–22.8°C) and bubbled with a 98% O2–2% CO2 gas mixture. Short segments of tendon at the ends of the muscle bundle were tied to a rigid hook and to the arm of a servomotor/force transducer (Cambridge Technology, series 300 dual mode, model 350) using 5–0 silk suture; to minimize stray compliance the tendon was pulled tight against the pins so that no length of suture extended between the two. Curare (17 mg/l) (22) was added to the saline in the chamber. Platinum stimulating electrodes were placed along the length of the muscle on each side and connected to a battery-driven current source gated by a Grass SD9 stimulator. The stimulator and servomotor were controlled with a computer using custom software written in LabView (National Instruments, Austin, TX). Muscle length and force signals were recorded on the computer at 5 kHz using a 12-bit analog-to-digital card (PCI-MIO-16E-4, National Instruments) and custom software written in LabView. The servomotor had a critically damped, step response time of ~500 µs. The force transducer had a resonant frequency of 830 Hz.

Establishing experimental parameters. The muscle’s length was adjusted to remove visible slack and then carefully measured using a microscope and calibrated ocular micrometer. The stimulus voltage was adjusted to 150% of that required to elicit a maximal isometric twitch (1-ms stimulus pulse). The muscle’s length was then systemically altered in 0.5-mm steps until maximal isometric twitch force was achieved. This length was used throughout experiments. For tetanic stimulation, a train of stimulus pulses was delivered at 100 Hz for 150 ms, long enough to ensure that maximal isometric tetanic force (P0) was attained.

To measure muscle stiffness (Fig. 1), the muscle was stretched (step function) by 0.3% of its length during the plateau of an isometric tetanus, held for 10 ms, and then released back to the original length. Another 20 ms was allowed to elapse for force to recover before the isokinetic shortening protocol described below began. From the stretch amplitude and the force response, stiffness was calculated as the ratio of the change in force to the change in length.

Immediately after the stiffness recording, isotonic force during isokinetic shortening at 2 muscle lengths/s was determined (Fisk) (Fig. 1). This velocity was selected as it is close to that at which frog muscle produces maximal power at room temperature (personal observation). During the plateau of an isometric contraction, the muscle was quickly shortened by ~1% muscle length; this caused force to drop close to the level at which it would stabilize during subsequent isokinetic shortening. After the shortening step the muscle was then ramp shortened at 2 muscle lengths/s, and isotonic force was recorded. Stimulation continued throughout the step and isokinetic shortening. The step shortening amplitude determined to be appropriate to measure Fisk during prefatigue (control) conditions was also used during fatigue and recovery. There was not time during the fatigue/recovery recordings to continually reestablish the ideal step size; however, it remained adequate throughout the experiment.

The work loop technique was used to measure cyclic work output as may occur during cyclic movement in animals (26). Muscle length was cycled in a sinusoidal fashion while imposing phasic stimulation. The length cycle frequency was set to 4 Hz, and the amplitude of the length cycle was set to 15% of muscle length (peak to peak). These conditions yield close to maximal power at room temperature in frog muscle (unpublished observation). The stimulus duration and phase required to maximize net work output were determined at the beginning of each experiment and were always close to 100 ms and 58°, respectively (where 90° is the point in the sine cycle where the muscle is at maximum length and 270° is the point of minimum length). The muscle’s length was cycled, and it was so stimulated for three consecutive cycles, and work was measured from the second or third cycle in the set. When the muscle fatigued it became progressively stiffer with each cycle in the set of three, and so force and work per cycle failed to stabilize; in these cases work was taken from the third cycle, which was the closest to a steady-state condition. Three different measures of cyclic work were assessed: 1) shortening work is the integral of muscle force with respect to muscle length over the shortening portion of the cycle (i.e., the work done by the muscle while it shortened), 2) lengthening work is the integral taken over the lengthening portion of the cycle (i.e., the work required to stretch the muscle), and 3) net work is the integral over a complete lengthen/shorten cycle (i.e., the net work done by or absorbed by the muscle...
during a complete cycle) and is the difference between shortening work and lengthening work. A positive value for net work indicates the muscle is imparting energy to the system, while a negative value indicates the muscle is absorbing more work than it contributes.

