Changes of surface and t-tubular membrane excitability during fatigue with repeated tetani in isolated muscle fast- and slow-twitch muscle

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Cairns SP, Taberner AJ, Loiselle DS. Changes of surface and t-tubular membrane excitability during fatigue with repeated tetani in isolated muscle fast- and slow-twitch muscle. J Appl Physiol 106: 101–112, 2009. First published 23 October 2008; doi:10.1152/japplphysiol.90878.2008.—We investigated whether impaired sarcolemmal excitability causes severe fatigue during repeated tetani in isolated mouse skeletal muscle. Slow-twitch soleus or fast-twitch extensor digitorum longus (EDL) muscles underwent intensive stimulation (standard protocol: 125 Hz for 500 ms, every second, parallel plate electrodes, 20 V, 0.1-ms pulses). Interventions with altered stimulation characteristics were tested either on the entire fatigue profile or after 90- to 100-s stimulation. n-tubocurarine did not alter the fatigue profile in soleus thereby eliminating impaired neuromuscular transmission. Lower stimulation frequencies partially restored peak force, especially in soleus. The twitch force-stimulation strength relationship shifted towards higher voltages in both muscle types, with a much larger shift in EDL. Augmenting pulse strength restored tetanic force from 29% (4.4 V) to 79% (20 V), or slowed fatigue in soleus. Increasing pulse duration (0.1 to 1.0 ms) restored tetanic force from 8 to 46% in EDL and from 41 to 90% in soleus; 0.25-ms pulses restored tetanic force to 83% in soleus. Switching from transverse wire to parallel plate stimulation increased tetanic force from 34 to 63%, and fatigue was exacerbated with wires compared with plates in soleus. The combined data suggest that impaired excitability (disrupted action potential generation) within trains is the main contributor (~50% initial force) to severe fatigue in both muscle types, the surface rather than t-tubular membrane is the main site of impairment during wire stimulation, and extreme fatigue in EDL includes an increased action potential threshold leading to inexcitable fibers. Moreover, mathematical modeling discounts anoxia as the major contributor to fatigue during our stimulation regime in isolated muscles.

skeletal muscle fatigue; fiber type; sarcolemma; electrolytes; anoxia

THE MECHANISMS underlying muscle fatigue during repeated brief tetanic stimulation could, in principle, involve impairment of: 1) myofilament function, with reduced cross-bridge activity; 2) excitation-contraction (E–C) coupling, with reduced Ca2+ release from the sarcoplasmic reticulum (SR); 3) sarcolemmal excitability, with disrupted action potentials in the sarcolemmal membrane; and/or 4) neuromuscular transmission. Much of the force decline with severe stimulation-induced fatigue of isolated muscle preparations appears to result from a diminished myoplasmic Ca2+ transient (tetanic [Ca2+]i) (1, 11, 12, 16). This in turn may be caused by direct impairment of E–C coupling through inhibitory effects on the SR or diminished t-tubular charge movement, or indirectly via impairment of sarcolemmal excitability so that fewer action potentials are available to trigger Ca2+ release from the SR. Whether diminished excitability causes fatigue during repeated tetani is controversial (23), with some challenges to this hypothesis (19, 51, 53). In particular, Zhang et al. demonstrated that isolated whole muscles from mice fatigued more rapidly than single muscle fibers, but when exposed to cyanide (to inhibit mitochondrial function) the fatigue profile was similar in both preparations (53). Since cyanide accelerated the decline of tetanic [Ca2+]i during fatigue, they proposed that fatigue in whole muscles results from an anoxic core which promotes impaired Ca2+ release from the SR. They further inferred that fatigue is unrelated to impaired excitability, although they did not record action potentials in support of their contention.

Several, but not all, studies show that with repeated intermittent stimulation, the compound extracellular muscle action potential (M-wave), undergoes a large decline of amplitude/area, consistent with impaired excitability (21, 27, 43, 46). However, the relationship between the M-wave and fatigue is complex. Force depression can occur before any decline of the surface M-wave (27, 46). The M-wave may not detect changes in t-tubular membranes (33). Individual M-waves can disappear during tetanic stimulation without any associated decrement of force (37) and M-waves may also reflect a change in neuromuscular transmission (23). Furthermore, a smaller M-wave does not discriminate between reduced action potential amplitude and inexcitable fibers; this distinction requires measurement of intracellular action potentials.

Attempts to make intracellular action potential recordings in the surface membranes of muscle fibers during repeated stimulation have proven to be difficult because glass recording electrodes commonly dislodge during contraction (2, 5, 37). An alternative approach is to impale fibers after the cessation of stimulation (39, 44), but with an unavoidable time delay, any action potential changes are likely to be underestimated due to rapid recovery (2, 34, 37, 39, 50). Moreover, the single action potentials recorded in fatigued muscle (39, 43) relate directly to the twitch but not necessarily to tetanic contractions, which are activated by trains of action potentials. Measurements of action potential trains have been made in fatigued fibers from amphibian muscle (2, 44, 50) but not from mammalian muscle. Whereas trains have been recorded in mammalian fibers using either microelectrodes in the surface membrane (5, 27, 38) or voltage-sensitive dyes in t-tubular mem-

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branes (17), this has been done only in nonfatigued preparations. To further complicate the issue, a drastic reduction of action potential amplitude or skipping during trains (i.e., an intermittent failure to generate action potentials) does not necessarily diminish force (5, 23, 37). Also the presence of normal action potentials in the surface membrane of fatigued muscle does not exclude the possibility of impaired t-tubular membrane excitability (2, 29, 33, 48). The only measurements which provide certainty that action potential changes lead to force loss include the occurrence of inexcitable fibers (5, 31, 46) or complete failure within trains (2, 5, 32). Therefore, another approach is needed to address the role of impaired sarcolemmal excitability during fatigue.

Recent work has identified the various sites of excitation when isolated muscles are stimulated with different pulse parameters or electrode configurations (6). We now consider that this approach can be exploited to probe mechanisms of fatigue. For example, an increased stimulus pulse charge (strength and/or duration) enhances excitation via voltage-dependent Na⁺ channels (see Fig. 1), which may help maintain action potential generation in the sarcolemma of fatigued muscle. Another example involves examining fatigue with different electrode configurations. Transverse wire stimulation excites a small area of surface membrane near a stimulation electrode leading to action potential propagation along the surface and then down t-tubular membranes (41). In contrast, parallel plate stimulation excites action potentials simultaneously all along the surface membrane to effectively bypass surface propagation (6, 41, Fig. 1). A comparison of the force responses evoked with these contrasting electrode configurations can thus be used to test for action potential failure in the surface membrane when fatigue is induced with wire stimulation.