**Fatigue and recovery.** After all parameters had been established (see previous section), a set of control recordings of $P_0$, twitch force, stiffness, $F_{sk}$, and cyclic work were made. These measurements were taken several times over a period of ~20 min to ensure the preparation was stable. The muscle was then given isometric tetanic stimulation (100 Hz for 150-ms duration) repeatedly with 2- or 5-s intervals to induce fatigue; most muscles did not show reversible fatigue unless stimulated with a 2-s interval. Such stimulation was continued until $P_0$ declined to 50% of its control value. $P_0$ was recorded on the computer each time it declined by ~5% of its initial level. Recordings of cyclic work, stiffness, $F_{sk}$, and twitches were made each time $P_0$ declined by ~5%. Measurements of either cyclic work or the combination of $(F_{sk} + \text{stiffness} + \text{twitch})$ were alternated to prevent excessive recovery or fatigue of the muscle between records of $P_0$. Thus a typical sequence would include several measures of $P_0$, then cyclic work, then several more of $P_0$, then $(F_{sk} + \text{stiffness} + \text{twitch})$, then more of $P_0$, then cyclic work, etc. Once $P_0$ had fatigued to 50% of control, the muscle was allowed to recover. During recovery the muscle was stimulated less frequently: every 30 s initially and up to 5 min later stages of recovery. Measurements of $P_0$ were interspersed with recordings of twitches, cyclic work, $F_{sk}$, and stiffness, lengthening work and measures of the twitch duration were made in all cases and paired with alternate measurements of cyclic work or $(F_{sk} + \text{stiffness} + \text{twitch})$. After the muscles had recovered, they were removed from the chamber, trimmed of any tendon and loose tissue, blotted on filter paper, and weighed.

**Data analysis.** All of the measurements for each muscle were initially plotted as a time series. The relative changes with fatigue and recovery were found to be strikingly consistent between experiments for all but a few of the measurements (see below). The datasets were thus combined for analysis using the following procedures. To control for differences in the sizes of the preparations, all data were first standardized as a percentage of the control (i.e., prefatigue) values, where control is 100%; note that some measurements actually exceed 100% of control by a small amount due to potentiation during the very early stages of fatiguing stimulation. Despite standardizing the data so, it was not possible to then simply average measurements across preparations because not all measurements were made at the same time after the onset of stimulation nor at precisely the same level of fatigue. Furthermore, such a presentation is biased by the specific stimulation protocol chosen to fatigue the muscle, complicating interpretation. We therefore expressed all the data relative to the level of $P_0$ during fatigue and recovery as described next.

In all experiments $P_0$ started at 100% and declined to 50% before recovering again; thus the data are expressed as a function of declining $P_0$, during fatigue and increasing $P_0$, during recovery. Recordings of $P_0$ were interspersed with recordings of twitches, cyclic work, $F_{sk}$, and stiffness. As all the measurements could not be made simultaneously, we estimated their values at each moment that $P_0$ was recorded using linear interpolation as follows. Associated with every measurement of $P_0$, a value for each of the other measurements was calculated using recorded values that bracketed the measure of $P_0$, and assuming a linear change with time between them. For example, if the recording of $P_0$ occurred at a time exactly half way between two recorded values of data, the value of the predicted data point (associated with the time that $P_0$ was recorded) would be exactly half way between the two bracketing values. The result was a series of values for $P_0$ recorded during fatigue and recovery and associated values for each of the other measurements as predicted to have occurred if measured at the same moment as $P_0$. The data from all experiments were then combined and binned based on 5% changes of $P_0$, during fatigue and during recovery. Data were not normally distributed and were compared using a Kruskal-Wallis one-way ANOVA on ranks and Dunn’s multiple comparisons. Differences from control were considered significant with $P < 0.05$. All data are presented as means ± SE.

**Stiffness and corrected stiffness.** The proportion of the cross-bridge population in the attached state during contraction can be estimated from muscle stiffness. This requires knowledge of the stiffness of the muscle during rigor, where it is assumed that 100% of the available cycling cross bridges are attached, and knowledge of the proportion of the recorded stiffness that originates in the cross bridges themselves. It was assumed that the stiffness of unfatigued frog muscle in rigor is 1.63-fold greater than during maximal tetanus (29), 2% of the total compliance resides in the cross bridges, which agrees well with an estimate of 43% (29), 3 that each myosin head contributes the same increment to stiffness in active contraction as it does in rigor (40), 4 that this stiffness is independent of the state of fatigue. The proportion of attached cross bridges during isometric tetanus was then estimated as $(S_A/S_R)/(2 - S_A/S_R)$, where $S_A$ is the stiffness during active tetanic contraction (as measured during fatigue and recovery) and $S_R$ is the stiffness in rigor.

These assumptions are not entirely valid. The origin and distribution of compliance in muscle is not entirely certain; thus stiffness may not be a quantitatively reliable predictor of the absolute proportion of attached cross bridges (21). However, these are not likely to change appreciably with fatigue, and thus changes in corrected stiffness should be indicative of changes in the population of attached cross bridges. A greater hurdle to interpretation is that the stiffness and force-generating capacity of individual cross bridges are sensitive to their nucleotide state, they may be differentially so, and that the stiffness of a cross bridge in rigor may depend on how the rigor state is achieved (e.g., 8, 34, 38). For example, force falls more rapidly than the level of phosphate rises; thus the relationship between force and stiffness may not be linear during fatigue and would violate an assumption above. Values and relative changes in proportions of attached cross bridges so estimated should thus be interpreted with caution; as such we refer to our estimate as corrected stiffness but imply that it approximates the proportion of attached cross bridges.

**RESULTS**

Data are presented for muscles from seven animals. $P_0$ was 216 ± 21 kN/m² before the fatigue protocol. Young’s modulus at maximum force was 5.08 ± 0.48 MPa before fatigue. Net cyclic work output was 15.5 ± 4.25 J/kg, and power was 61.9 ± 17.0 W/kg at a cycle frequency of 4 Hz and 15% strain, typical for frog muscle at room temperature (44, 45).

With the fatigue protocol used, $P_0$ declined by 50% and then recovered toward initial levels over an ~30-min period; fatigue lasted ~10 min and recovery ~20 min. There was no obvious decline in the rate of fatigue of $P_0$ at the height of fatigue, while the rate of recovery slowed as recovery progressed. Muscles recovered to an average of 88.3 ± 1.6% of control $P_0$ after 20 min, with the poorest recovery being 83%. This level of recovery is similar to that reported by Thompson et al. (50) and Fitts and Holloszy (20) in frog sartorius and semitendinosus after ~30–40 min of recovery from a 50–90% reduction in $P_0$. No attempt was made to leave the muscles in the chamber for prolonged periods to determine if full recovery would eventually occur as the gradual and unavoidable deterioration of isolated preparations would render such evidence inconclusive. Thus, while most of the decline in force was due to reversible fatigue, we cannot rule out a small contribution from cell death or damage.

Two exemplary datasets are plotted vs. time during fatigue and recovery to assess patterns (Fig. 2). $P_0$, stiffness, $F_{sk}$, and shortening and net work all declined during fatigue and then recovered with trajectories that were similar across preparations. Lengthening work and measures of the twitch duration
increased during fatigue and then decreased back toward initial levels during recovery. However, two distinct patterns for recovery of twitch relaxation were noted in different preparations. In three of seven preparations, the period of twitch relaxation declined rapidly during early recovery and then continued to decline more gradually as recovery continued (Fig. 2D). The other four preparations showed a similar early rapid recovery in the period of twitch relaxation but then a substantial rebound in duration as recovery continued, reaching values similar to those at the height of fatigue (Fig. 2B). Twitch relaxation eventually returned toward control levels in all preparations. The transient increase in twitch duration during recovery noted in some preparations resulted from the development of a pronounced secondary slowing of relaxation about midway through the relaxation phase and thus does not reflect a simple reversal of the recovery process.

Twitch force initially increased and then decreased back toward control levels during fatigue (Fig. 2, A and C). During recovery there was a transient increase in twitch force, the magnitude of which varied between preparations from virtually none (Fig. 2A) to levels that rivaled the maximum attained during fatigue (Fig. 2C). Of note, in muscles that showed the recovery/rebound/recovery of both twitch force and the period of twitch relaxation, the transients were not synchronized with one another. Also, in some cases one measure but not the other showed the pattern of rebound during recovery.

In the remaining graphs, data are expressed relative to $P_0$ as described in MATERIALS AND METHODS. Two lines are shown on each graph, one showing data during fatigue (downward filled triangles with solid lines) and one during recovery (upward open triangles with dashed lines). The line of unity is a thin dotted line; data falling above this line indicate the relative change in a particular measurement was less than the decrease in $P_0$, and data falling below the line indicate the change was more than the decrease in $P_0$.

Results for the two steady-state measures, stiffness and $F_{isk}$, are described first (Fig. 3). As $P_0$ fatigued toward 80% of control, corrected stiffness remained unchanged. With further fatigue of $P_0$ to 50%, corrected stiffness decreased in an approximately linear fashion to ~75% of initial levels, eventually changing to become parallel to the line of unity with $P_0$. Corrected stiffness recovered with a trajectory that slightly lagged that during fatigue. $F_{isk}$, the force produced during isokinetic shortening, reflects the muscle’s ability to do work under conditions of maximal sustained activation. $F_{isk}$ declined linearly with fatigue of $P_0$ but declined to ~20% of its control level despite $P_0$ dropping only 50%. $F_{isk}$ recovered with a trajectory that slightly lagged that during fatigue.