Therefore, the primary aim of this study was to utilize altered stimulation characteristics to quantify the contribution of impaired sarcolemmal excitability to fatigue during repeated tetani in isolated fast- and slow-twitch mammalian muscle. A secondary aim was to distinguish between the surface and t-tubular membranes as the major site of impairment of excitability during fatigue induced by such stimulation regimes.

METHODS

Muscle Preparations and Solutions

Adult female mice (Swiss CD-1), aged 4–12 wk and weighing 20–35 g, were killed by cervical dislocation, and intact soleus or extensor digitorum longus (EDL) muscles were dissected. The fiber type composition of these muscles, based on myosin ATPase assays and when expressed as percentage muscle cross-sectional area, was 51% type I, 49% type IIa/x for soleus and 1% type I, 31% type IIa/x, 68% type IIb for EDL (10). Muscles were dissected in a Krebs solution of composition (mM): 122.2 NaCl, 2.8 KCl, 1.2 KH₂PO₄, 25.1 NaHCO₃, 1.2 MgSO₄, 1.3 CaCl₂, and 5 d-glucose, and maintained at pH 7.4 by continuous bubbling with 95% O₂-5% CO₂ at room temperature. Several experiments were undertaken in Krebs solutions at elevated [K⁺] (8–11 mM K⁺), in which NaCl was replaced with equimolar KCl to maintain osmolarity. In some experiments, 30 μM d-tubocurarine (Sigma Pharmaceuticals) was present to abolish neuromuscular transmission (29). The temperature of the Krebs solution used in all experiments was 25°C. All studies were approved by the Animal Ethics Committee of the University of Auckland.

Stimulation and Force Recording

For a full description of the experimental arrangement, see setup 2 in Cairns et al. (6). In brief, muscles were mounted vertically in a thermostatically controlled chamber containing ~100 ml of Krebs solution (bubbled from the base with 95% O₂-5% CO₂). Electric field stimulation was usually delivered via two parallel plate platinum electrodes (separated by 7.7 mm) that flanked the muscle. In some experiments stimulation occurred via two fine wire electrodes that transversed the muscle (separated by 4–6 mm) about midway along its length and were located between the plate electrodes. Rectangular stimulation pulses (bipolar) were initiated from an Apple Macintosh PowerPC 7100/80 with custom-written Labview software and delivered to the electrodes via a purpose-built power amplifier (MOSFET). Pulse duration and strength were adjusted via the Labview program. Pulse strength could also be adjusted rapidly via the power amplifier. The electrode configuration (plates or wires) could be changed through a switch on the power amplifier. The standard pulses (20 V, 0.1 ms) were supramaximal for the twitch (6). The stimulus strength (V), measured between dry electrodes, was the same for plates and wires, and increased linearly up to 26 V, at which point the amplifier saturated. Note that it is the electric field strength that ultimately determines the current density necessary to activate the sarcolemma (this is 26 V/cm between the plates with our standard 20 V pulses, i.e., 20 V/0.77 cm). Peak twitch and tetanic forces for isometric contractions were recorded via a KSP-2-E3 force transducer (Kyowa, Japan) on a chart recorder (Gould model 244) or saved on the computer. The fatigue profile was determined from chart recordings.

Experimental Protocol

The muscle length was first adjusted until maximum tetanic force was achieved, followed by a 30- to 60-min period of equilibration during which tetani were evoked every 5 min. Control nonfatigued contractile parameters were then determined before inducing fatigue.
We define fatigue as any reversible decline of peak force evoked by repeated tetanic stimulation of muscle. Our standard fatigue stimulation regime (125 Hz for 500 ms, evoked once every second for 100 s) was utilized because it generated tetani that produced near maximal force (10). This regime could be modified with Labview software, by varying the stimulation frequency (30, 50, or 125 Hz), rest period duration (0.5 or 2.5 s), or number of contractions (20 or 100). Test tetani (using altered stimulation frequency, pulse strength, pulse duration, or electrode configuration) were usually elicited in fatigued muscle at 2 s after the cessation of a fatigue run (e.g., Fig. 2). On some occasions this was done after 20 or 90 tetani (e.g., Fig. 5A). After each fatigue run the tetanic force was monitored for at least 60 min to ensure that recovery had occurred and that the preceding loss of force had not been irreversible. For experiments involving elevated [K⁺]o, the contractile measurements were assessed after at least 50 min equilibration, by which time the peak tetanic force had reached a new steady-state value. Muscles were rejected as being nonviable if the decrement of peak tetanic force was greater than 0.2%/min, or the peak tetanic force evoked with 1.0 ms pulses was 20% greater than that with 2.0 ms pulses (see “% control” (site 1)). By contrast, parallel plate stimulation triggers an action potential simultaneously all along the length of the fibers in either the surface membrane or at a short distance down the t-tubular membranes (site 3). We capitalize on this distinction by switching to parallel plate electrodes to bypass surface propagation, having previously induced fatigue using our transverse wire configuration.

The twitch force-stimulation strength relationship can be used to test for a change of action potential threshold (5, 7, 18). The rationale is that whereas individual fibers have varying thresholds, each is activated in an all-or-none manner. The effect of increasing stimulus strength is to cause a graded increase in twitch force in whole muscles until all fibers have been recruited. Thus, a right shift of the twitch force-stimulation strength relationship towards higher voltages indicates that an increase of action potential threshold has occurred in at least some fibers. This interpretation must be tempered by the knowledge that excitation with the weakest pulses (less than 4.4 V, 0.1 ms) occurs via nerve terminals (site 1). To infer that the muscle action potential threshold has increased, it is required to demonstrate a large shift of this relationship over the voltage range where the sarcolemma is excited directly. Hence, we determined the relationship by means of twitches evoked first with standard pulses (20 V, 0.1 ms), then with the stimulus strength varied in a random sequence, and again with standard pulses. At each reduced stimulus strength we averaged the peak force of two consecutive twitches and expressed it as a percentage of the mean peak force of bracketing twitches evoked with standard pulses. The pulse strength that elicited 50% of maximum twitch force was determined by linear interpolation between points. In fatigued muscle, twitches were evoked every 5 s, commencing 10 s after fatiguing stimulation. Normalizing to bracketing twitches evoked at the standard pulse strength accounted for any recovery of twitch force.

Statistical Analysis

Data given in the text and figures are mean ± SE for the number (n) of muscles tested. Data were assessed by ANOVA with the software package Statistica 7.1. When the data sets (typically large but occasionally sparsely populated) permitted, repeated measures designs were employed. Otherwise, nested designs were adopted (despite some loss of power). In all cases in which the F ratio arising from the ANOVA was adjudged to be significant (P < 0.05), differences among levels of interventions (e.g., stimulation frequency, method of stimulation, etc.) were sought by means of a suitably constrained matrix of post hoc contrasts. All differences reported in the text are statistically significant unless stated otherwise.

RESULTS

Changes of the Force-Frequency Relationship with Fatigue

Figure 2A shows an original force record of the severe fatigue evoked with our standard protocol (125 Hz, 500 ms, delivered once every second for 100 s) in a slow-twitch soleus muscle. On average, peak force declined to 40 ± 2% (n = 32) of its initial value over 100 s. In four experiments, the fatigue profile evoked with standard stimulus pulses and parallel plate stimulation electrodes was unaltered in the presence of 30 μM α-tubocurarine; specifically, peak force was reduced to 39 ± 3% of its initial value at 100 s. Test contractions were elicited at different stimulation frequencies in fatigued muscle to de-
The averaged data for soleus (Fig. 3A) evoked 2 s after fatigue, caused a partial recovery of peak force (40 Hz, parallel plate electrodes. Test tetanus was evoked at lowered stimulation frequency (40 Hz, A) or with longer stimulation pulses (1.0 ms at 200 Hz, B). Note, increase of resting force seen in A has been attributed to slowing of late phase of relaxation (10).

TERMINE WHETHER HIGH-FREQUENCY FATIGUE HAD OCCURRED (see METHODS). Lowering the frequency to 40 Hz, for a test tetanus evoked 2 s after fatigue, caused a partial recovery of peak force (Fig. 2A). The averaged data for soleus (Fig. 3A) show that the entire force-frequency relationship changed with severe fatigue so that greater forces were achieved at 30–80 Hz than at 125 Hz. The largest restoration of peak force was to 52 ± 3% of initial at 50 Hz (n = 10). Moreover, stimulation at 200 Hz caused an extra loss of force to 33 ± 5% of initial (n = 5). When a similar fatigue protocol was used, except that the frequency was 50 Hz, the decline of peak force after 100 s was attenuated to 47 ± 3% (n = 9) of that for the 125-Hz tetanus in nonfatigued muscle. When test tetani were subsequently evoked at 125 Hz in these muscles that had been fatigued at 50 Hz, the peak force was reduced to 39 ± 3% of the nonfatigued level, which did not differ from that achieved in muscles fatigued at 125 Hz.

In fast-twitch EDL muscles, the fatigue induced with our standard protocol was extreme (Fig. 2B), falling on average to 5.9 ± 0.4% (n = 11) of the initial level. Varying the frequency either below or above 125 Hz did not cause any peak force recovery in these fatigued muscles (Fig. 3B). Nevertheless, there was an ancillary effect of frequency. Fade (i.e., the decline of peak force within a 500-ms tetanus), which normally occurred during fatigue at 125 Hz (relative force 0.60 ± 0.05, n = 6), did not occur at 80 Hz (relative force 0.97 ± 0.03, n = 4). The effect of reducing frequency was also tested after fatigue was induced with 20 tetani in EDL (Fig. 3B), at which time the extent of tetanus depression was similar to that in soleus after 100 tetani. The resulting peak force at 80 Hz increased by a greater extent than at 125 Hz when tested with repeated fatigue runs in the same four EDL muscles. These data confirm that high-frequency fatigue occurs with our stimulation regimes in whole muscles.

Changes of the Twitch Force-Stimulation Strength Relationship with Fatigue

We tested whether fatiguing stimulation causes an increased threshold for single action potentials by means of the twitch force-stimulation strength relationship. In soleus muscles fatigued with the standard protocol (peak tetanic force 39% initial, n = 9), the peak twitch force fell to 62 ± 2% initial at 10–15 s post-stimulation, then recovered to 78 ± 2% at 50–60 s and 87 ± 2% at 90–110 s. In consequence of this recovery, the twitch force-stimulation strength relationship was assessed.
separately over 10–60 s and 60–110 s post-stimulation. Figure 4A shows the relationship at 10–60 s post-stimulation, when a small, albeit significant, right shift towards higher voltages occurred for stimulation strengths below 5 V. The pulse strength needed to generate 50% of maximum twitch force increased significantly from 2.1 ± 0.1 to 2.8 ± 0.3 V (n = 9, paired t-test). However, the relationship did not differ from the control when assessed over 60–110 s.

In EDL muscles (peak tetanic force 8% initial, n = 5), peak twitch force was depressed to 56 ± 7% initial at 10–15 s post-stimulation, but in contrast to soleus remained at 54 ± 3% initial at 90–110 s. During this 2-min post-stimulation period, the twitch force-stimulation strength relationship exhibited a large right shift towards higher stimulation strengths at values less than 13 V (Fig. 4B). The pulse strength that generated 50% of maximum twitch force increased from 2.1 ± 0.3 to 8.0 ± 0.9 V (paired t-test). This fatigue effect then fully reversed by 30 min post-stimulation. The relationship was also assessed in EDL after fatigue with 20 tetani (peak tetanic force 38% initial, n = 3). Standard twitches (20 V) were potentiated to 133 ± 15% at 10–15 s post-stimulation, but this effect then reversed to 96 ± 13% at 90–110 s. With this severe but slightly lesser extent of tetanic fatigue, the relationship was unaffected (○ and broken line, Fig. 4B). These combined data implicate an increased threshold for sarcolemmal action potentials but only with extreme fatigue, and most especially in EDL.

We compared quantitatively the effects of fatiguing stimulation with that of raised bath [K+] on nonfatigued muscle to test whether a reduced trans-sarcolemmal K+ gradient could cause this fatigue. In soleus the right shift of the twitch force-stimulation strength relationship with severe fatigue (Fig. 4A) was mimicked quantitatively by 9 mM K+ (Fig. 4C), where the pulse strength needed to evoke 50% of maximum twitch force increased from 2.4 ± 0.1 to 3.2 ± 0.7 V (n = 5, paired t-test). However, in contrast to the twitch depression observed with severe fatigue, the peak twitch force at 9 mM K+ was 102 ± 5% (n = 5) of the control value (peak tetanic force 79 ± 4% control). There was no significant change of this relationship at 8 mM K+*, whereas a much larger shift occurred at 10 mM K+ (Fig. 4C). In EDL the larger right shift of the twitch-force-stimulation strength relationship with fatigue at 100 s (Fig. 4B) was similar to the effect of 11 mM K+ (Fig. 4D), where the pulse strength that evoked 50% of maximum twitch force increased to 8.6 ± 1.2 V (n = 6, paired t-test). However, at 11 mM K+ the decline of the twitch to 83 ± 5% (n = 6) and of the tetanus to 55 ± 5% (n = 5) were both smaller effects than that of severe fatigue.