The work done by the muscle during cyclic activity, as might occur during locomotion or other repetitive movements, was measured using the work-loop technique. Before and during early fatigue, the force produced by the muscle during shortening (while the muscle was stimulated) was greater than...
the force required to lengthen it (while it was not stimulated),
resulting in a work loop that was traversed in a counterclock-
wise direction and net work production by the muscle during a
cycle (Fig. 4, inset). As fatigue ensued, the force produced by
the muscle during shortening decreased and the force required
to lengthen the muscle increased, the latter due to relaxation
after activation becoming progressively less complete (Fig. 4,
inset); the force during lengthening eventually exceeded the
force during shortening so that the work loops were traversed
in a clockwise direction and the muscle absorbed net energy
during each cycle (i.e., more work was required to lengthen the
muscle than was done by the muscle when it shortened). Of
note, as a result of incomplete relaxation in fatigued muscle,
the force during the early stages of muscle shortening (i.e.,
immmediately after stretch) exceeded the force at this point in
unfatigued muscle (Fig. 4, inset). This resulted in more work
being done by the fatigued muscle than unfatigued muscle
during the initial stages of shortening. As such, while shorten-
ing work during cyclic activity did decrease with fatigue, to
~70% of control as Po declined to 50%, it rose further above
the line of unity with Po as fatigue progressed (Fig. 4),
reflecting the progressively incomplete relaxation and in-
creased force at the onset of shortening.

Also as a result of this progressively incomplete relaxation,
the work required to lengthen the muscle increased rapidly
during the early stages of fatigue, reaching 600% of control
when Po had declined by only 20% (Fig. 5). Lengthening work
peaked at ~800% of control as Po continued to decline toward
50%. During the initial recovery of Po (from 50 to ~70%),
lengthening work remained steady at ~500% of control, after
which there was a rapid and almost complete recovery of
lengthening work as Po recovered from 70 to only ~80% of
control. Interestingly, the entire fatigue of lengthening work
occurred as Po declined from 100 to 80% of control, while
almost the entire recovery of lengthening work occurred as Po
recovered from 70 to 80% of control. Thus the major effects
occurred at relatively low levels of fatigue and over a very
small range of Po.

Fig. 3. Steady-state measures of muscle performance during fatigue and
recovery. Corrected stiffness (top) represents a relative proportion of
attached cross bridges (see MATERIALS AND METHODS). Isokinetic force
(Fisk) while shortening at 2 muscle lengths/s (bottom) was measured after
the plateau of isometric tetanic contractions had been attained and is
equivalent to work during shortening. Values are expressed relative to the
control (prefatigue) condition. Solid lines and ▲ are during fatigue; dashed
lines and ⊳ are during recovery. The line of unity is a dotted line. Data are
means ± SE. *Values that are significantly different from control (P <
0.05).

Fig. 4. Shortening work done during cyclic contractions vs.
isometric tetanic force during fatigue and recovery. Work was
measured using the work loop method; cycle frequency was 4
Hz and strain was 15% of muscle length. Values are expressed
relative to the control (prefatigue) condition. Solid lines and ▲
are during fatigue; dashed lines and ⊳ are during recovery. The
line of unity is a dotted line. Data are means ± SE. *Values that
are significantly different from control (P < 0.05). Inset:
exemplary work loops before fatigue (solid line), midway
through fatigue (dashed line), and at the height of fatigue
dotted line). Arrows indicate the direction the loops are tra-
versed; note the control loop runs counterclockwise while the
fatigued loops run clockwise.
The duration of the twitch during force development is the time from 10 to 90% peak force, which displayed the same pattern during fatigue as did the period from stimulus to peak force just described (Fig. 8B). The duration of the twitch during relaxation, measured as the time from 90 to 10% of peak force, also increased nearly linearly with fatigue of $P_o$, but to over 400% of control (Fig. 8C). Like the previous measures, the duration of relaxation recovered along a trajectory very similar to that during fatigue, with the notable exception of some preparations in which there was a large, transient increase during the late stages of recovery (as noted above).

**DISCUSSION**

While we do not address directly the cellular mechanisms of fatigue in this study, we present here a brief summary of proposed mechanisms that may be supported or refuted by, and are relevant to understanding, changes in mechanical performance with fatigue. The changes that occur during fatigue are held to follow a series of stages, each reflecting its own characteristic failure of some element in the chain of excitation through cross-bridge activity. The initial fall in force during fatigue from high-frequency stimulation, from rest (100%) toward ~75%, is attributed to an impairment of the ability of cross bridges to generate force, with little or no change in the free calcium transient (activation) and by crude inference the number of attached cross bridges (17, 19, 54). Acidification of frog muscle reduces force and unloaded velocity of shortening as well as slowing twitch kinetics (18, 43) but does not explain entirely the effects of fatigue (32). The effects of acidification appear to be quite limited in mammalian muscle at normal temperatures (e.g., 37, 39).

The net cyclic work output, which is the net mechanical work produced by the muscle during a complete lengthen/shorten cycle, fatigued very rapidly, reflecting the steady decline in shortening work and the rapid rise in lengthening work. Net work declined to zero when $P_o$ had fatigued by only 15–20% (Fig. 6). Net work continued to decline with further fatigue, becoming negative and then reaching a minimum as $P_o$ fatigued to only ~70% of control. Net work recovered somewhat more rapidly than it fatigued relative to $P_o$; when $P_o$ had recovered to 80% of control, net work had recovered to ~50%, despite being zero at the same level of $P_o$ during fatigue. Net work was always below the line of unity with $P_o$ during fatigue and recovery.

Isometric twitch force was well above the line of unity with $P_o$ at all times during fatigue and recovery (Fig. 7). Twitch force showed an initial increase of ~50% with fatigue and then gradually declined back toward control levels with further fatigue. The extent of the increase of twitch force and the level to which it returned at the height of fatigue were quite variable; in some preparations twitch force was still substantially above its control level at the height of fatigue. During recovery, twitch force also showed a transient increase in all preparations, the magnitude of which varied from very little to levels that equaled the same high forces achieved during early/mid fatigue (Fig. 2A and C). However, because twitch force fell below control levels during recovery in some preparations, when averaged across all preparations the transient rise was not readily apparent or statistically different from control (Fig. 7). While difficult to discern in the averaged plots, the transient rise of twitch force during both fatigue and recovery occurred over the same range of values of $P_o$.

The duration of the twitch during force development, measured as the time from the stimulus to peak force, increased in an approximately linear fashion to ~180% of control as $P_o$ declined by 50% (Fig. 8A). It recovered along a remarkably similar trajectory. An alternate measure of the duration of the twitch during force development is the time from 10 to 90% peak force, which displayed the same pattern during fatigue as did the period from stimulus to peak force just described (Fig. 8B). The duration of the twitch during relaxation, measured as the time from 90 to 10% of peak force, also increased nearly linearly with fatigue of $P_o$, but to over 400% of control (Fig. 8C). Like the previous measures, the duration of relaxation recovered along a trajectory very similar to that during fatigue, with the notable exception of some preparations in which there was a large, transient increase during the late stages of recovery (as noted above).

**DISCUSSION**

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42, 57), making P_i a prime candidate as the inhibitor of not only force and shortening velocity during fatigue but also intracellular calcium handling and myofibrillar calcium sensitivity (1, 10, 11, 27, 54, 55). High-energy phosphate depletion during fatigue does not appear to be a factor that limits cross-bridge function (12, 19, 35). If activity continues past the early stages of fatigue, the muscle suffers a subsequent fall in force that is attributed to reduced calcium release from the sarcoplasmic reticulum and thus reduced activation and fewer cross bridges, again perhaps due to accumulation of P_i (2, 14, 27, 53, 54, 56, 58) and failure of the inward spread of activation (17, 22). Sustained activity may also be associated with physical damage at the triads and ryanodine release channels, leading to impairment of calcium handling that recovers with a time course of hours or days (56).

**Effects on tetanic force.** During the early rapid fall of force with fatiguing stimulation (or the initial linear fall of force in cane toad muscle), the internal free calcium transient remains normal or may actually increase (27, 51, 52, 54). Therefore, excepting a potential effect of fatigue on troponin affinity for calcium (reviewed in Ref. 19), the level of activation and hence number of cross bridges should not be greatly impaired in early fatigue. Our stiffness results (Fig. 3) and those of others (9, 16, 17) support this notion. Thus, after moderate fatigue, there is only a small reduction in the number of cross bridges, and the fall in force is attributed largely to a reduction in the average force per attached cross bridge. Here we also report stiffness during the very initial stages of fatigue (from rest to ~80% P_o) and found that it remained unchanged (Fig. 3). Hence, the initial fatigue of P_o appears to be due entirely to less force per cross bridge. A reduced ability of cross bridges to generate force has been attributed to accumulation of phosphate (19, 36, 55) and more so to protons in frog muscle (e.g., 18, 19, 23, 39). Fatigue below 80% P_o was accompanied by a steady decline in stiffness (Fig. 3) (17), with the rate of decline eventually matching the rate of fatigue of force. This supports suggestions that during more severe fatigue there is failure of activation and thus impaired recruitment of cross bridges via accumulation of protons and phosphate associated with inhibition of calcium release and reuptake by the sarcoplasmic reticulum (2, 14, 19, 27, 51, 54, 55, 56), impairment of the inward spread of excitation (17, 22), and failure of excitation contraction coupling at the triads (6).