**Influence of Stimulation Pulse Strength on Peak Tetanic Force in Fatigued Muscle**

The effect of increasing pulse strength during fatigue was predicted to maintain action potential generation better throughout train stimulation. We stimulated at 4.4 V, which excites muscle entirely via nerve terminals (Fig. 1, site 1), as opposed to 20 V, which causes excitation along the entire extent of the surface membrane of muscle (Fig. 1, site 3). The peak tetanic force generated with 4.4-V pulses was 95 ± 2% (*n* = 9) of that evoked with 20-V pulses in nonfatigued muscle. Fig. 5A shows an experiment with a soleus muscle in which fatigue was induced with 4.4-V pulses before stimulation was switched to 20-V pulses after 90 s. On average this increase in stimulus strength restored the peak force from 29 ± 4 to 79 ± 9% initial (*n* = 4) for the 91st tetanus. When soleus muscles were fatigued with 20-V pulses and stimulation was then switched to 4.4-V pulses after 100 s, the peak tetanic force was reduced further to 9 ± 1% (*n* = 5). When fatigue was induced at 20 V and then, after 90 s, stimulus strength was increased to

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**Fig. 4. Changes of twitch force-stimulation strength relationships with fatigue or raised [K+] in soleus and EDL muscles. Each point is mean ± SEM. Peak twitch force at each pulse strength (0.1-ms duration) was expressed as percentage of that for bracketing twitches evoked with standard 20-V, 0.1-ms pulses delivered via plate electrodes. Control relationships were the average of values recorded before and after intervention. Fatigue protocol: 125 Hz for 500 ms, once every second for 100 or 20 s. A: influence of fatigue in soleus (*n* = 9; ○, control; ■, 10–60 s after 100 tetani); ○, 60–110 s after 100 tetani). B: influence of fatigue in EDL (○, control; ■, 10–60 s after 100 tetani, *n* = 5; ○, 10–90 s after 20 tetani, *n* = 3). C: influence of raised [K+] in soleus (○, control; ■, 10–60 s after 100 tetani, *n* = 5; ○, 10–110 s after 100 tetani, *n* = 5; ○, 10–90 s after 20 tetani, *n* = 3). D: influence of raised [K+] in soleus (○, control; ■, 11 mM K+). In values given above, * indicates significant shift relative to control (ANOVA). With dashed lines, error bars were omitted for clarity. In C and D, muscles had been fully equilibrated for at least 50 min at raised [K+].
The peak force increased to more physiological stimulation frequencies of 30 and 50 Hz), tetani or with different fatigue protocols (in particular at the (125 Hz, 1.0-ms pulses) was repeated in soleus after just 20 control in soleus (125 Hz) (Table 1). When the test contraction average to 46% of control in EDL (200 Hz) and to 90% of tetanus evoked with 1.0-ms pulses restored peak force on
When muscles were fatigued with the standard protocol, a test contraction to 60% of the force lost during fatigue (relative to the initial tetanus), while 0.25-ms pulses achieved 89% restoration. Table 1 illustrates these data in soleus when expressed relative to control tetani obtained before and after recovery from fatigue.

To determine whether the recovery of force with longer pulses is due to the greater delivery of stimulus charge (proportional to strength × duration), test tetani were evoked at different voltages while holding the total pulse charge constant, i.e., pulses of 0.15 ms × 13.3 V, (n = 3) vs. those of 0.1 ms × 20 V (n = 8). This resulted in similar relative forces in fatigued muscle of 46% and 41% control, respectively (Table 1). Hence it is the stimulus pulse charge per se that is the important determinant, rather than pulse duration. These combined results from varying pulse durations indicate a large contribution from impaired sarcolemmal excitability during severe fatigue.

### Influence of Stimulation Pulse Duration on Peak Tetanic Force in Fatigued Muscle

The effect of increasing pulse duration during test tetani was also predicted to enhance the triggering of sarcolemmal action potentials and to restore force in fatigued muscle. Such an experiment is shown for a fatigued EDL muscle in Fig. 2B. When muscles were fatigued with the standard protocol, a test tetanus evoked with 1.0-ms pulses restored peak force on average to 46% of control in EDL (200 Hz) and to 90% of control in soleus (125 Hz) (Table 1). When the test contraction (125 Hz, 1.0-ms pulses) was repeated in soleus after just 20 tetani or with different fatigue protocols (in particular at the more physiological stimulation frequencies of 30 and 50 Hz), the peak force increased to ~90% of control in each case. Since stimulation with 1.0-ms pulses causes some direct activation of t-tubular voltage sensors in addition to triggering action potentials (Fig. 1, site 4), we tested for a contribution by this additional mechanism. Tetani elicited at 200 Hz (1.0-ms pulses), which more strongly activate the voltage sensors than does 125 Hz (6, 7), did not cause significantly greater force restoration in fatigued soleus (Table 1). The effect of a briefer prolongation of stimulation pulses (from 0.1 to 0.15 or 0.25 ms) was tested since these pulses excite only via voltage-dependent Na+ channels (6). Tetani with 0.15 ms pulses restored 60% of the force lost during fatigue (relative to the initial tetanus), while 0.25-ms pulses achieved 89% restoration.

### Influence of Electrode Configuration on Peak Tetanic Force During Fatigue

We postulated that comparing the fatigue responses with different electrode configurations would reveal whether the force impairment occurs in surface or t-tubular membrane (Fig. 1, sites 2 and 3). To prevent any contribution to fatigue from impaired neuromuscular transmission, the muscles were exposed to d-tubocurarine and pulse strength was increased to the maximum of 26 V. The peak tetanic force evoked via wires relative to that via plates was 97 ± 2% (n = 6) in nonfatigued soleus, which was not significantly different. The fatigue profile obtained subsequently with repeated tetani but different electrode configurations was similar until ~50 s, when fatigue became slightly greater with wires than with plates (Figs. 6, 7). For example, at 100 s (n = 6) the relative force was 42 ± 2% initial with wires but 52 ± 3% initial with plates. Moreover, test tetani (125 Hz) evoked with 0.25-ms pulses after 100 s resulted in recovery of peak force from 41 ± 3 to 80 ± 2% control with wires, and from 49 ± 4 to 83 ± 2% control with plates in the same four muscles (e.g., Fig. 6). The final intervention involved switching electrode configurations during severe fatigue. Figure 7A shows that changing from wires to plates after 90 tetani caused an immediate and notable restoration of peak force. In six soleus muscles, the peak force increased from 34 ± 4% initial with wires at the 90th tetanus, to 63 ± 6% initial with plates at the 91st tetanus. When the converse experiment was performed in three soleus muscles (Fig. 7B), the peak force was reduced from 43 ± 4% initial at the 90th fatiguing tetanus with plates, to 21 ± 10% initial at the 91st tetanus with wires. Each of these findings via altered electrode configurations implicates a large decline of excitability in the surface membrane during severe fatigue induced with transverse wire stimulation.

### DISCUSSION

#### Role of Impaired Sarcolemmal Excitability During Fatigue

We present evidence from five different types of intervention that implicates diminished sarcolemmal excitability as the
Table 1. Influence of altered stimulation pulse duration on peak tetanic force during test contractions evoked after fatigue had been induced with various stimulation regimes

<table>
<thead>
<tr>
<th>Stimulation regime</th>
<th>Test contraction characteristics</th>
<th>Fatigue Index (% initial)</th>
<th>Test force (% control)</th>
</tr>
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<tbody>
<tr>
<td>EDL</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>standard</td>
<td>(200 Hz, 0.1 ms, 20 V)</td>
<td>7±1%</td>
<td>8±1%</td>
</tr>
<tr>
<td>standard</td>
<td>(200 Hz, 1.0 ms, 20 V)</td>
<td>6±1%</td>
<td>46±3%*</td>
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<tr>
<td>Soleus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>standard</td>
<td>(125 Hz, 0.1 ms, 20 V)</td>
<td>37±3%</td>
<td>41±3%</td>
</tr>
<tr>
<td>standard</td>
<td>(125 Hz, 1.0 ms, 20 V)</td>
<td>39±3%</td>
<td>90±1%*</td>
</tr>
<tr>
<td>20 tetani</td>
<td>(125 Hz, 1.0 ms, 20 V)</td>
<td>84±1%</td>
<td>94±1%†</td>
</tr>
<tr>
<td>50 Hz</td>
<td>(125 Hz, 1.0 ms, 20 V)</td>
<td>67±8%</td>
<td>90±2%†</td>
</tr>
<tr>
<td>30 Hz</td>
<td>(125 Hz, 1.0 ms, 20 V)</td>
<td>85±3%</td>
<td>93±1%†</td>
</tr>
<tr>
<td>2.5 s rest</td>
<td>(125 Hz, 1.0 ms, 20 V)</td>
<td>67±2%</td>
<td>88±1%†</td>
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<tr>
<td>standard</td>
<td>(200 Hz, 1.0 ms, 20 V)</td>
<td>40±3%</td>
<td>92±2%†</td>
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<tr>
<td>standard</td>
<td>(125 Hz, 0.15 ms, 20 V)</td>
<td>43±3%</td>
<td>74±3%*</td>
</tr>
<tr>
<td>standard</td>
<td>(125 Hz, 0.25 ms, 20 V)</td>
<td>45±3%</td>
<td>82±1%*</td>
</tr>
<tr>
<td>standard</td>
<td>(125 Hz, 0.15 ms, 13.3 V)</td>
<td>40±7%</td>
<td>46±8%§</td>
</tr>
</tbody>
</table>

Data are mean ± SE obtained from n muscles. Standard stimulation regime used to induce fatigue: 125 Hz for 500 ms, evoked once every second for 100 s, with (20 V, 0.1 ms) pulses delivered via parallel plate electrodes. Different stimulation regimes (left-hand column) involved changing a single aspect with respect to our standard regime. These included: 20 tetani (instead of 100 tetani); 50 Hz (instead of 125 Hz); 30 Hz (instead of 125 Hz); 2.5 s rest (instead of 0.5 s rest). Fatigue index is peak force at end of fatigue run expressed relative to that of first tetanus, i.e., “% initial”. Test contractions (for 1 s in EDL or 2 s in soleus) were evoked 2 s after fatigue stimulation. Peak force achieved was expressed relative to average of those for control tetani (under identical stimulation conditions) obtained before fatigue run and after maximum recovery from it, i.e., “% control”. *Test force at increased pulse duration was significantly different from that evoked with 0.1-ms pulses (ANOVA); †Test forces not significantly different from one another (ANOVA).

major cause of severe fatigue during repeated tetani in isolated mammalian skeletal muscle (Figs. 2–7, Table 1). These findings are consistent with other studies showing a large reduction of M-wave area/amplitude during similar intensive stimulation protocols (21, 27, 43, 46). However, we extend the previous work by testing for a role of inexcitable fibers, distinguishing between impairment in surface and t-tubular membranes, and quantifying the contribution of impaired excitability to fatigue in different muscle-types. We also show that the decline of force in response to repeated brief tetani can exhibit symptoms of high-frequency fatigue (Figs. 2–6), as first described by Edwards (20), but is usually associated with prolonged continuous tetani (6, 19, 29, 51). Such fatigue has previously been attributed to an altered action potential profile (29, 34, 46) and impaired excitability (20, 32, 37) in t-tubular membranes (19, 51).

We confirmed that the decline of force with our standard protocol was localized to muscle per se, since blockade of neuromuscular transmission with ν-tubocurarine did not alter the fatigue profile, at least in soleus. Our lines of evidence for impaired sarcolemmal excitability come from interventions employing altered stimulation characteristics, which do not change action potential profile (Cairns, unpublished). These include the following observations: 1) lowering the stimulation frequency partially restored peak force in severely fatigued soleus and EDL muscles (Figs. 2A, 3) and reversed the fade during extreme fatigue in EDL; 2) the twitch force-stimulation strength relationship shifted towards higher voltages in extremely fatigued EDL muscles (Figs. 4B), reflecting an increased threshold for muscle action potentials; 3) Increasing stimulus pulse strength from 4.4 to 20 V impressively restored peak tetanic force from 29 to 79% initial (see Fig. 5A) and markedly attenuated the rate and extent of late fatigue in soleus (Fig. 5B); similarly, tetanic force was also restored from 8 to 50% of initial with greater pulse strengths in fatigued rat fast-twitch muscle (35); 4) switching stimulation from wire to plate electrode configurations (Fig. 7A) partially restored peak tetanic force (from 34 to 63% of initial) in soleus; and 5) changing the stimulus pulse duration from 0.1 to 1.0 ms increased peak tetanic force from 8 to 46% of control in EDL (Fig. 2B, Table 1) and from 41 to 90% of control in soleus (Table 1). Importantly, this restoration was also observed after fatigue had been induced at the more physiological frequencies of 30 and 50 Hz (Table 1). Clearly, the SR was still capable of releasing considerable Ca2+ in late fatigue in both muscle types when activation of the voltage sensors was adequate. We consider that using test tetani with 0.25-ms pulses is our best approach to quantify the impairment of excitability (Table 1, Fig. 6), since only voltage-dependent Na+-channels are excited; 1.0-ms pulses also directly activate the voltage sensors (6). Such test contractions generated 80–83% of the control force in severely fatigued soleus muscle, whether delivered from plate or wire electrodes. This provides strong indirect evidence that most late fatigue involves impaired excitability.