The ratio, P_o/corrected stiffness, is an index of the average force produced per cross bridge. During fatigue, the force per cross bridge declined in an exponential fashion (Fig. 9), being near the line of unity with P_o during early fatigue (control < 0.05). The 2 outliers are preparations that showed unusually high-twitch durations late in recovery.
Fatigue commences, accounting entirely for the decline in force during early fatigue; 2) an eventual lower limit to the force per cross bridge with advanced fatigue (~69% of control this study), and 3) the decline in P_o during fatigue (e.g., impaired activation). Again, potential nonlinearities between stiffness and force or proportion of attached cross bridges as a result of fatigue leave these conclusions tentative, less so during early and more so during later fatigue.

During recovery, force per cross bridge increased more rapidly while the number of cross bridges increased more slowly than during fatigue (Figs. 3 and 9). Further, the force per cross bridge fatigued exponentially but recovered linearly with respect to P_o. Further still, at levels of P_o between 80 and 100% of control, stiffness remained unaltered during fatigue but was depressed during recovery. These differences strongly suggest that the mechanisms responsible for altering the number of attached cross bridges vs. the force per cross bridge are independent (such as accumulation of protons or phosphate vs. impaired activation), that they fail and recover at different rates, and that P_o itself cannot be used directly to infer the cause of fatigue.

Effects on twitches. We use the term activation to refer to most aspects of excitation-contraction coupling, including the time course and magnitude of the calcium transient and the associated activation of thin filaments. While this use is imprecise, we intend it only as an ellipsis of the processes involved. The amplitude of the intracellular free calcium transient increases during the early stages of fatigue (saturation of myoplasmic calcium buffers) and then gradually declines as fatigue progresses (reduced calcium release from the sarcoplasmic reticulum) (1, 19, 51). The duration of the free calcium transient also becomes prolonged during fatigue (52, 59), and the resting intracellular calcium levels rise, causing a prolongation of the duration of the twitch, particularly the relaxation phase [1, 51, 59; see McIntosh et al. (30) for a discussion of the effects of fatigue on twitch duration and its interpretation]. Twitch force and kinetics should reflect these changes in activation (50), which in turn should influence the ability of muscle to power locomotion.

While there is commonly a decline in twitch force during fatigue (e.g., 20, 50), a transient rise during early fatigue is not novel (20). In the present study, twitch force increased during fatigue and reached a maximum ~50% above control (Figs. 2 and 7), this in the face of a 20% decline in force produced per cross bridge (Fig. 9) and no change in corrected stiffness during tetanus (Fig. 3). Other factors affecting peak twitch force include the magnitude and time course of activation, cross-bridge kinetics, and the series compliance. We assumed the series compliance to be passive and not prone to fatigue; however, it is possible that in a muscle or fiber bundle its distribution and thus characteristics could change during fatigue. Regarding cross bridges, slowing of the cross-bridge detachment rate with fatigue as reflected in a slowing of the maximal velocity of shortening (both \(V_{\text{max}}\) and \(V_c\)) (9, 18) would cause an increase in the number of attached cross bridges and hence twitch force. However, there is also a slowing of twitch \(dP/dt\) (20, 50), suggesting a slowing of the attachment rate, which would result in less force (see also Ref. 19), and there was no change in stiffness during early fatigue (Fig. 3), suggesting no change in the number of cross bridges. As cross-bridge kinetics during the plateau of a tetanic contraction are different than during the early portion of a twitch, conclusions about twitches based on results from tetanic contractions (stiffness, \(V_{\text{max}}\)) should be interpreted with due caution. Last, an increase in amplitude of the twitch free calcium transient during early fatigue (see above) could result in more force. Likewise, prolongation of the calcium transient would lead to a longer twitch (50), allowing more time for cross bridges to form and extend the series compliance [as suggested by Metzger and Fitts (33)]. In support of this idea, Sun et al. (48) noted a close relationship between the rate of decay of the calcium transient and peak twitch force. Similarly, during the early stages of fatigue (100–80% \(P_o\)), we noted a highly significant linear relationship (slope = 0.90, \(P < 0.001\)) between peak twitch force and twitch duration (measured as the time available for force development, 10–90% peak force); however, a low \(r^2\) for this relationship (0.39) suggests other factors are also involved. Thus the increased twitch force during early fatigue seems attributable to the increased time available to develop force.