Each of our interventions permits more action potentials to be triggered either at rest or within short trains (i.e., they test for impaired excitability), but they do not address the role of reduced action potential amplitude (23, 27). However, exceedingly small action potentials have been recorded in the surface membrane during 125-Hz stimulation for 2 s in nonfatigued soleus (action potential peak falls from +30 mV to less than -30 mV), but despite this, the peak force remains maximal (5). This argues strongly against a detrimental effect from reduced action potential amplitude, at least in the surface membrane.

Inexcitable fibers. Our evidence for inexcitable fibers comes from the large right shift of the twitch force-stimulation strength relationship towards higher voltages, in combination with twitch depression, when our standard pulses were used. These effects were detected in EDL muscles after 100 s (peak tetanic force 8% of initial) but not after 20 s (peak tetanic force 38% initial) (Fig. 4B). The much smaller shift of this relationship in soleus at 100 s (Fig. 4A) may reflect the lesser extent of tetanic fatigue, together with some force recovery. This is unlikely to involve inexcitable fibers when our standard pulses...
were used, since peak twitch force had reached a plateau. The occurrence of inexcitable fibers in EDL seems to require extreme fatigue, which is in line with microelectrode recordings showing that fibers become inexcitable with repeated tetani in fast-twitch preparations only when peak tetanic force declines to less than 10% initial (31, 46).

**Diminished excitability during short trains.** The relative decline of the tetanus markedly exceeded that of the twitch during severe fatigue, and peak tetanic force was also partially restored on reducing the frequency, both of which suggest that tetanic force loss occurs within short trains. This is likely to involve severe skipping and/or complete termination of action potentials, as is seen during intermittent tetanic stimulation of amphibian fibers (2, 44) or continuous stimulation of mammalian fibers (32). From the force-frequency relationship obtained in fatigued soleus (Fig. 3A), we suggest that moderate skipping with the loss of 2 out of 3 action potentials at 125 Hz (i.e., effectively 40–50 Hz stimulation) would help maintain force, but severe skipping with the loss of 5 out of 6 action potentials (i.e., effectively less than 20-Hz stimulation) is needed to depress peak force to a level similar to that with 125-Hz stimulation.

**Impairment in surface vs. t-tubular membranes.** It is frequently postulated that the t-tubular membranes are more suscept as a site of failure during repetitive stimulation (9, 14, 18, 25, 29, 48). However, we present results that are suggestive of a failure to generate and propagate action potentials along the surface membrane when inducing fatigue with transverse wire electrodes in soleus fibers. This is most evident when switching from wire to plate stimulation, which markedly restored peak tetanic force during severe fatigue (Fig. 7A). A similar response is seen with a switch from 4.4- to 20-V plate stimulation during late fatigue (Fig. 5A); these weaker pulses also trigger a surface-propagated action potential (6). Conversely, a switch from plates to wires further depressed the tetanus in fatigued muscle (Fig. 7B). The fatigue profiles diverged during late fatigue, showing greater force loss with wire than plate stimulation (Figs. 6, 7). There were also identical abilities of test tetani with 0.25-ms pulses to restore force when evoked with wires or plates. Note that if action potentials are not triggered in the surface membrane when wire stimulation is used, then the t-tubular membranes are not activated rather than being directly impaired; hence they still have ability to generate action potentials in response to plate stimulation. Moreover, sarcolemmal impairment during fatigue with plates cannot involve propagation along the surface membrane and therefore involves problems with t-tubular membrane propagation (or surface initiation). In fact, parallel plate electrodes have been deliberately used to avoid excitation failure in the surface membrane (34).

**Contribution of electrolytes.** Stimulation regimes comparable to ours induce a diminution of K\(^{+}\), Na\(^{+}\) and Cl\(^{-}\) and Ca\(^{2+}\) gradients across the sarcolemma (1, 9, 30, 36), which may cause the impaired excitability that we observe during fatigue. When testing for a possible role of K\(^{+}\), it was observed that the right shift of the twitch force-stimulation strength relationship...
with fatigue (Figs. 4A, B) was quantitatively mimicked by 9 mM K+ in soleus and 11 mM K+ in EDL (Figs. 4C, D). Nevertheless, these [K+]o did not depress peak twitch or tetanic forces by the same extent as fatiguing stimulation, making it unlikely that K+ causes the entire extent of fatigue. However, K+ may interact with diminished Na+ and Cl− and Ca2+ gradients to impair excitability, thereby contributing to fatigue (9). Moreover, the exacerbated fatigue seen at 125 vs. 50 Hz (Table 1) may have arisen, in part, because the sarcolemma was unable to respond to each stimulus at the higher frequency due to K+ extending the refractory period (18), rather than simply greater K+ shifts at 125 Hz. This may explain our finding that the 125-Hz test tetanus was depressed equally after fatiguing stimulation at 50 and 125 Hz.

Role of Other Muscle Processes During Fatigue

Impaired myofilament function. The tetanic force evoked with 1.0-ms pulses fell by ~10% during severe fatigue in soleus (Table 1). We consider that this force level represents maximum cross-bridge activity, since caffeine, which enhances Ca2+ release from the SR without increasing maximum Ca2+-activated force (11), does not potentiate these 1.0-ms tetani in nonfatigued soleus muscle (7). Hence, we suggest that there is a 10% decline of maximal cross-bridge activity during fatigue, much of which occurs within 20 s of stimulation (Table 1, Refs. 1, 16). The magnitude of this effect parallels the extent of early fatigue estimated by curve-fitting to fatigue profiles in both soleus and EDL muscles (10). Stimulation protocols comparable to ours lead to increased inorganic phosphate levels and decreased pH, [ATP], and muscle glycogen levels (1, 12, 16, 36), any one of which may depress maximum myofilament function (1, 12, 22, 26). However, inorganic phosphate is the only metabolite to change dramatically during early fatigue (1, 16), and it achieves a 10–15% reduction of maximum Ca2+-activated force in skinned fibers (22, 26). It is therefore favored as the candidate responsible for early fatigue and depressed maximum cross-bridge activity. Note that 1.0-ms test tetani cannot be used equivalently in EDL because these tetani do not fully restore force in K+ -depressed fast-twitch muscle (7), yet maximum cross-bridge activity is unaffected by a reduced trans-sarcolemmal K+ -gradient (18).