As fatigue progressed further, twitch force tended back toward control levels (Fig. 7). Associated with this drop in twitch force was a 30% decline in corrected stiffness (fewer cross bridges) (Fig. 3) and a 10% decline in force per cross bridge (Fig. 9); together these approximately account for the drop in twitch force. On average, twitch force remained remarkably stable near control levels during the entire period of recovery (Fig. 7). Thus the near linear increase in corrected stiffness (Fig. 3) and force per cross bridge (Fig. 9) during recovery, which would increase twitch force, appeared to counteract the effects of a near linear decline in twitch duration (Fig. 8), which would decrease twitch force. Of note, the large separation between fatigue and recovery of twitch force when expressed relative to \(P_o\) (Fig. 7) is strong evidence that differ-
dent mechanisms are responsible for the impairment of tetanic force vs. twitch force. The present results suggest that during early fatigue the continued slowing of calcium kinetics augments twitch force, and this overshadows the reduced force per cross bridge, while later in fatigue the combination of reduced force and number of cross bridges begins to dominate. During recovery, cross-bridge force and number and activation kinetics interact such that force remains relatively stable. Given that fatigue of twitch force involves changes in the force per cross bridge, number of cross bridges, the kinetics and level of activation, as well as the effects of potentiation, it may not be surprising to find extreme variability in the response of twitch force to fatiguing stimulation, both between preparations and fatigue protocols.

Slowing of the twitch during fatigue is commonly observed (see also 11) and is attributed to a slowing of the calcium transient (reviewed in 19). The periods of both twitch contraction and relaxation showed an increase during fatigue and decrease during recovery and were nearly linearly related to $P_o$ at all levels of fatigue, and there was a striking similarity during fatigue and recovery to their relationship with $P_o$ (Fig. 8). This linear and consistent relationship between twitch duration and $P_o$ has not been reported previously and implies that either a single element acting via different mechanisms or a single mechanism itself is responsible for changes in both $P_o$ and twitch duration. While there is evidence that failure of activation results in reduced tetanic force during severe fatigue, it does not appear to be an important factor during early fatigue (14) and is thus unlikely to account for the similar changes in both twitches and $P_o$ at all stages of fatigue. Both twitch kinetics and $P_o$ are influenced by the interactions of calcium with troponin and by cross-bridge kinetics (41); however, we are not aware of compelling evidence that either could explain the correlated changes observed. Alternatively, proton or Pi accumulation may be single elements that could similarly influence both twitches and $P_o$ via different mechanisms; they simultaneously reduce force and slow intracellular calcium kinetics (5, 55, reviewed in 19), although a clear effect on the calcium transient is not yet demonstrated. Changes in $P_o$ are a complex function of changes in both the number of and force generated by cross bridges, which we have noted above appear to involve separate mechanisms; likewise twitch duration is a complex function of compliance and the kinetics of activation and troponin affinity, which are all independent. Thus whether a sole agent or many mechanisms acting in concert are responsible, the linear tracking of twitch duration and $P_o$ during fatigue and recovery seems extraordinary.

Effects on work. When fatigue is assessed in the context of cyclic animal movement, changes in twitch duration and mechanical work or power are of primary concern. Prolongation of the twitch will severely hinder movements that require rapid oscillations in force, even in the face of a maintained ability to produce force (41, 49). Stevens and Syme (46) first highlighted the marked difference between fatigue of $P_o$ and the ability to perform cyclic work and conclude that $P_o$ is a poor index of a muscle’s ability to do work in a system undergoing cycles of flexion and extension. In accord, we note here that net work output declined precipitously to zero as $P_o$ declined by only 20%, and continued to decline to $-50\%$ of control (net work absorption) when $P_o$ dropped by less than half (Fig. 6). Thus, although the muscle was capable of producing considerable amounts of tetanic force at the height of fatigue, more work was required to lengthen the muscle than was done during shortening. This would be disastrous for certain movements, particularly during undulatory swimming, where the muscles used for propulsion are also the antagonists of contralateral muscles. If muscles on both sides of the animal fatigue at the same rate, as may be expected, the animal would become paralyzed when net work reached zero, despite their muscles retaining most of their ability to produce force. While the experimental use of isolated preparations precludes selective and graded recruitment of motor units as employed by animals, it is unlikely that the tendency for net work to become negative with fatigue could be avoided without a drastic curtailment of power output. Regardless of the strategy used to activate the muscle, rapid failure of the system to power locomotion will ensue.

Even animals with dedicated antagonistic muscles, where one muscle powers movement and is extended by an antagonist that does not contribute directly to movement, likely do not fair a great deal better. In such cases it is only the work done by the power producer while shortening that is relevant to propulsion; the work to extend them is done by their antagonist and does not detract from propulsion. Yet the work to extend the power producer increased to near 600% of control as $P_o$ fatigued by only 20% (Fig. 5). The power producer’s antagonist will then fatigue against this high load and suffer the same escalation in work required to extend it, work that comes at the expense of its antagonist, which is the muscle powering movement itself. Hence, it is again net work that is relevant; the energy available for propulsion is the difference between the shortening work done by the muscle powering movement and the work required to extend its antagonist. Thus the power available for propulsion fatigues much more rapidly than either the ability to produce force or shortening work. Further, the energetic cost to sustain movement during fatigue will be extreme, including the cost of powering movement and the rising costs of extending...
both the propulsive muscle and its antagonist. Paradoxically, the height of energy consumption could occur under conditions where mechanical power available for movement is minimal; antagonistic muscles would expend most of their energy simply extending one another, with little or nothing left to power locomotion.