Impaired E–C coupling. The large force recovery with 1.0-ms pulses in soleus (Table 1) also largely discounts four putative mechanisms contributing to fatigue during our intensive stimulation regimes. These include diminished opening of the SR Ca2+ release channel (1), reduced Ca2+ loading of the SR (1), calcium phosphate precipitation within the SR (1, 22), and decreased maximum voltage sensor activity (4). Of course this interpretation does not exclude the possibility that such mechanisms contribute to fatigue during exercise or during other stimulation regimes. Furthermore, an impairment of E–C coupling, as seen in amphibian fibers (4, 25), may also contribute to fatigue in EDL muscles. Indeed, peak tetanic force does not recover fully (~20% deficit) after fatigue with our standard protocol in EDL, although it does in soleus muscles (10). This incomplete recovery of force depression in fast-twitch fibers is credited to a long-term impairment of SR Ca2+ release (11). Moreover, fast-twitch fibers undergo inactivation of E–C coupling with prolonged depolarization (1, 13, 42), whereas slow-twitch fibers resist this process (13). We hypothesize that inactivation of the voltage sensors (4, 13, 25) contributes to some of the force decline during late fatigue in EDL.

Evaluation of Our Model of Fatigue

The experimental approaches used to study fatigue, including the type of muscle preparation, the muscle environmental conditions, and the stimulation regime, all have a considerable bearing on the mechanisms involved in fatigue (1, 8). Indeed, utilizing a reductionist approach with an isolated muscle permits interventions to study peripheral fatigue but necessarily eliminates any contribution from impaired motor drive from the central nervous system, as can occur during exercise (1, 24). Other aspects of our fatigue model with potential to influence the mechanisms of force loss are now discussed.

Diffusion limitations. When isolated whole muscle preparations are used, one concern is that they may develop an anoxic core at some point during fatiguing stimulation due to insufficient diffusion of O2 from the bathing solution (1, 3, 8, 53). To test the degree to which anoxia may contribute to fatigue in our isolated mouse muscles, we duplicated the mathematical model of O2 diffusion developed by Barclay (3). We solved the diffusion equation (Eq. A1) as applied to our standard protocol under the harsh assumption that O2 demand remains maximal throughout the period of stimulation, despite the progressive diminution of force (see APPENDIX). Under this scenario, the model (Fig. 8A) predicts that central (axial) fibers do indeed become anoxic (i.e., PO2 < 1 Pa), after ~20 s in EDL and ~40 s in soleus muscles. By contrast, peripheral fibers located in layer 2 remain sufficiently oxygenated (i.e., PO2 > 15 kPa) throughout the entire stimulation period. Note that this value of PO2 far exceeds the P50 for mitochondrial ATP production: 0.26 Pa (47, 52).

The solution of Eq. A1 yields the PO2 profile across the muscle. Using Eq. A3 allows prediction of the extent of the muscle cross-sectional area that is rendered anoxic during fatiguing stimulation. The result of this calculation (Figs. 8B, C) makes it unlikely that any substantial decline of force occurs as a consequence of anoxia in our model of fatigue. The peak tetanic force had fallen by ~65% in EDL and ~35% in soleus prior to any fibers becoming anoxic (see vertical dashed lines in Figs. 8B, C). The proportion of muscle cross-sectional area calculated to become anoxic during stimulation predicts a markedly lesser decrement of force than was measured (Figs. 8B, C). Increasing the stimulus pulse charge or switching from wire to plate stimulation (Figs. 2, 5–7) both partially restored peak force during severe fatigue, up to 90% control (Table 1), at a time when anoxia was predicted to prevail (Figs. 8A).

These combined theoretical and experimental results argue against a large detrimental role of anoxia in our model of fatigue in either muscle-type. Nevertheless, we cannot exclude the possibility that anoxia makes some contribution to the impairment of excitability, although its known consequence involves a depression of myofilament function (22, 26). Our data are consistent with the recent observation that an anoxic environment does not exacerbate fatigue during continuous tetanic stimulation in isolated rat soleus muscle (40).

Oxygen is not the only diffusion-limited chemical species to be considered. Isolated whole muscles also have a long diffusion pathway for electrolytes between axial fibers and the
bulk extracellular solution. This limitation is predicted to exaggerate changes of interfiber electrolyte concentrations during stimulation (1, 7, 8). Ion-sensitive microelectrode measurements reveal that the interfiber [K⁺] value can reach 10 mM between surface and layer 2 fibers in isolated mouse soleus muscles during repeated tetanic stimulation (30). Notably, such measurements have not been made adjacent to deeper fibers where larger electrolyte changes may be expected to occur.

Relevance to exercise. Repeated electrical stimulation of isolated muscle removes any possibility that the neural strategies seen during voluntary exercise (1, 8, 24, 28) can protect against fatigue, as highlighted in a recent review (1). Indeed, activation of muscle via the motor cortex usually involves trains of action potentials with an initial doublet, and then some decline of frequency, so that the average in vivo firing rates are less than 60 Hz (1, 8, 24, 28). In contrast, our standard stimulation regime, which involves constant high-frequency (125 Hz) trains, is likely to exaggerate the trans-sarcolemmal electrolyte fluxes and to promote a scenario where the sarcolemma is unable to respond to stimuli. Hence, our model does not directly mimic the neural activation strategy which occurs during exercise. Nevertheless, large electrolyte disturbances do occur during intense exercise (9, 49), possibly due, in part, to the fact that muscles contract 2–5 times per second during rapid locomotion (8), which exceeds the single tetanus per second in the present study. The consequent very brief intercontraction rest periods during exercise diminish the time for recovery processes, and hence promote electrolyte disturbances. Moreover, stimulation at the lower, more physiological frequencies of 30 or 50 Hz in soleus resulted in fatigue with a component that also involved impaired excitability (Table 1). Despite this, the additional force loss when fatigue was induced at 125 Hz may involve extra mechanisms not seen during voluntary exercise. Perhaps a question of more importance (8) is whether severe force fatigue (i.e., to less than 50% initial) ever occurs during intense exercise in vivo? At least in the present study, impaired excitability seems to become more prevalent the greater the loss of force. Ultimately, to mimic a more realistic exercise scenario when stimulating isolated muscles, it would be necessary to account for the protective physiological effects mediated by hormones, such as the catecholamines, as well as by repeated excitation, both of which confer some protection via enhancing the activity of sarcolemmal Na⁺-K⁺ pumps (9, 14, 15).

Summary: the Quantitative Contribution of Different Processes to Fatigue

**Soleus.** The fall of peak tetanic force (~60% initial) can be adequately described by two mechanisms: early fatigue involving reduced maximum cross-bridge activity (~10% initial force), and late fatigue involving impaired sarcolemmal excitability within trains (~50% of initial force). When stimulation has been delivered via transverse wires electrodes, much of this impaired excitability occurs in the surface membrane.

**EDL.** The larger decline of peak tetanic force (~95% initial) can be explained by four mechanisms. The early fatigue involves ~10% decline of maximum cross-bridge activity, as in soleus (10). The late fatigue incorporates three processes: 1) impaired sarcolemmal excitability within trains (~45% initial force); 2) impaired E–C coupling, which may be linked to voltage sensor desensitization and which does not recover after fatigue (~20% initial force, Ref. 10); and 3) the occurrence of inexcitable fibers due to an increased action potential threshold (~20% initial force).
Taking all our evidence into consideration, a large component of the severe stimulation-induced decline of peak tetanic force observed in isolated whole muscles is due to impaired sarcoplemmal excitability (~50% of initial force). The greater extent of late fatigue in fast-twitch EDL than in slow-twitch soleus muscles (~35% of initial force) (Fig. 2, Ref. 10) appears to be a consequence of greater K- and Na+ shifts (14, 30, 36), leading to the extra fatigue processes of voltage sensor desensitization and the occurrence of inexcitable fibers.

APPENDIX

We investigated whether fibers become anoxic during our standard stimulation regime by modeling the profile of oxygen partial pressure (PO2) between the periphery and the central axis of isolated soleus and EDL muscles of mice. When muscles are bathed in Krebs solution and equilibrated with 95% O2 at 25°C (see METHODS), the measured PO2 in our muscle chamber is 94.2 ± 4.1 kPa (45). In modeling the PO2 as a function of time and radial distance within the muscle, we adopted an equivalent of the governing diffusion equation published by Barclay (3):

\[
\frac{\partial}{\partial t}PO_2(r,t) = D \frac{\partial^2}{\partial r^2}PO_2(r,t) + \frac{1}{r} \frac{\partial}{\partial r}PO_2(r,t) - D_{MB} \left( \frac{\partial}{\partial r}S(PO_2) + \frac{1}{r} \frac{\partial}{\partial r}S(PO_2) \right) - m(PO_2,t)
\]

(A1)

where \( t = \) time (s); \( r = \) radial distance within the muscle \( (m, 0 < r < r_o, r_o = 440 \mu m); D = \) diffusion constant for O2 in muscle \( (1.09 \times 10^{-5} \text{mol m}^{-1} \text{s}^{-1} \text{Pa}^{-1} \text{ at 25°C}); S = \) saturation of myoglobin with O2; \( \sigma_{MB} = \) total concentration of myoglobin plus oxymyoglobin in tissues \( (0.4 \text{ mol m}^{-3}); D_{MB} = \) diffusion constant for myoglobin in muscle \( (1.25 \times 10^{-11} \text{ m}^2 \text{s}^{-1}); \) and \( m = \) metabolic rate of O2 consumption in the tissues \( (\text{mol m}^{-3} \text{s}^{-1}).\)

Equation A1 accounts for diffusive O2 supply (both the simple and myoglobin-facilitated components) and O2 demand \( [\text{PO}_2(t)]\). The geometries of mouse muscles are approximated as long cylinders of circular cross section (3). A muscle radius \( (r_o) \) of 0.44 mm was used since our average radii were 438 \( \mu m \) for soleus and 435 \( \mu m \) for EDL (10). In accord with Barclay (3), we assumed a sigmoidal dependency of the normalized metabolic rate \( \gamma \) on \( \text{PO}_2 \) (Eq. A2) and adopted the identical Hill coefficients and \( \text{P}_{50} \) values: \( n_m = 1.5, \text{P}_{50} = 0.26 \text{ Pa} \) (corresponding to 1.5 mmHg) (47, 52).

\[
Y(\text{PO}_2) = \frac{\text{PO}_2(r,t)^{n_m}}{\text{P}_{50} + \text{PO}_2(r,t)^{n_m}}, \quad 0 \leq Y \leq 1
\]

(A2)

We use the rates of basal \( (0.05 \text{ m}^{-1} \text{s}^{-1} \text{g}^{-1} \text{ for both muscles}) \) and active \( (4 \text{ and } 13.5 \text{ m}^{-1} \text{s}^{-1} \text{g}^{-1} \text{ for soleus and EDL, respectively}) \) O2 consumption given in Table 1 of Barclay (3). We adopt the ultra-conservative assumption that the O2 demand remains maximal over the period of fatigueing stimulation, notwithstanding the progressive decline of tetanic force that was observed experimentally and which probably attenuates O2 demand. Solutions for \( \text{PO}_2 \) were computed at each of 250 equal time steps and 250 equidistant radial steps by means of Mathcad v.14 (Mathsoft Inc, Cambridge, MA). Figure 8A shows the values of \( \text{PO}_2 \) in fibers on the central axis and in layer 2 fibers, of muscles at rest, and then during the stimulation and recovery periods.

Fatiguing stimulation led to a progressively enlarging region of simulated anoxia, which encroaches radially outward from the central axis, as the stimulation continues. We calculated the shape of the muscle cross section that remains oxygenated (A) by:

\[
A(r) = 2\pi \int_0^r Y(\text{PO}_2) \cdot dr
\]

(A3)

where it can be seen that \( Y(\text{PO}_2) \) acts as a weighting function, which progressively diminishes the contribution to \( A \) as local \( \text{PO}_2 \) approaches the \( \text{P}_{50} \). With Eq. A3, it is straightforward to calculate the proportion of the cross-sectional area of a muscle that is rendered anoxic as a function of stimulation time (Figs. 8B, C). Furthermore, if anoxia results in complete force loss in individual fibers [which is an extreme assumption (26)], then the curves shown in Figs. 8B and C provide a direct assay for the proportion of muscle force lost due to anoxia during fatigue, under the unlikely assumption that O2 demand remains maximal throughout fatigueing stimulation.

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