Quite unlike net work, cyclic shortening work actually failed less than $P_o$ as fatigue progressed (Fig. 4) (see also Ref. 46). This reflects the progressive lack of complete relaxation of the muscle during fatigue (Fig. 4, inset) (see also Ref. 46). Incomplete relaxation led to much higher forces during the stretch portion of a work cycle and thus higher forces and more work done at the subsequent onset of shortening, more than might be expected based on $P_o$. This failure of relaxation and enhancement of cyclic shortening work also caused a notable difference between the effects of fatigue on the dynamic measure of shortening work ($F_{\text{sk}}$). Work during isokinetic shortening (proportional to $F_{\text{sk}}$) (Fig. 3) declined much more than shortening work during cyclic contractions (Fig. 4), to the extent that isokinetic work was only 30–50% of cyclic work at the height of fatigue (Fig. 10). This was initially unexpected, as unlike $F_{\text{sk}}$, cyclic work is additionally limited by activation kinetics (7); we anticipated that these limitations would be exacerbated as fatigue progressed. However, as noted above, cyclic shortening work was actually enhanced by the failure of the muscle to relax fully when fatigued, while $F_{\text{sk}}$ is independent of activation kinetics and was not. The enhancement was so pronounced as to bias cyclic work upward by two- to threefold over what was predicted based on steady-state measurements using $F_{\text{sk}}$. This again reinforces the important difference between cyclic and steady-state measures of performance, even when both measures are of the work done when a muscle shortens.

The rate of doing work is power, which is the product of force and shortening velocity. A shift in the force-velocity relationship of muscle after fatigue has been reported (e.g., 25, 58) such that when maximum power from force-velocity curves is expressed relative to the product of $V_{\text{max}}$ and $P_o$, there is a 10–50% increase in relative power output with fatigue (i.e., the decrease in the ability to produce power with fatigue is less than the decrease in the product of $P_o$ and $V_{\text{max}}$) (3, 9, 13). The decline in $P_o$ with fatigue has been noted to be similar to, somewhat less than, or greater than the decline in $V_{\text{max}}$ (3, 9, 18, 20, 25, 50, 58). $F_{\text{sk}}$ in the present study is one point on the force-velocity relationship and reflects power during isokinetic shortening with maximal activation. While we did not specifically select $F_{\text{sk}}$ to produce maximal power, we did select it to be close (see MATERIALS AND METHODS); accepting this, and based on the observation that at the height of fatigue $P_o$ declined to 50% and $F_{\text{sk}}$ (power) to 25%, then $V_{\text{max}}$ would also have to decline by at least 50% for relative power to increase, similar to reports above. The approximately equivalent drop in ability to produce force and shorten with fatigue, and the linear and virtually identical relationship between $P_o$ and $F_{\text{sk}}$ during both fatigue and recovery (Fig. 3), suggest a common mechanism may be responsible for changes in force production and the ability to shorten during fatigue.

In summary, early fatigue was attributed entirely to a reduction in the ability of cross bridges to generate force and was followed and accompanied by a reduction in the number of cross bridges with further fatigue. The force per cross bridge reached a lower limit at about 69% of maximum, whereby further reductions in force with fatigue were attributed entirely to a loss in the number of cross bridges. Fatigue was accompanied by a slowing of contraction kinetics, which appears to be the primary factor responsible for differences between dynamic vs. steady-state measures of performance during fatigue. Because slowed relaxation has no influence on steady-state performance such as $P_o$ or $F_{\text{sk}}$, changes in these measures with fatigue reflect only changes in cross-bridge function. They insinuate the effects of fatigue on animal movement, but in an indirect and often misleading fashion. Slowed relaxation was a major contributor to all aspects of changes in dynamic performance. The drastically reduced net work output and increased lengthening work during cyclic contractions, the augmentation of work done while shortening during cyclic contractions above what would be predicted based on $P_o$ or $F_{\text{sk}}$, the longer twitch duration, and the increased twitch force despite fewer and weaker cross bridges all have a basis in slowed activation kinetics and are all relevant to animal movement. That failure of $P_o$ by a mere 10% would signify that antagonistic pairs of muscles have been rendered almost incapable of producing any net power or that despite this the same muscles are actually doing more work when they shorten than $P_o$ would predict, is evidence that dynamic measures of performance are uniquely important to understanding the effects of fatigue on animal movement. Steady-state measures of muscle performance, which do not reflect activation kinetics and dynamic properties of contraction, will not provide accurate information about the impact of fatigue on a muscle’s ability to power movement.

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REFERENCES


